ASSESSMENT OF *Plasmodium falciparum* RESISTANCE TO PIPERAQUINE AND OTHER FRONTLINE ANTIMALARIALS IN KENYA USING GENOMIC ANALYSES AND GROWTH INHIBITION ASSAYS

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Master of Science Degree in Biochemistry of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this University or any other for the award of a degree.

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Signature  Date: 2/06/2023

Recommendation

This thesis has been submitted with our approval as the University supervisors.

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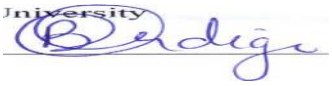
Signature  Date: 02 JUNE 2023

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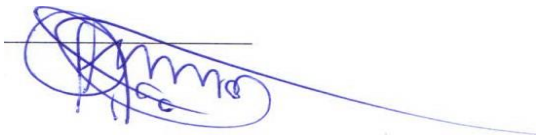
Signature  Date: 02 JUNE 2023

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DEDICATION

It is with sincere gratitude that I dedicate this work to my loving and caring parents, Tomas and Rose Wakoli whose kind words of hope and inspiration ring in my mind. Also, to my brothers Kevin and Simon, my sisters Belinda and Ebby, my uncle Benard Karani who stood by my side always even when things were overwhelming. My grandma Loice and Elinah, and in loving memory of my late grand dads Mark and Ben who always inspired me to put the best in everything I do.

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ABSTRACT

Dihydroartemisinin-piperaquine (DHA-PPQ) is an alternative first-line antimalarial to artemether-lumefantrine in Kenya. However, recent reports on emergence of PPQ resistance in Southeast Asia threaten its continued use in Kenya and Africa. In line with the policy on continued deployment of DHA-PPQ, it is imperative to monitor susceptibility of Kenyan parasites to PPQ and other frontline antimalarials in order to clarify its field expedience at this period of intensified deployment. Parasites isolates from samples collected between 2008 and 2021 from individuals with naturally acquired *P. falciparum* infections presenting with uncomplicated malaria were tested for *in vitro* susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine using the malaria SYBR Green I method. A subset of the 2019-2021 samples was further tested for *ex vivo* susceptibility to PPQ using piperaquine survival assay (PSA). Each isolate was also characterized for mutations associated with antimalarial resistance in *Pfcrt, Pfmdr1, Pfpm2/3, Pfdhfr,* and *Pfdhps* genes using real-time PCR and Agena MassARRAY platform. Associations between phenotype and genotype were also determined using the Kruskal-Wallis H-test and Mann-Whitney U test. The PPQ median IC_{50} interquartile range (IQR) remained stable during the study period, 32.70 (IQR 20.2-45.6) nM in 2008 and 27.30 (IQR 6.9-52.8) nM in 2021, (P=0.1615). The median *ex vivo* piperaquine survival rate (IQR) was 0 (0-5.27) %, at 95% CI. Five isolates had PSA survival rate of ≥10%, consistent with the range of PPQ resistant parasites, though they lacked polymorphisms in *Pfmdr*1 and *Plasmepsin* genes. Lumefantrine and artemether median IC_{50}s rose significantly to 62.40 (IQR 26.9-100.8) nM, (P = 0.0201); 7.00 (IQR 2.4-13.4) nM, (P = 0.0021) in 2021from 26.30 (IQR 5.1-64.3) nM; 2.70 (IQR 1.3-10.4) nM in 2008, respectively. Conversely, chloroquine median IC_{50}s decreased significantly to 10.30, (IQR 7.2-20.9) nM in 2021 from 15.30 (IQR 7.6-30.4) nM in 2008, coinciding with a decline in prevalence of *Pfcrt* 76T allele over time from 39.3% to 0% (P = 0.0357). The proportions of piperaquine-resistant markers including *Pfpm2/3* and *Pfmdr1* did not vary significantly. However, a significant association was observed between PPQ IC_{50} and *Pfcrt* K76T allele (P=0.0026). Circulating Kenyan parasites have remained sensitive to PPQ and other antimalarials, though response to artemether and lumefantrine is declining. This study forms a baseline for continued surveillance of current antimalarials for timely detection of resistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

ACD	Acid citrate dextrose
ACT	Artemisinin combined therapy
AL	Artemether-lumefantrine
CITI	Collaborative Institutional Training Initiative
CMS	Complete medium with serum
CQ	Chloroquine
DHA- PPQ	Dihydroartemisnin and Piperaquine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamininetetraacetic acid
HCL	Hydrochloric acid
IC_{50}	50% inhibitory concentration
IQR	Interquartile range
KEMRI	Kenya Medical Research Institute
LM	Lumefantrine
MDR	Malaria Drug Resistance
MOH	Ministry of Health
MQ	Mefloquine
Mrdt	Malaria rapid diagnostic test
MSP 1 & 2	Merozoite surface protein 1&2
PCR	Polymerase chain reaction
NACOSTI	National Commission for Science, Technology and Innovation
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
Pfexo	*Plasmodium falciparum exonuclease* gene
Pfk13	*Plasmodium falciparum kelch* 13
Pfmdr1	*Plasmodium falciparum multi-drug resistance gene I*
Pfpm2	*Plasmodium falciparum plasmepsin* 2
Pfpm3	*Plasmodium falciparum plasmepsin* 3
PSA	Piperaquine survival assay

QN	Quinine
RPMI 1640	Rosewell Park Memorial Institute 1640
RSA	Ring survival assay
SEA	Southeast Asia
SNP	Single nucleotide polymorphism
SP	Sulphadoxine and Pyrimethamine
SSA	Sub-Saharan Africa
USAMRD-A	US Army Medical Research Directorate-Africa
WHO	World Health Organization
WWARN	Worldwide Antimalarial Resistance Network

CHAPTER ONE
INTRODUCTION

1.1 Background information

Malaria remains a fatal parasitic disease if not treated. It infects over 200 million people causing more than 600,000 deaths annually (WHO, 2022). Sub-Saharan Africa (SSA) accounts for 95% of the disease burden, with most cases occurring in children below five years and pregnant women (WHO, 2022). In Kenya, malaria is a major public health problem with over 70% of its population at risk, especially in the endemic Lake region of western Kenya, while it has been suggested that a decline in prevalence proffer increased risk of severe disease across all age groups (Malaria indicator survey, 2020). The high burden of malaria worldwide is sustained partly by the rapid development of *P. falciparum* resistance to antimalarial drugs among other factors (WHO, 2019, 2021).

Malaria is caused by the apicomplexan *Plasmodium* parasites which are transmitted to human host through bites of infected female Anopheles mosquito that exclusively feeds on blood for her reproduction (WHO, 2019). Over 120 *Plasmodium* species exist but only six namely; *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae*, *Plasmodium ovale wallickeri*, *Plasmodium ovale curtisi* and *Plasmodium knowlesi* are of human health significance (Ashley & Phyo, 2018; WHO, 2015; 2018). *Plasmodium falciparum* and *Plasmodium vivax* are the most deadly species globally, responsible for both severe and complicated malaria (Bartoloni & Zammarchi, 2012). *P. falciparum* is the most widespread species and it accounts for the highest malaria burden in sub-Saharan Africa (WHO, 2021). Malaria infection can be a mono-infection (infection by single species) or a coinfection (infection by mixed species) (Sagara *et al*., 2018).

The high malaria burden in sub-Saharan Africa is attributed to first, presence of *Anopheles gambiae,* a highly efficient and ubiquitous mosquito vector. Secondly, the warm climatic condition that favor breeding of mosquitoes. Thirdly, the predominantly lethal and aggressive *P. falciparum* parasites. Lastly, the scarce resources that hinder efficient malaria case management, and control (Mbacham *et al*., 2019; Stratton *et al*., 2008).

Vector control, effective vaccine and chemotherapy are the preferred tools of malaria control. However, declining vector control efforts driven partly by insecticides resistance is a major public

health concern. Thus, antimalarial drugs alongside the recently approved RTS,S vaccine remains central interventions for malaria control (Martin *et al*., 2018; WHO, 2022). The recent decrease in the global malaria burden has been attributed partly to the deployment of ACTs and other interventions (Inoue *et al*., 2018; Martin *et al*., 2018). Recommended ACTs include; artemether-lumefantrine (AL), dihydroartemisinin-piperaquine (DHA-PPQ), artesunate-amodiaquine (AS-AQ), artesunate plus sulphadoxine-pyrimethamine (AS-SP), artesunate-mefloquine (AS-MQ) and artseunate-pyronaridine (AS-PND) (Ashley & Phyo, 2018; WHO, 2021). Though, ACTs have had marked success for the last two decades (Ashley *et al*., 2018), the recent emergence of artemisinin resistance in Southeast Asia and emerging signals of the same from Rwanda and Northern Uganda is a threat to the continued use of ACTs in SSA (Balikagala *et al*., 2021; Uwimana *et al*., 2021).

Dihydroartemisinin-piperaquine combination is the recommended alternative first-line drug to artemether-lumefantrine (AL) and artesunate-amodiaquine (AS-AQ) in SSA (Inoue *et al*., 2018). This treatment was adopted by the Kenya Ministry of health 2009 (ACTwatch group, 2017; Ogutu *et al*., 2014). PPQ confers a 60-day prophylactic effect after treatment (White NJ., 2005), and has never been used as a monotherapy in Africa (Basco *et al*., 2003). Recent studies show the emergence, development and spread of DHA-PPQ resistance in Cambodia, Vietnam, and Thailand (Ashley & Phyo, 2018; Duru *et al*., 2015; Imwong *et al*., 2017). The emergence of resistance warrants monitoring of the efficacy of DHA-PPQ in SSA (Amato *et al*., 2017; Inoue *et al*., 2018; van der Pluijm *et al*., 2019), since historical patterns of antimalarial drug resistance emergence show that once reports emerge in any part of the world often reaches Africa (Mita *et al*., 2009).

Resistance to DHA-PPQ is thought to be due to the drug stress of PPQ due to its longer half-life, thus exposing the parasites to this drug for a long time (Duru *et al*., 2015). Studies suggest that resistance to PPQ is caused by genetic mutations within the Plasmepsin-2/3 (*Pfpm2/3*) complex, *Pfmdr*1, and non-synonymous SNPs in *Pfcrt*, *PfK*13, and *Pfexo* genes (Amato *et al*., 2017; Ansbro *et al*., 2020; Diakite *et al*., 2019). There is a scarcity of genotypic and phenotypic data that can inform on piperaquine resistance in Kenya currently as the Country continues to deploy DHA-PPQ alternative treatment. To address this gap, this study assessed polymorphisms in *Pfpm2*, *Pfpm3*, *Pfexo*, *PfK13*, *Pfcrt*, and *Pfmdr1* genes that confer resistance to frontline antimalarial drugs in circulating *Plasmodium falciparum* parasites in Kenya using genomic analyses. Profiled *P. falciparum* isolates susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether,

and chloroquine using the growth inhibition assays. Lastly, established the associations between mutations in genes associated with piperaquine resistance and *ex vivo/in vitro* phenotype using the Kruskal-Wallis H-test and Mann-Whitney U test. Findings from this study underscore parasite susceptibility to antimalarials including DHA-PPQ before its widespread use. Additionally, this will form a baseline to support tracking how natural infections will respond to DHA-PPQ as the use of this treatment becomes more widespread.

1.2 Statement of the Problem

Malaria is a life threatening and devastating parasitic disease that claims approximately half a million lives globally every year. Evolution of drug resistant *P. falciparum* parasites at a faster rate than the pace of discovering and bringing new drugs into use is a major problem for global malaria control. Resistance to the scarce currently used antimalarial drugs has emerged in SEA. This threatens the continued use of frontline antimalarials in sub-Saharan Africa, since this resistance could emerge in the region in a matter of time rendering widespread use of DHA-PPQ and other frontline antimalarials a short-lived solution. The paucity of phenotypic and genotypic data for ongoing monitoring of antimalarial drug resistance in Kenya warrants assessment of the susceptibility of *P. falciparum* to current antimalarials using genomic analyses and growth inhibition assays.

1.3 Objectives

1.3.1 General objective

To establish *Plasmodium falciparum* resistance to piperaquine and other frontline antimalarials in Kenya using genomic analyses and growth inhibition assays.

1.3.2 Specific objectives

i. To determine polymorphisms in *Pfpm2, Pfpm3, Pfexo, PfK13, Pfcrt* and *Pfmdr1* genes that confer resistance to frontline antimalarial drugs in circulating *Plasmodium falciparum* parasites in Kenya.

ii. To determine *P. falciparum* isolates susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine using the malaria SYBR Green I based method.

iii. To determine *P. falciparum* isolates susceptibility to piperaquine using the Piperaquine survival assay (PSA).

iv. To establish associations between mutations in genes associated with piperaquine resistance and *ex vivo*/*in vitro* phenotype using the Kruskal-Wallis H-test and Mann-Whitney U test.

1.4 Hypotheses

1.4.1 Null hypotheses

i. Polymorphisms in *P. falciparum Pfpm*2, *Pfpm*3, *Pfexo*, *PfK13*, *Pfcrt* and *Pfmdr1* genes do not confer resistance to frontline antimalarial drugs

ii. Kenyan *P. falciparum* field isolates are not susceptible to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine

iii. Kenyan *P. falciparum* field isolates are not susceptible to piperaquine.

iv. There is no significant association between *Pfpm2*, *Pfpm3*, *Pfexo*, *PfK13*, *Pfcrt* and *Pfmdr1* polymorphisms, and *in vitro*/*ex vivo* phenotype among the Kenyan *P. falciparum* isolates.

1.5 Justification

World health organization (WHO) emphasizes on the importance of sustained surveillance of resistance to all frontline antimalarial drugs in malaria endemic regions (WHO, 2021). Kenya ministry of health (MOH) recommends the use of dihydroartemisinin-piperaquine (DHA-PPQ) as an alternative first-line antimalarial drug for treatment of uncomplicated malaria. This shift of prescription coincides with a period when quinine and artemether-lumefantrine (AL) efficacy is declining in the region (Dimbu *et al*., 2021; Ebong *et al*., 2021). However, despite reports of DHA-PPQ resistance in Southeast Asia (SEA) (Boonyalai *et al.,* 2020*;* Duru *et al.,* 2015*;* Imwong *et al*., 2020; Witkowski *et al.,* 2017), there is scanty empirical data on *P. falciparum* susceptibility patterns and molecular markers for monitoring piperaquine resistance in Kenya. This raises a serious concern on the continued use of DHA-PPQ in Kenya since resistance established in SEA might spread to Africa putting millions of lives at risk as it was the case with chloroquine and sulphadoxine-pyrimethamine resistance (Mita *et al.*, 2009). This study assessed polymorphisms in *Pfpm2*, *Pfpm3*, *Pfexo*, *PfK13*, *Pfcrt*, and *Pfmdr1* genes that confer resistance to frontline antimalarial drugs in circulating *Plasmodium falciparum* parasites in Kenya using genomic analyses. Profiled *P. falciparum* isolates susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine using the growth inhibition assays. Lastly, established the associations between mutations in genes associated with piperaquine resistance and *ex vivo/in*

vitro phenotype using the Kruskal-Wallis H-test and Mann-Whitney U test. The molecular, *in vitro*, and *ex vivo* data generated by this research study underscore parasite susceptibility to antimalarials including DHA-PPQ before widespread use of DHA-PPQ in Kenya. Furthermore, these findings form a baseline that supports tracking how natural infections respond to DHA-PPQ as drug pressure increase on it.

1.6 Study limitations

This study is subject to several limitations. First, the PSA assay was not designed to analyze PPQ sensitivity to different parasite stages through drug pulsing (different drug pressures) hence it doesn't exhaustively unravel the mechanism of drug action and resistance. PSA was limited to isolates collected from high malaria endemic ecological zone and these results might not reflect the susceptibility of parasites from low malaria transmission setting. Secondly, DNA degradation amid sample storage, handling, and freeze-thawing might possibly have biased the tests towards detection of wild type over mutants in case of parasites genome obtained infections with low density of strains that harbor mutations that confer resistance (Gupta *et al.*, 2020). Thirdly, Genotype-phenotype association study was limited by the small sample size of isolates with genotypic data for *Pfcrt, Pfdhps, Pfdh*fr and *Pfmdr1* SNPs. Lastly, Screening methods for genetic polymorphisms associated with PPQ resistance left out genotyping analysis of SNPs in other genes namely, *Pfcrt, Pfexo* and *Pfk13* that have been implicated in PPQ resistance.

CHAPTER TWO
LITERATURE REVIEW

2.1. History of malaria

Plasmodium falciparum is the most virulent species that causes malaria especially in sub-Saharan Africa. Malaria infection has infected human for thousands of years yet it is still a major public health burden affecting half of the global population. The history of this disease dates back to 2700 BC, this is evidenced by the existence of Chinese documents, clay tablets in Mesopotamia, Egyptian papyri and Hindu texts (Bruce-Chwatt *et al*., 1988). *P. falciparum* is thought to have originated from gorilla and spread to humans in West Africa and later on due to population migration dispersed to other continents (Liu *et al*., 2010). Hippocrates and other early Greeks in 400 BC understood and described the symptoms associated with malaria, they included; fever marked by environment and seasons, splenomegaly (enlargement of the spleen) in people living in the swamps (Cox, 2010). During the 16th century in Rome, it was believed that malaria was caused by spoiled air from swamp waters earning it the name "mal'aria" (Dagen, 2020). This speculation continued until when bacteria was discovered in 1800 as a result of intensified research in microbiology by Robert Koch and Louis Pasteur.

At around 1880, Charles Louis Alphonse Levaran a French army doctor discovered *Plasmodium* parasites in the blood of an infected soldier while in Algeria for war (Bruce-Chwatt *et al*., 1981). Later on, Italian Scientists Camillo Golgi and co-workers discovered that *Plasmodium* genus consisted of different species that caused malaria (Cox, 2010). Glovanni Grassi and colleagues classified *Plasmodium parasites* into *Plasmodium malariae* and *P. vivax*. Welch and co-workers further characterized malaria parasites into *P. falciparum* and *P. ovale*. In 1890 another novel discovery on how *Plasmodium* parasites were transmitted to avian was made by a group of scientists, namely; Manson and Sir Ronald Ross. They noted that these parasites are spread by female Anopheles mosquitoes (Cox, 2010). Similarly, in 1898 Grassi and co-workers using comparative studies discovered that human malaria parasites are transmitted by female Anopheles mosquitoes (Cox, 2010; Majori, 2012).

2.2. Life cycle of *Plasmodium falciparum*

This parasite exhibits a complex life cycle whose thriving in the two hosts (Human and Mosquito) largely depends on differentiated protein expression. These proteins are responsible for malaria

pathogenesis. *Plasmodium falciparum* life cycle comprises of the following three stages: pre-erythrocytic, intra-erythrocytic and sporogonic stages as shown in Figure 1. To realize malaria elimination and eradication goal this cycle remains the potential target (Delves *et al*., 2012a). However, the parasite life cycle poses a major challenge to chemotherapy and chemoprophylaxis, and it's an obstacle to circumvent in order to unleash an effective malaria vaccine (Arama *et al*., 2015).

2.2.1. Pre-erythrocytic stage (Tissue schizogony)

During a blood meal, an infected female Anopheles mosquito inoculates sporozoites from its salivary glands slowly into the human host (intermediate host) blood circulation. Sporozoites reach the liver and invade the hepatocytes (Delves *et al*., 2012a; Guerrant *et al*, 2011). This invasion is facilitated by the thrombospondin domain receptors on the circumsporozoite protein and thrombospondin-related adhesive protein which bind specifically to the liver heparan sulfate proteoglycans. After 8-27 days sporozoites mature into schizonts which rapture releasing infective merozoites into the blood circulation (Ansbro *et al*., 2020).

2.2.2. Intra-erythrocytic stage (Asexual blood stage)

After replication in the liver, merozoites invade the erythrocytes through a complex process that entails specific molecular interaction of parasite surface protein receptors and erythrocyte ligands. Once in the erythrocytes, they transform into mature schizonts through ring and trophozoite stages 48 hours later (Ansbro *et al.*, 2020). Mature schizonts contains up to 20 merozoites each, they rapture releasing merozoites, some of which commit to sexual erythrocyte stages (gametocytes) and others reinvade more uninfected erythrocytes. This intra-erythrocytic cycle continues in a synchronous manner releasing uniform merozoites almost the same time of the day. Erythrocyte content that leaks after lysis induce the secretion of cytokines which causes the clinical signs and symptoms of the disease (Guerrant *et al.*, 2011). Gametocytogenesis occurs randomly and immature gametocytes (stage I-IV) sequester into internal organs for up to 12 days before they develop into mature gametocytes (stage V). Mature gametocytes re-enter peripheral blood circulation and are vital for bridging the infection to the next host through the female Anopheles mosquitoes (Gebru *et al*., 2017).

2.2.3 Sporogonic cycle

Some bloodstream merozoites ~1-5% differentiate into immature trophozoites (ring stage) which in turn transforms into the female and male gametocytes instead of developing into the schizonts (Delves *et al*., 2012a). Female Anopheles mosquito ingests the gametocytes (Macrogametocytes and Microgametocytes) into its midgut during a blood meal. In the midgut microgametocytes exflagellate into microgamete and macrogametocytes transform into macrogametes. Microgametes and macrogametes fuse forming zygotes (Delves *et al*., 2012a). Zygotes transform into ookinetes which invade the mid gut cell developing into oocyst. Mature oocyst rapture releasing numerous sporozoites which make their way to the salivary glands for infecting the next host. Sporogony stage takes 10-18 days and the sporozoites within the mosquito remains viable for 1-2 months (Gebru *et al*., 2017). Malaria cycle continues when the sporozoites are inoculated into a new susceptible human host by the mosquito (Guerrant *et al.*, 2011).

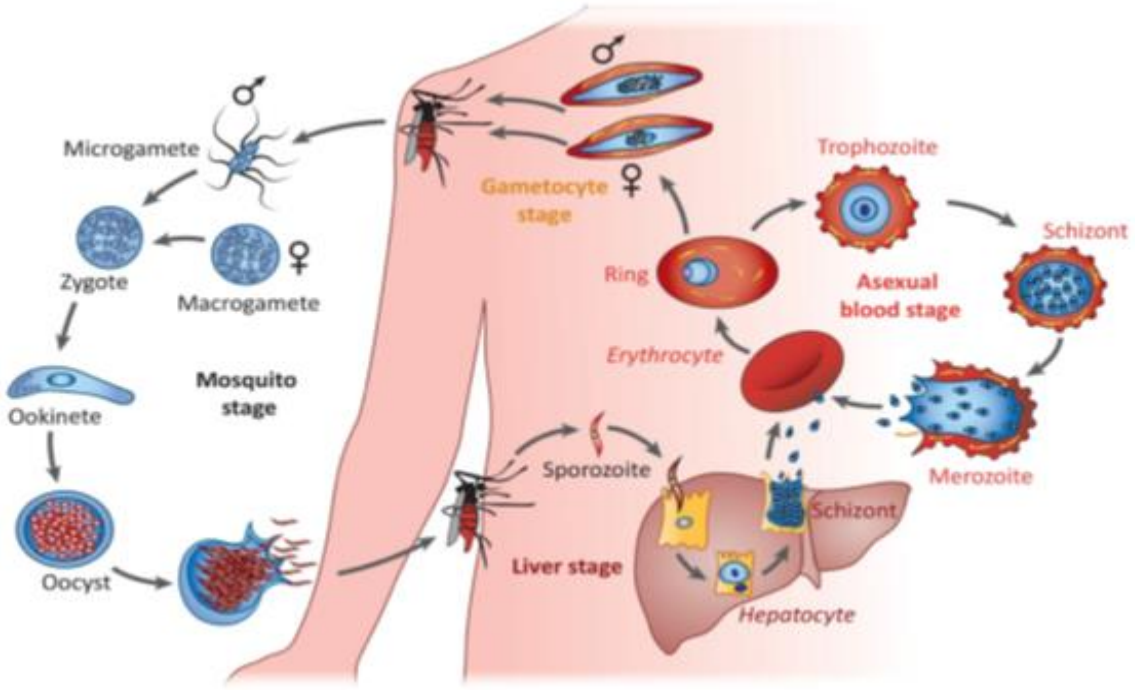

Figure 1: Outlines the life cycle of *P. falciparum* (Maier *et al*., 2019).

2.3. Diagnosis of *P. falciparum* malaria

Effective malaria treatment relies greatly on diagnosis, a main support of malaria control and elimination efforts (Achonduh *et al*., 2013). According to the WHO guidelines for malaria treatment, all suspected malaria cases should be tested, treated and monitored (WHO, 2021). Patients in malaria endemic regions or those with history of having visited the endemic areas and are presenting with fever, headache, chills and malaise are suspected to be infected with malaria parasites (WHO, 2015). Malaria diagnosis is based on clinical manifestations of the disease and parasite confirmatory tests. Microscopic examination is the gold standard method for malaria testing. However, other methods for malaria diagnosis exist and, they include; Clinical diagnosis, Immunochromatic technique (mRDT), serological method and parasite species specific PCR method. WHO recommends use of parasite confirmatory methods for diagnosis in order to avoid unnecessary treatment with antimalarial drugs hence providing improved management of non-malarial fevers, slow down evolution of resistant parasites and surveillance of treatment outcomes. In most African malaria screening sites Microscopy and malaria rapid diagnostic tests (mRDT) are the most common parasitological methods employed for malaria detection (Ngasala & Bushukatale, 2019).

2.3.1 Microscopy

It's a method of choice in malaria prone areas because of its cost effectiveness, specificity and sensitivity (50-500 parasites/µl of blood) (Ngasala & Bushukatale, 2019). Giemsa stained thick and thin blood films are used for parasites quantification, speciation and distinction between different parasite stages (Guerrant *et al.,* 2011). Giemsa stain component binds to the DNA phosphate group while other components stain the cytoplasm and nucleus differently making them distinct under the light microscope. Since the red blood cells lacks DNA, white blood cells DNA and *Plasmodium* infected red blood cells are stained. However proper identification of *Plasmodium* species depends on factors such as technical experience, proper stained slides, microscope maintenance and slide reading time. Despite microscopy being a gold standard method of diagnosis it is limited by: inability to detect low parasitemia in asymptomatic patients (10-30 parasites/µl of blood) and incorrect speciation (Achonduh *et al*., 2013). Microscopy is unavailable to some settings which have no access to electricity, clean water, standard reagents, laboratory facilities and trained personnel. In such settings, immunochromatic methods (mRDT) of detection are applicable (universal access).

2.3.2 Malaria rapid diagnostic tests (mRDTs)

This is an immunochromatographic technique used for rapid detection of specific *Plasmodium* antigens in finger prick blood samples. This rapid assay is designed to detect the presence of parasite antigens such as histidine rich protein-2 (HRP2), *Plasmodium* aldose and *Plasmodium* lactate dehydrogenase (pLDH) activity in blood sample after a few minutes (Kasetsirikul *et al.*, 2016). Monoclonal antibodies directed against the parasite antigen(s) are impregnated on the strip to detect *Plasmodium* antigens. There are three types of mRDTs namely; first, those which detect *P. falciparum* spp alone (Paracheck-Pf® and Paramax-3), secondly, those which detect non *P. falciparum* spp only (pan-LDH only RDTs) and lastly the combined RDTs which detect both *P. falciparum* and non-*P. falciparum* spp (Pan/*Pf* RDT, [Zephyr Biomedicals, Verna Goa, India]) (Gatton *et al*., 2020; Kasetsirikul *et al*., 2016). *P. falciparum* HRP-2 RDTs are the most preferred one because of their better sensitivity and thermo stability (Verma *et al*., 2018). This simple immunochromatic technique has increased access to parasitological malaria diagnosis due to its good sensitivity (100 parasites/µl of blood), applicability to wide range of setups from hospitals to remote field laboratories, minimum training is needed for users unlike microscopy (Achonduh *et al*., 2013), faster results and it is easy to handle (Verma *et al*., 2018). However, this method is limited by deletion of Hrp-2/3 genes giving false negative results, inability to quantify parasites and parasite antigen lingers in the circulation for up to two weeks after clearance giving false-positive results hence it should be used alongside microscopy (Berzosa *et al*., 2018; WHO, 2021). In most cases non-Pan/*Pf* mRDTs are used because they are cheap. Moreover, these HRP-2 mRDTs are limited to *P. falciparum* parasite leaving non-*P. falciparum* parasites undetected (Berzosa *et al*., 2018).

2.3.3 Quantitative polymerase chain reaction (qPCR)

It's a molecular based method that detects *Plasmodium* spp genetic material in patient's sample (blood, fecal, urine and saliva for *P. falciparum* and *P. vivax*). PCR method is based on a known oligonucleotide sequence (primer) annealing to the unknown DNA sequence targets of the parasite (18S ribosomal RNA [rRNA], mitochondrial DNA, cytochrome b) and subsequently amplifying them. Successful amplification signifies presence of the *Plasmodium* parasite (Kasetsirikul *et al*., 2016). This method is highly sensitive and specific; its detection range is between 0.5-10 parasites/µl of blood sample especially asymptomatic patients which test negative by microscopy. However, it is limited by need for a special equipment which is expensive and high level of training

required (Achonduh *et al*., 2013). Copy number variation in the target genes affects the sensitivity of PCR method greatly since sensitivity and specificity of this detection method depends on complementary nucleotides between the primer and target gene (Kasetsirikul *et al*., 2016). Additionally, this method does not offer rapid results as mRDT and microscopy and is not applicable in the field setting (Berzosa *et al*., 2018).

2.3.4 Serological method

This method detects specific antibodies that are produced by the human host against malaria parasites in the blood sample (Bell *et al*., 2016). Immunofluorescence antibody testing (IFA) is the widely used serological method for malaria diagnosis (Tangpukdee *et al*., 2009). IFA is widely used for screening blood donors to prevent transfusion of malaria infected blood. However, this method is not widely used for diagnosis of acute malaria infection since antibodies against the malaria parasite take long (within two weeks) to be secreted by the host. Once antibodies against malaria parasites are produced, they last for 3-6 months in circulation (Tangpukdee *et al*., 2009). Presence of antibodies in the host blood signifies past infection. IFA is sensitive and easy to perform. Moreover, it is time consuming because sample processing takes time and cannot be automated, relatively expensive, cannot be automated hence can be time consuming, it requires florescence microscopy and trained personnel (Tangpukdee *et al*., 2009).

2.3.5 Clinical diagnosis

This method relies on the clinical manifestations of the disease, namely, fever, headache, abdominal pain, nausea, vomiting, myalgia, sweats and fatigue. It is commonly applied in poor remote malaria endemic settings which are under resourced and lacks parasitological detection methods set up. This method results to presumptive treatment which sometimes might lead to overtreatment due to the nonspecific nature of the malaria signs and symptoms. Malaria can co-exist with other life-threatening infections and fevers might be due to other infections. However, this method is cheap, easy and fast to carry out (Ngasala & Bushukatale, 2019).

2.4. Pathogenesis of *P. falciparum* malaria

Malaria disease is classified into three categories, namely; asymptomatic, uncomplicated and severe malaria. The manifestation of each of these forms depends on the host immunity levels,

inherited host factors (human malaria resistance factors such as sickle cell trait, G6PD deficiency and Thalassemia) and age (Laishram *et al*., 2012).

2.4.1 Asymptomatic malaria

This form of the disease does not manifest with symptoms. However, it can be transmitted by mosquito to other hosts due to the presence of circulating parasites in the patient's blood. Sometimes asymptomatic cases display with undetectable parasites by microscopy (submicroscopic parasite level) (Laishram *et al*., 2012). The host immune system and other factors play an important role in suppressing clinical manifestation of this form of the disease (Chen *et al*., 2016). Asymptomatic malaria is common in malaria endemic regions. This form of the disease is majorly caused by *P. falciparum* but also other species, namely; *P. ovale* and *P. vivax* have been shown to cause asymptomatic malaria (Laishram *et al*., 2012).

2.4.2 Uncomplicated malaria

Unlike asymptomatic cases uncomplicated malaria is characterized by the following classical signs and symptoms; fever, headache, abdominal pain, nausea, vomiting, myalgia, sweats and fatigue. Fevers are associated with the intra-erythrocytic stage of the parasite life cycle (Milner, 2018). Patients with this form of the disease are tested and treated with the appropriate antimalarials (Milner, 2018). The sporogonic and pre-erythrocytic stages are not characterized by any symptom apart from the mild inflammation at the biting site during sporozoite injection by the mosquito. In *P. falciparum* fevers occurs after every 2 days (tertian) because it's intra-erythrocytic stage takes 48 hours. Sometimes these fevers can be irregular; they are as a result of the host immune response to the rupturing red blood cells. Malaria infection symptoms are non-specific; they are similar to those caused by diseases such as dengue, chikungunya, typhoid and bacterial septicaemia. However, this calls for diagnosis of the disease using the standard methods described earlier for effective treatment.

2.4.3 Severe malaria

This complicated form of the disease is fatal and can lead to death. Most severe malaria cases in endemic areas are caused by *P. falciparum*. Other *Plasmodium* species (*P. vivax* and *P. knowlesi*) can cause severe malaria (Guerrant *et al*., 2011). Symptoms for this disease manifestation include all the uncomplicated malaria signs and multi organ complications such as brain, kidney and

pulmonary system damage. It is characterized by metabolic acidosis, severe malaria and cerebral malaria. Severe malaria is caused by the intra-erythrocytic asexual stages of the parasites and 48 hours after disease presentation death occurs. Severe malaria pathogenesis is caused by; cytoadherence (adherence of the infected red blood cells to the endothelial cells of blood vessels including those in the brain leading to cerebral malaria) and sequestration of infected red blood cells into vital organs which affects blood perfusion leading to seizures, cerebral malaria, hypoglycemia, severe anemia, renal failure, metabolic acidosis, pulmonary edema. However, this disease can be treated by stage specific drugs such as quinine and artesunate.

2.5 Malaria prevention and control approaches

Prevention of malaria infections can be achieved through vector control, use of effective vaccines and chemoprevention approaches (WHO, 2018).

2.5.1 Vector control

The main goal of vector control is to reduce the chances of mosquitoes biting human host. Vector control is the main approach for preventing transmission of malaria in endemic sub-Saharan Africa region (World Health Organization, 2019). Effective vector control intervention for the risk group of people (pregnant mothers and children <5 years) in the population reduces malaria transmission and mortality greatly. Insecticide treated mosquito nets (ITNs) and indoor residual spraying (IRS) are the two main forms of vector control approach that depends on insecticides (WHO, 2021).

Insecticide treated mosquito nets (ITNs) offers a protective barrier against late night and indoor mosquito bites. WHO recommends that all people at risk of malaria should sleep under properly maintained long lasting insecticidal nets, to achieve this public health programs such as free distribution of nets to allow equal access have been implemented in malaria endemic areas (WHO, 2018). ITNs reduce the mortality of children aged 5 years by 80% in sub-Saharan high malaria transmission region (Guerrant *et al.*, 2011).

Indoor residual spraying (IRS) with insecticides also reduces malaria transmission greatly by targeting the indoor resting mosquitoes. This method is effective when 80% of the households in targeted areas are covered. Indoor spraying offers protection against mosquitoes for 2-3 months depending on the sprayed surface and the insecticide formulation. In some areas multiple spraying is conducted to offer protection for the whole malaria season. It is implemented where ITNs are

used routinely to prevent insecticide resistance; an insecticide with different mode of action to that used on ITNs is employed. Moreover, despite the great progress in reducing malaria burden in sub-Saharan Africa that is attributed partly to vector control, insecticide resistant mosquitoes have emerged posing a great challenge to this approach.

2.5.2 Chemoprevention

Antimalarial drugs are administered to vulnerable groups such as children aged less than 5 years to reduce malaria burden and transmission. This intervention suppresses blood stage malaria parasites preventing the disease. WHO recommends seasonal chemoprevention, where monthly doses of amodiaquine and sulphadoxine-pyrimethamine are administered to group of individuals living in the Sahel sub-region of Africa to prevent seasonal malaria transmission during high transmission season (WHO, 2018).

In sub-Saharan Africa malaria chemoprophylaxis intervention (intermittent preventive treatment) is used in pregnant women and infants. Intermittent preventive treatment in pregnant women (IPTp) involves administering curative doses of sulphadoxine-pyrimethamine (SP) starting from 2nd trimester at each antenatal visit to reduce severe anaemia in this group of women and low birth weight infants. Pregnant women in these high malaria transmission areas should receive at least three doses of sulphadoxine-pyrimethamine (SP) (WHO, 2019).

Chemoprophylaxis approach is also used in the protection of travellers against malaria. Travellers from malaria free region visiting malaria endemic areas are deficient of natural acquired partial immunity against malaria. Antimalarial drugs such as doxycycline are administered in doses of 100mg/day starting from one day before getting into malaria region, the whole period they are in the region and for 28 days after leaving the high malaria transmission region (Guerrant *et al.*, 2011). Malarone® (Atovaquone-proguanil) is also another malaria prophylactic regimen recommended for travellers from malaria free regions (Blasco *et al.*, 2017).

2.5.3 Vaccine development

Drug and insecticide resistance have emerged reducing the effectiveness of vector control and chemotherapy as the main tools for controlling malaria in sub-Saharan Africa. The recent recommendations by WHO to use RTS, S vaccine to complement the already existing tools of malaria control will ensure that malaria elimination goal is achieved (WHO, 2021). In this

approach malaria parasite antigens are introduced into the body, they stimulate the immune system to produce antibodies and T-cells against them (partial acquired immunity). This prevents invasion of the human host cells by the malaria parasites (Molina-Franky *et al*., 2020). Several potential malaria vaccines have been developed and are in the clinical trial. However, this process of development has taken more than three decades due to the challenges posed by; complex malaria parasite life cycle and inability of the malaria infections to confer sterile immunity (Molina-Franky *et al.*, 2020). Three distinct approaches based on the malaria parasite life cycle are key in the development of an effective malaria vaccine. From earlier research done by Hoffman and colleagues in 2002, it has been demonstrated that attenuated sporozoite stage confer immunity against malaria infection although this approach was not cost effective and applicable on large scale.

Today several improvements have been made on Hoffman's discovery to develop numerous potential malaria vaccines which are in the pipeline. Pre-erythrocytic vaccines target to block sporozoites from invading liver cells or destroying the infected liver cells. The main hurdle for these vaccines is the short time frame (<1 hour) taken by the sporozoites to reach the hepatocytes after being released by mosquitoes. Consequently, the immune system has limited time to eliminate these parasites. Moreover, most pre-erythrocytic vaccines are in phase I and II, only one vaccine (RTS, S/AS01 [mosquirix^{TM}]) was rolled out by WHO in 2021 for preventing malaria infections among children in sub-Saharan Africa (WHO, 2021).

RTS, S/AS01 (mosquirix^{TM}) is a recombinant vaccine developed by fusing sporozoite antigen (circumsporozoite protein) with the hepatitis B surface antigen to improve its immunogenicity. Furthermore, an adjuvant (A01S) was conjugated to the vaccine subunit to boost immune systems response to the antigen (Laurens *et al*., 2020). RTS, S is the most promising vaccine developed so far in the history of malaria vaccinology. The recent pilot studies in Malawi, Ghana and Kenya have proved that this vaccine offers 40% and 30% protection against uncomplicated and severe malaria respectively in children (WHO, 2021).

Blood stage vaccines targets to block rapid invasion and asexual reproduction of malaria parasites in the erythrocytes. Its goal is to reduce the number of merozoites invading the red blood cells

rather than completely blocking replication. Most of erythrocytic vaccines are in phase I and II and none of them have shown great success rates as RTS, S/AS01 vaccine (Targett *et al.*, 2015).

Transmission blocking vaccines (TBV) aims at the sexual stage reproduction of the parasite that occurs in the mosquito gut. *PfS25* antigen vaccine is an example of transmission blocking vaccine that is in the pipeline. Ideally this vaccine stimulates the immune system to produce antibodies against it; these antibodies are sucked by the mosquito taking a blood meal. In the mosquito gut the antibodies encounters the parasite and mosquito antigen preventing further development of the parasites and subsequently killing them (Targett *et al*., 2015). Efforts to develop a more effective vaccine than RTS, S/AS01 are on-going and this might call for combined multiple approaches; targeting antigens from all malaria parasite stages alongside the application of mathematical and computational biology tools (Frimpong *et al*., 2018).

2.6 Malarial chemotherapy

Plasmodium falciparum treatment guidelines vary geographically depending on the severity of the disease, cause and resistance patterns. Currently the frontline antimalarials globally for treatment of *P. falciparum* uncomplicated malaria includes the artemisinin combination therapies (ACTs) (WHO, 2021). The principle of ACTs involves using two drugs with different mechanisms of action. A rapid acting artemisinin derivative drug which has a short half-life is combined with a longer half-life partner drug (Martin *et al*., 2018). The first ACT to be synthesized was artemisinin-lumefantrine (Coartem®), it was developed by Prof Zhou Yiqing of China. The ACTs have impacted positively on the global malaria control efforts by reducing malaria burden since they were rolled out (Tse *et al*., 2019). However, *P. falciparum* parasites that are resistant to all known frontline antimalarials have been reported in some regions such as SEA and this poses a great concern to global malaria elimination plan since scarce antimalarials are available.

Antimalarial drugs are grouped into four categories; artemisinin derivatives/endoperoxides (artemether, arteether, artemisone, artesunate), antifolates (Pyrimethamine, sulphadoxine, proguanil, chlorproguanil, trimethoprim), hydroxynaphthaquinone (atovaquone) and quinolines (chloroquine, piperaquine, mefloquine, quinine, amodiaquine) (Guerrant *et al.*, 2011; Nsanzabana, 2019; Slater *et al*., 2021). Most of these drugs have been used as monotherapy (single drugs) in the past, but because of the wide spread antimalarial drug resistance, WHO recommends

combination therapy (Guerrant *et al.*, 2011). Combining two or more antimalarial drugs increases cure rates and scale down drug resistance development due to the different modes of action and unrelated biochemical targets (Muangnoicharoen *et al.*, 2009). Antibiotics (Sulfonamides and sulfones, tetracyclines, lincosamides, macrolides, and chloramphenicol) have antimalarial activity, but due to their slow action they are used in combination with antimalarial drugs or for chemoprophylaxis exclusively on visitors from malaria free regions (Guerrant *et al.*, 2011). In Kenya, artemether-lumefantrine (Coartem®) and dihydroartemisinin-piperaquine (Duo-cotecxin®) are the recommended first-line and alternative first-line regimens respectively for the treatment of uncomplicated *P. falciparum* malaria (ACTwatch group *et al*., 2017; Chebore *et al*., 2020).

2.6.1 Quinine sulfate

Quinine was the first antimalarial to be discovered on earth, it is an alkaloid molecule that was obtained from the bark of cinchona tree in 1820 by French scientist, Joseph Pelletier and Jean Biename Coventry (Achan *et al.*, 2009). It was used in the treatment of chills and fever. It belongs to the aryl-alcohol group of quinolines (Ménard *et al*., 2013). Quinine is an effective antidote against complicated and uncomplicated malaria. However, its widespread application in malaria case management has declined since the discovery of chloroquine, a more safer molecule (Guerrant *et al*., 2011). In the second trimester of pregnancy quinine remains the drug of choice for the treatment of uncomplicated malaria. In malaria endemic regions, quinine forms part of the second-line regimen for the treatment of uncomplicated malaria. Currently, quinine is used in the treatment of severe malaria, and as second-line treatment of multidrug resistant parasites in combination with antibiotics (Petersen *et al*., 2011; Tse *et al*., 2019). However, quinine resistant parasites have been reported in Southeast Asia, Africa and South America (Guerrant *et al*., 2011). Over ten years ago, QN resistance was reported in Uganda, Africa (Achan *et al.*, 2009). This catastrophic scenario and the adverse effect of QN have contributed to a shift of prescription towards DHA-PPQ regimen (Duo-cotecxin®) as a first-line alternative antimalarial for treating uncomplicated malaria in sub-Saharan Africa (Chebore *et al*., 2020).

a). Mode of action

Quinine acts on the erythrocytic stage of the malaria parasite and its mode of action is partially known. Quinine is a weaker base than chloroquine and its potency is augmented by the host

immune system. Similar to chloroquine it accumulates in the digestive food vacuole of the parasite thus inhibiting the heme detoxification process (Petersen *et al*., 2011). Additionally, earlier studies have demonstrated that quinine also inhibits parasite nucleic acid, protein synthesis and glycolysis metabolic pathways.

b). Resistance

Quinine resistance was first documented in Brazil over one century ago; subsequently it has spread to other malaria endemic regions notably Southeast Asia, South America and currently Africa (remains low). *Pfnhe*1 in chromosome 13 encodes for a transmembrane protein that is responsible for the efflux of hydrogen (H+) ions to maintain the neutral pH (7.4) within the parasite cell, this prevents acidification due to anaerobic glycolysis (Antony & Parija, 2016). Polymorphisms in *Pfcrt*, *Pfmdr1* and *Pfnhe1* transporter encoding genes have been associated with quinine resistance (Petersen *et al*., 2011). QN failure has also been reported in Africa, specifically Uganda (Achan *et al*., 2009).

2.6.2 Chloroquine

Chloroquine (CQ) is a 4-aminoquinoline antimalarial. It was first discovered in Germany in 1934 and synthesized by chemists. The efficacy of this molecule was further improved by the American scientists. Its use inclined during World War II by US Army soldiers (Ansbro *et al*., 2020). This drug is active against the blood stage parasites (blood schizonticide). Since its discovery chloroquine has been used in supporting global malaria elimination and eradication goal of WHO launched in 1955 (Petersen *et al.*, 2011). Malaria burden declined globally proving that this disease is treatable. Chloroquine is safe in all groups of people (pregnant women, children and adults), it's cheap, readily available and it can be used for prophylaxis and treatment of uncomplicated *P. falciparum* malaria (Ansbro *et al*., 2020).

a). Mode of action

Chloroquine is an alkaline molecule that enters the acidic food vacuole through simple diffusion physiologic process. Inside the food vacuole CQ is protonated to facilitate its accumulation (Saifi *et al*., 2013). This drug kills *Plasmodium* parasites by blocking haem detoxification step in the haemoglobin metabolism pathway (Guerrant *et al.*, 2011; Müller & Hyde, 2010). Asexual blood stage parasites digest haemoglobin to globin (amino acid source) and free haem (toxic) in the acidic

food vacuole. Haem is subsequently polymerized into a non-toxic, inert, crystalline hemozoin (malaria molecule) (Pussard & Verdier, 1994; Wellems & Plowe, 2001). Chloroquine binds to haem and adsorbs to the growing hemozoin forming toxic CQ-haeme complex which accumulates in parasite food vacuole destroying parasite membrane. This causes the lysis of infected red blood cells and subsequently destruction by the immune system. Haeme accumulation is thought to inhibit nucleic acid biosynthesis (Wellems & Plowe, 2001). Chloroquine increases the pH of the food vacuole, this denature the activity of proteases that digest amino acids sourced from host erythrocyte haemoglobin thus blocking protein metabolism by the parasite.

b). Mechanism of chloroquine resistance

Chloroquine (CQ) mechanism of resistance is well described globally (Bloland, 2001), it originated from Combodia-Thailand boarder in 1950s and spread further to other continents (Mita *et al*., 2009). In Africa it was reported in 1978 at the Eastern coast (Kenya-Tanzanian boarder) and later spread to the entire African continent (Petersen *et al*., 2011). CQ mechanism of resistance involves K76T point mutations and M74I, N75E, A220S, Q271E, N326S, I356T, R371I mutations in *P. falciparum* chloroquine transporter gene (*Pfcrt*) in chromosome 7. K76T point mutation is the key molecular maker for CQ resistance, this mutation causes a shift in amino acid from lysine (positively charged) to threonine (neutral), this affects the conformation of the *Pfcrt* protein facilitating the expulsion of positively charged chloroquine base from the food vacuole at a faster rate that cannot allow its accumulation to parasite inhibition levels (Bloland, 2001; Müller & Hyde, 2010).

Plasmodium falciparum multidrug resistance 1 gene (*Pfmdr*1) in chromosome 5 encodes for Pgh-1 P-glycoprotein transporter, pumps drugs into the food vacuole. *Pfmdr1* mutations (N86Y, S1034C, N1042D and D1246Y) hinder drug influx ability thus they are implicated for CQ resistance (Wellems & Plowe, 2001). However, *Pfmdr*1 polymorphisms alone are not adequate to confer chloroquine resistance but rather alter the degree of resistance expressed by resistant parasites harboring *Pfcrt* mutant. They occur due to parasite fitness cost (Martin *et al*., 2018).

2.6.3 Sulphadoxine and pyrimethamine (Antifolates)

Sulphadoxine-pyrimethamine (SP) combination was rolled out in 1970 for malaria chemotherapy and prophylaxis after CQ resistance (Nsanzabana *et al*., 2018). SP is a single dose, highly effective,

cheap and well tolerated drug combination that acts on the *P. falciparum* erythrocyte stage. Resistance to this regimen emerged rapidly after its roll out and currently it is mainly used for intermittent preventive malaria treatment in pregnancy and endemic areas where parasites are susceptible to it (Petersen *et al*., 2011).

a). Mode of action

These antifolates target the folate metabolic pathway of the parasite, which is vital in DNA biosynthesis (Müller & Hyde, 2010). These drugs are competitive inhibitors of selected substrates in the parasite folate biosynthesis pathway (Nsanzabana, 2019). They sequentially and synergistically block the activity of *dihydropteroate synthetase* (*DHPS*) and *dihydrofolate reductase (DHFR)* respectively, key enzymes in the pathway (Kasturi *et al*., 2012). This leads to inhibition of de novo synthesis of pyrimidine, purines and amino acids (glycine and methionine) which are essential for the parasite survival (Saifi *et al*., 2013).

b). Mechanism of resistance

Like CQ, SP resistance originated from SEA and spread to other malaria endemic regions, notably Africa through the Eastern coast (Mita *et al*., 2009; Nsanzabana, 2019). Resistance to SP is associated with the build-up of point mutations in *Pfdhps* and *Pfdhfr* genes that codes for folate biosynthesis enzymes *dihydropteroate synthetase* and *dihydrofolate reductase* respectively (Müller & Hyde, 2010). A combination of three point mutations (SNPs) in the *Pfdhfr* gene (N51I, C59R and S108N) and two point mutations (A437G and K540E) in the *Pfdhps* gene forms quintuple mutant markers for SP resistance surveillance (Nsanzabana, 2019).

2.6.4 Mefloquine

Mefloquine (Lariam®) is an aryl-amino alcohol quinoline that was discovered by the Walter Reed Army Institute of Research (WRAIR) just after the Vietnamese war (Trenholme *et al*., 1975). It was synthesized as a derivative of quinine and introduced in 1970s to treat chloroquine resistant *P. falciparum* parasites. Mefloquine has a longer half-life of up to 20 days thus it is recommended for both treatment and prophylaxis. However, 5 years later after its introduction first cases of resistance were reported in SEA (Trenholme *et al*., 1975; Tse *et al*., 2019).

a). Mode of action

Mefloquine targets the *Plasmodium falciparum* multidrug resistant 1 gene (*Pfmdr1*) transporter; it inhibits these transporters interfering with their physiological functioning thus blocking influx of useful molecules into the parasite digestive vacuole. Additionally there are suggestions that mefloquine targets other asexual parasite stage processes namely: haem formation and the cytoplasmic ribosome (Pf80s) (Petersen *et al.*, 2011).

b). Mechanism of resistance

Wild type *Pfcrt* and *Pfmdr*1 amplification have been implicated for mefloquine resistance: they enhance mefloquine influx into the digestive vacuole away from its cytosol target. Mutations in *Pfmrp*1/2 genes have been associated with decreased parasite susceptibility to Mefloquine: they facilitate increased export of glutathione-drug complex away from its cytoplasmic target (Martin *et al*., 2018). Chloroquine resistant parasites have reverted sensitivity to MQ, this postulates that the two drugs have opposing selective pressure on the parasite (Kasturi *et al.*, 2012).

2.6.5 Amodiaquine

Amodiaquine is a 4 amino quinoline pro-drug that was rolled out after its synthesis in 1948 to treat chloroquine resistant *P. falciparum* malaria (Tse *et al*., 2019). It remains to be effective against African parasites. The active metabolite of this drug is monodesethylamodiaquine (Sa & Twu, 2010). Additionally, amodiaquine was used for malaria chemoprophylaxis in travelers from non-malaria endemic regions. This drug was later withdrawn from the WHO list of essential medicines due to its adverse effects of hepatitis and agranulocytosis when used for long by some patients (Aweeka & German, 2008). Currently it is used in combination with artesunate (Camoquine®/Coarsucam^{TM}) to treat uncomplicated malaria in endemic regions of sub-Saharan Africa (Lacaze *et al*., 2011).

a). Mode of action

Amodiaquine has similar mechanism of action to chloroquine. Briefly, it inhibits the degradation of haemoglobin in the parasite food vacuole (Tse *et al*., 2019). Further, it has been suggested that this drug impedes the detoxification of ferriprotoporphyrin IX whose build up is detrimental to the malaria parasite (Aweeka & German, 2008).

b). Mechanism of resistance

Genetic studies in sub-Saharan Africa suggests a role of *Pfcrt* and *Pfmdr1* polymorphisms in amodiaquine resistance. Recrudescence in patients with parasites harboring *Pfcrt* T76 alongside *Pfmdr*-1 Y86 mutations has been reported after treatment with amodiaquine implying the role of these mutations in amodiaquine resistance (Tinto *et al*., 2008). *In vitro* amodiaquine resistant parasites have been shown to carry *Pfcrt* CVIENT haplotype and *Pfmdr*-1 Tyr 86 allele at the molecular level by a study in Nigeria (Folarin *et al*., 2011). Selection of parasites for SVMNT, SNPs from codon 72-76 in *Pfcrt* gene has been noted in areas where amodiaquine has been used in combination with artesunate suggesting evolution of resistance to the former drug (Sa & Twu, 2020). The markers highlighted here can be used for amodiaquine resistance surveillance in areas where the drug is used.

2.6.6 Lumefantrine

Lumefantrine also known as benflumetol is a synthetic aryl-amino alcohol drug with a longer half-life of 3-5 days (Petersen *et al*., 2011). This drug was first synthesized in 1979 under the "Chinese antimalarial project 523" that led also to the discovery of artemisinin (Tse *et al*., 2019). Studies in China, Gambia and Tanzania have reported good lumefantrine efficacy against chloroquine resistant parasites (Basco *et al*., 1998; Meier's *et al*., 2016). In Thailand, this drug has shown declined efficacy against *P. falciparum* compared to mefloquine. This drug has been in use for more than a decade as a first-line antimalarial in combination with artemisinin in most endemic areas. This combination has demonstrated continued efficacy contributing to the great gains that have been made towards malaria control. However, recently decreased lumefantrine efficacy has been reported in sub-Saharan countries namely; Angola, Nigeria, Zambia and Tanzania (Chebore *et al*., 2020; Dimbu *et al*., 2021; Ebohon *et al*., 2019; Ebong *et al*., 2021; Sitali *et al*., 2020).

a). Mode of action

The mechanism of action of this drug is not well understood. However, it is speculated that in addition to the unknown primary mode of LM action in the cytosol, it kills parasites by the following mechanism; lumefantrine acts by binding to β-hematin in the ratio of 1:2 to form μ-oxo dimer complex partially interfering with haem detoxification and haemoglobin degradation pathway (Wicht *et al*., 2020). Consequently, blocking the detoxification process and inhibiting nucleic acid and protein synthesis (Tse *et al*., 2019).

b). Mechanism of resistance

Declining lumefantrine efficacy has been linked to the mutations in the food vacuole Pfmdr1 transporter. *Pfmdr1* N86, 184F and D1246 mutations have been associated with lumefantrine resistance (Chebore *et al*., 2020; Malmberg *et al*., 2013; Sitali *et al*., 2020). These mutations enhance the transportation of LM away from its primary targets of action in the cytosol to the food vacuole (Wicht *et al*., 2020).

2.6.7 Antibiotics

Antibacterial drugs targeting protein and nucleic acid synthesis have antimalarial activity against *P. falciparum*. Most of these antibiotics have low antimalarial activity and they are used in combination with other antimalarial drugs except trimethoprim-sulfamethoxazole (Guerrant *et al*., 2011). Trimethoprim-sulfamethoxazole mode of action is similar to that of pyrimethamine and its continued use selects parasite against antifolates hence it is not recommended for malaria treatment. Other antibacterials with significant antimalarial activity include: Tetracycline (Doxycycline), Azithromycin, Chloramphenicol, Rifampicin, Clindamycin and sulfonamides (Guerrant *et al*., 2011).

2.6.8 Artemisinin derivatives

Artemisinin was discovered by the Chinese group of Scientists led by Prof YouYou Tau during the Vietnamese war. It was extracted from the Artemisia annua plant commonly known as wormwood plant by Chinese scientists in 1970s (Ansbro *et al*., 2020). Historically it was used to treat fever in China (Saifi *et al*., 2013). Artemisinin has been applied globally beyond China for the treatment of multidrug resistant *P. falciparum* malaria (Muangphrom *et al*., 2016). Artemisinin and its derivatives have a broad spectrum action and rapid therapeutic responses against the asexual parasite stages (Guerrant *et al*., 2011). Artemisinin derivatives are highly active against trophozoite stage due to the presence of haem (Blasco *et al*., 2017). Artemisinin and its derivatives (dihydroartemisinin, artemether and artesunate) have been used in combination with other antimalarial partner drugs, to form an ACT regimen that has demonstrated rapid parasite clearance. Success in reducing malaria burden in the last one decade has been attributed partly to deployment of ACTs in Malaria endemic regions as recommended by WHO (Blasco *et al*., 2017). However, reports of emergence of resistance to artemisinin in SEA and recently in East Africa (Rwanda and

Uganda) threaten the continued use of this magic drug (Balikagala *et al*., 2021; Duru *et al.*, 2015; Uwimana *et al*., 2021).

a). Mode of action

ACTs mode of action is not well established. Activation of the artemisisnin endoperoxide ring through the mitochondrial, haem-mediated degradation pathway and Cysteine protease hemoglobinases (falcipains) brings into play the activity of endoperoxides (Muangphrom *et al*., 2016). This generates reacting oxygen species (ROS) that induce alkylation of haem, proteins and lipids subsequently leading to parasite cytotoxicity through cellular damage and oxidative stress (Blasco *et al.*, 2017; Chebon *et al.*, 2016)

Artemisinin binds covalently to ornithine aminotransferase, pyruvate kinase, L-lactate dehydrogenase, Spermidine synthase and S-adenosyl-methionine synthetase) of the parasite inhibiting them thus interfering with proper functioning of metabolic pathways catalysed by these enzymes (Muangphrom *et al*., 2016). DHA mediates killing of the *Plasmodium* parasite by; blocking protein folding, unfolding and damaging proteins, and subsequently inhibiting proteasome degradation thus inducing endoplasmic reticulum (ER) stress, this leads to diminished translation (Bridgford *et al*., 2018). Continued induction of ER stress increases the build-up of polyubiquitinated proteins eventually resulting to cell death (Bridgford *et al*., 2018).

b). Mechanism of resistance

Mechanism of artemisinin (ART) resistance is not well understood (Müller & Hyde, 2010). Mutations in K13 protein domain are markers for ART resistance (Nsanzabana, 2019). They cause resistance by inducing cellular stress response that engages the response of unfolded proteins and ubiquitin proteasome pathway. This may lead to declined hemoglobin metabolism hence very low activation of artemisinin (Martin *et al*., 2018; Muangphrom *et al*., 2016). Parasite adjustment to stress at the ring stage prolongs its development period thus surviving the drug stress (Marti*n et al.*, 2018). Furthermore, K13 adapter mutations results to defective presentation of proteins for polyubiquitation, a marker of cell death hence enhancing parasite survival rate (Bridgford *et al*., 2018). Over ten single nucleotide polymorphisms (SNPs) in the *Pfk13* gene namely; F446I, N458Y, C469Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580, and A675V have been associated with artemisinin resistance (Balikagala *et al*., 2021; Uwimana *et al*., 2021). All these resistance markers have been reported in SEA, Similarly, R561H and P574L SNPS have

been noticed in Rwanda, and A675V and C469Y in Uganda in the recent past suggesting emergence of resistance to artemisinin in Africa (Balikagala *et al.*, 2021; Uwimana *et al*., 2021). Previous functional analysis studies argue that mutation in *Pfk13* gene reduces endocytosis of hemoglobin thus declining ART activation hence parasite resistance (Birnbaum *et al*., 2020). *Pfk13* mutants have been postulated to shorten the parasite trophozoite stage and prolong the ring stage, this has been associated with diminished drug exposure, hemoglobin degradation and subsequently decreased drug activation (Ross & Fidock, 2019). Mutations in genes encoding transporters on parasite food vacuole membrane have been associated with ART resistance (Muangphrom *et al.*, 2016). Mutated *Pfmdr1* (Y86N, Y1246D, Y184F) and *Pfmdr5* transports ART away from the cytosol (site of action) into the food vacuole making it unavailable to its target (Muangphrom *et al*., 2016).

2.6.9 Piperaquine

Piperaquine (PPQ) is a 4-amino bis-quinoline antimalarial compound (Delves *et al*., 2012b) with a very longer half-life (~30 days) than DHA (Muangnoicharoen *et al.*, 2009). PPQ is a highly lipophilic basic molecule with a molecular weight of 535.5 g/mole (Tärning *et al*., 2008) and structurally, it is an analog of chloroquine (Bawa *et al.*, 2010). This drug was first synthesized and used alone in China for the treatment and prevention of uncomplicated malaria on the wake of chloroquine resistant *P. falciparum* parasites (Tärning *et al*., 2008; WHO, 2010). Nonetheless, piperaquine resistance developed rapidly in less than a decade contributing to its ineffectiveness against *P. falciparum* (Muangnoicharoen *et al*., 2009). This led to a shift from monotherapy to combined therapy (ACTs) (Briolant *et al*., 2010; WHO, 2010). PPQ was combined with DHA that targets the asexual stage of the *P. falciparum* parasites and gametocytes (Delves *et al.*, 2012b; Liu *et al*., 2018). This combination has proved to be suitable and better than other antimalarial drugs (quinolines) in the control of CQ and mefloquine (MQ) resistant *P. falciparum* parasites (Bawa *et al*., 2010; Nsanzabana, 2019). Additionally, DHA-PPQ has proved to be effective in malaria prevention offering a prophylactic effect of 60 days (White, 2005). Today WHO recommends the use of dihydroartemisinin and piperaquine as a first-line and second-line drug for the treatment of *P. falciparum* uncomplicated malaria in endemic regions (Chebore *et al*., 2020).

a). Mechanism of action

Piperaquine mode of action is uncertain (Tarning *et al*., 2008). Nevertheless, it is proposed that piperaquine acts on *Plasmodium* parasites by blocking hemoglobin metabolism pathway

(Nsanzabana, 2019). Plasmepsin family of enzymes (aspartate proteases/hemoglobinases) breaks down hemoglobin to amino acid (globin) and a toxic compound haem (haematin) (Martin *et al*., 2018). Haem is further polymerized to non-toxic hemozoin (malaria molecule) by this group of enzymes (Martin *et al*., 2018). Lipophilic PPQ base readily penetrates through parasite food vacuole membrane and its accumulation here blocks one or more steps in hemoglobin degradation subsequently leading to; buildup of free haem, undigested hemoglobin in the digestive food vacuole leading to inhibition of haem detoxification pathway (Blasc*o et al.,* 2017*;* Martin *et al*., 2018).

b). Mechanism of resistance

PPQ resistance first emerged in China, and despite its wide spread use, its mechanism of resistance is not well elucidated (Kiboi *et al*., 2014; Muangnoicharoen *et al*., 2009). Piperaquine resistance is caused by mutations in more than one gene (multifactorial) (Kiboi *et al*., 2014; Martin *et al*., 2018). Amplification (overexpression/copy number variation) of *Plasmepsin*-2 (*Pfpm*2) and *Plasmepsin*-3 (*Pfpm*3) genes has been implicated in PPQ resistance (Leroy *et al*., 2019; Martin *et al., 2018)*. Duplication of these genes increases hemoglobin degradation resulting to peptide derived amino acids hence protein synthesis (Blasco *et al*., 2017), this incapacitates piperaquine ability to block hemoglobin degradation and haem detoxification (Amato *et al*., 2017). A recent study by Leroy and co-workers established increasing prevalence of *Pfpm*2 amplification in Africa suggesting PPQ resistance (Leroy *et al*., 2019). Furthermore, it has been hypothesized that polymorphisms in *Pfmdr1* and *Pfcrt* causes PPQ resistance. Single copy number variation in *Pfmdr1* (Duru *et al*., 2015) and SNPs in *Pfcrt* (H97Y, F145I, M343L, C350R and G353V) reduces the accumulation of PPQ in the food vacuole to effective levels that can block heme detoxification process (Martin *et al*., 2018). *Pfcrt* mutations have been thought to cause resistance by facilitating increased efflux of PPQ out of the digestive food vacuole and this decreases the inhibition of heme detoxification process and sensitizes the parasite to chloroquine (Ross & Fidock, 2019). A previous study has demonstrated that not all PPQ resistant *P. falciparum* parasites have single copy of *Pfmdr*1 on their genetic background (Ansbro *et al*., 2020). Mutations (SNPs) in *PfK*13 propeller domain (C580Y, I543T, P553L, R539T, Y493H and P574L) decreases parasite sensitivity to DHA leading to delayed parasite clearance (Duru *et al*., 2015). However this markers have not been reported in Africa (Leroy *et al*., 2019). Additionally, exo-nuclease gene E415G, a non-synonymous SNP has been implicated in increased PPQ IC_{50}. Nevertheless, the prevalence of this

SNP is increasing in Cambodia (Amato *et al.*, 2017), and in Mali it has been detected in a few field isolates (Diakite *et al.*, 2019). In Cambodia a study demonstrated that the presence of *Pfcrt* and *Pfexo* E415G SNPs alone confers PPQ resistance without *Pfpm2* amplifications (Boonyalai *et al*., 2020). *Plasmepsin*-2/3 gene disruption and overexpression studies have shown increased parasite susceptibility to PPQ and no effect on PPQ sensitivity respectively (Mukherjee *et al*., 2018). However, *Plasmepsin*-2/3 amplification on the background of parasites harboring *Pfcrt, Pfmdr1* and *Pfexo* polymorphisms confers PPQ resistance although its role in PPQ resistance modulation is not well known (Silva *et al*., 2020), and it has been suggested that amplification of *Plasmepsin*-2/3 genes generates low levels of PPQ resistant parasites and catalyzes the evolution of parasites with high grade resistance mediated by *Pfcrt* mutations (Ross & Fidock, 2019).

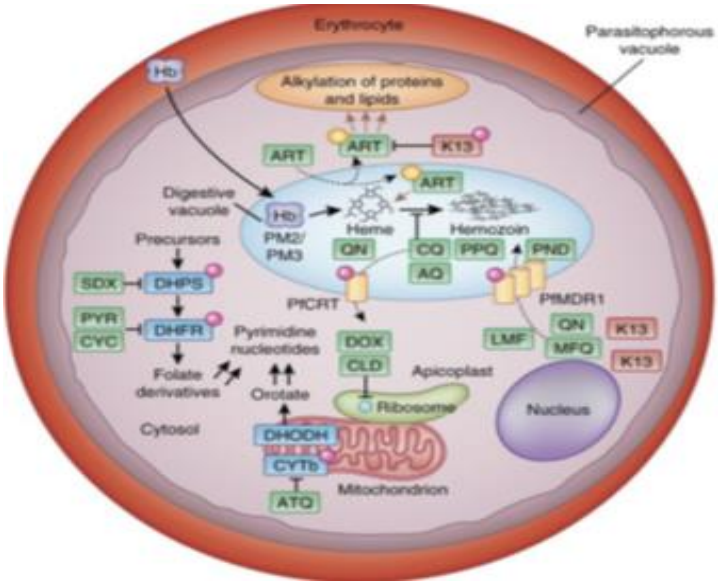

Figure 2: An infected human erythrocyte cell showing different antimalarials and their proposed mechanism of action against the asexual blood stage of *P. falciparum* parasite. Hemoglobin degradation process is shown in the parasite digestive vacuole (light blue). Standard antimalarials are shown in green near their proposed site of action. Chloroquine (CQ) and piperaquine (PPQ) are shown to be interfering with haem detoxification. *Pfcrt* (yellow) is shown on the digestive vacuole membrane with a mutation (red circle) that enables the efflux of CQ. *Pfmdr1* (yellow) is also shown on the digestive vacuole membrane (with increased copies) transporting mefloquine (MQ) into the vacuole. Artemisinin (ART) is depicted in its proposed mode of action that causes oxidative stress and alkylation of proteins and lipids (Wicht *et al*., 2020).

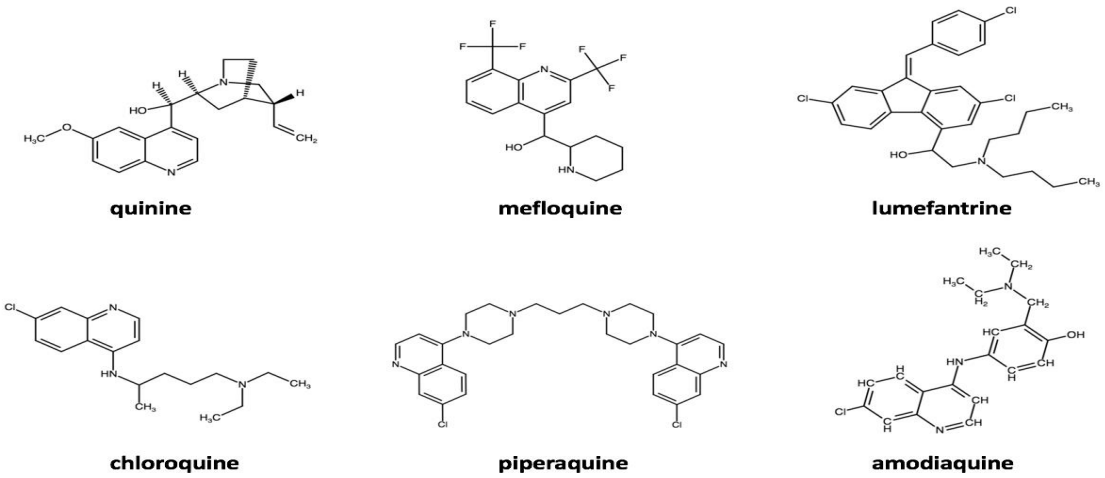

Figure 3: Shows different **s**tructures of antimalarial drugs and how they are related to each other (Ansbro *et al*., 2020).

Quinine was the first antimalarial drug to be discovered. It belongs to a class of antimalarials called quinolines. The 4 amino quinolines (chloroquine, amodiaquine and piperaquine) and aryl amino alcohols quinolines (lumefantrine, and mefloquine) were derived from quinine. Amodiaquine is an analog of chloroquine, and piperaquine is similar to two chloroquine molecules joined together (Figure 3) (Wiesner *et al*., 2003).

2.7. *In-vitro* drug sensitivity assay using SYBR Green 1 based method

This assay has been used universally alongside molecular analyses in monitoring *in-vivo* drug resistance. These assays exposes the parasites to drugs to determine their sensitivity and then later measuring drug inhibitory concentrations (IC_{50}s) that blocks half of the parasites from developing to mature schizont stage (Akala *et al*., 2011). This technique is most convenient, rapid, reproducible, cheap, less hazardous and less cumbersome hence preferred to the earlier used hypoxanthine uptake assay and very sensitive (can detect up to 0.019% parasitaemia) (Akala *et al.*, 2011; Cheruiyot *et al*., 2016). Inclined piperaquine (PPQ) IC_{50}s has been reported with isolates from DHA-PPQ treatment failures (Duru *et al*., 2016).

This assay uses a DNA fluorescent dye that binds to the parasite guanine (G) and cytosine (C) base pairs of the double stranded DNA. The SYBR Green I dye intercalates into the DNA after incubation with lysis buffer and the sample in the dark. The intercalated dye absorbs and emits light at different wave lengths indicating parasite replication activity (Duru *et al*., 2015).

Fluorescent intensity correlates with amount of DNA in cultures (Nsanzabana *et al.*, 2018). Lysis buffer destroys the RBCs and hemoglobin to improve the fluorescence ability of the dye *(Akala et al*., 2011). However, this method is limited by low fluorescence rates, high background noise (Derry *et al*., 2015), and lack of cut off values for defining PPQ resistance (Duru *et al.*, 2015). Non-interpretable dose response curves have been commonly reported with PPQ, IC_{50}s values range for PPQ resistance overlaps with PPQ sensitive parasites hence rendering this assay undependable for determining PPQ resistance (Boonyalai *et al*., 2020). However, these limitations can be circumvented by employing a new and robust assay known as the piperaquine survival assay (PSA) (Duru *et al*., 2016).

2.8. Piperaquine Survival Assay (PSA)

This is a new and robust drug inhibitory assay that examines susceptibility of the entire *Plasmodium* parasites asexual blood stage to PPQ (Duru *et al*., 2015). It entails two types of assays; the *in-vitro* and *ex-vivo* piperaquine survival assays. These assays mimic the *in-vivo* exposure of the parasites to PPQ by subjecting them to 200 nM of the drug for 48 hours and then assessing their survival 24 hours later. *In-vitro* PSA is conducted on 0-3-hour post-invasion tightly synchronized rings obtained from culture adapted parasites whereas ex-vivo PSA is performed on parasite isolates collected immediately from patients infected with *P. falciparum* malaria *(*Duru *et al*., 2015).

This assay adequately distinguishes PPQ resistant parasites from sensitive ones, with PSA $\geq$ 10% defined as the cut-off value for piperaquine resistance (Duru *et al*., 2016). It is superior to therapeutic efficacy studies and IC_{50}s methods of parasite drug susceptibility detection since it adequately distinguishes recrudescence from reinfections and rapidly detects PPQ resistance in real time. It has been used in Combodia as main support for the prediction of DHA-PPQ resistant markers and evaluation of drug efficacy. Alongside genotyping, It has proved to be a powerful and convenient tool for monitoring PPQ resistance where DHA-PPQ is used for chemotherapy (Duru *et al*., 2016). Nonetheless, PSA is not designed to determine susceptibility of different stages of the parasite through drug pulsing (Duru *et al*., 2015).

2.9 Estimation of *Pfmdr1, Pfpm2,* and *Pfpm3* copy number variation

Quantitative PCR detects target DNA in real time using a reporter dye as it is synthesized (Ansbro *et al*., 2020). TaqMan Realtime PCR methods using QuantStudio 6 pro real-time PCR system (Applied Biosystems Inc., Foster City, CA) is employed to assay for copy number variation (Lim *et al*., 2009). *Pfmdr1, Pfpm2/3* gene and single copy reference gene *P. falciparum* β-tubulin (normalizer) are amplified in triplicates using the hydrolysis TaqMan probes and oligonucleotide primers specific for the two genes respectively (Costa *et al*., 2017). *P. falciparum* reference strains 3D7 and Dd2 with *Pfmdr1* single copy number and multiple copies respectively are used for validating the assay. Amplification of the target gene and reference gene is done at the following PCR cycling conditions: initial step of 95^{o}C for 5 minutes followed by 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for 60 seconds (Ansbro *et al*., 2020). Amplified samples amplicons are analyzed with SDS software (version 2.0.6; Applied Biosystems Inc., Foster City, CA) (Ngalah *et al*., 2015), using the relative quantification method, $2^{-\Delta\Delta Ct}$ (Boonyalai *et al*., 2020). $\Delta\Delta Ct = (Ct_{TE} - Ct_{HE}) - (Ct_{TC} - Ct_{HC})$, where T is the test gene (either *pfmdr1*or *Pfm2/3*), H is the reference gene (β-tubulin), E is the experimental sample, and C is the control sample (3D7 single copy control) is used in the estimation of *Pfmdr1* copy number variation (Ansbro *et al*., 2020). Results are interpreted based on the relative quantification cut-off value to distinguish between amplified and single copy number (Costa *et al*., 2017). Samples with copy number ≥1.5 are interpreted as true amplification (Gupta *et al*., 2017, 2020). Unlike traditional methods of copy number variation detection, TaqMan real-time PCR is fast, simple and can be used to obtain specific, reproducible and easily interpretable results (Boonyalai *et al*., 2020).

2.10. SNP Analysis by Iplex sequenom MassARRAY technique

This assay is a multiplexed PCR and quantitative mass spectrometry based, it can be used to detect all the SNPs in *P. falciparum* genome (Chebon *et al*., 2016). Iplex massARRAY technique has also been applied in oncology, gene expression studies, methylation analysis, copy number variant analysis and molecular pathogen typing. This technique detects drug resistance markers (SNPs, CNVs) based on the following principle (Ellis & Ong, 2017).

2.10.1 Principle of Iplex MassARRAY platform

This genotyping platform is based on PCR reactions and matrix assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) (Chebon *et al*., 2016). The primary

PCR amplifies regions/ loci containing the SNP. The secondary PCR reaction extends the primer whose 3' end anneals to base proximal to the SNP site by a single mass modified terminator nucleotide (ddNTPs) complementary to the polymorphic site, the terminator base lacks a 3' hydroxyl group thus prevents further amplification of the DNA fragment (Ellis & Ong, 2017). The resulting multiplex product is dispensed onto the SpectroCHIP array using the Agena Bioscience RS1000 Nanodispenser robot. The analyte mixes with the matrix at the spectroCHIP spot. The spectroCHIP array is then transferred to the massARRAY mass spectrometer, UV light laser is fired at each spectroCHIP spot containing the co-crystallized matrix-analyte mixture leading to desorption and ionization. This soft ionization technique allows for the analysis of large, non-volatile and temperature sensitive biomolecules such as nucleic acids, proteins and inorganic substances. Processes such as excited proton transfer, ion molecule interaction and desorption of pre-formed ions causes Ionization and desorption (Nyasinga *et al.*, 2019). The resulting positively single charged gaseous DNA ions move upwards through a high voltage electric field vacuum to the detector based on their mass to charge ratio values (m/z) (Ellis & Ong, 2017). Lighter ions reach the detector faster than the heavier ones hence described as 'electrophoresis in a vacuum'. The time of flight of the analyte is recorded and is used to calculate its mass and further identify the added nucleotide base (Oeth *et al*., 2007). This assay is robust, cost effective and it gives a high throughput because large number of samples with different SNPs up to 40 targets per pool/reaction can be analyzed at the same time (Chebon *et al*., 2016). The workflow of massARRAY technique is shown in Figure 4 below.

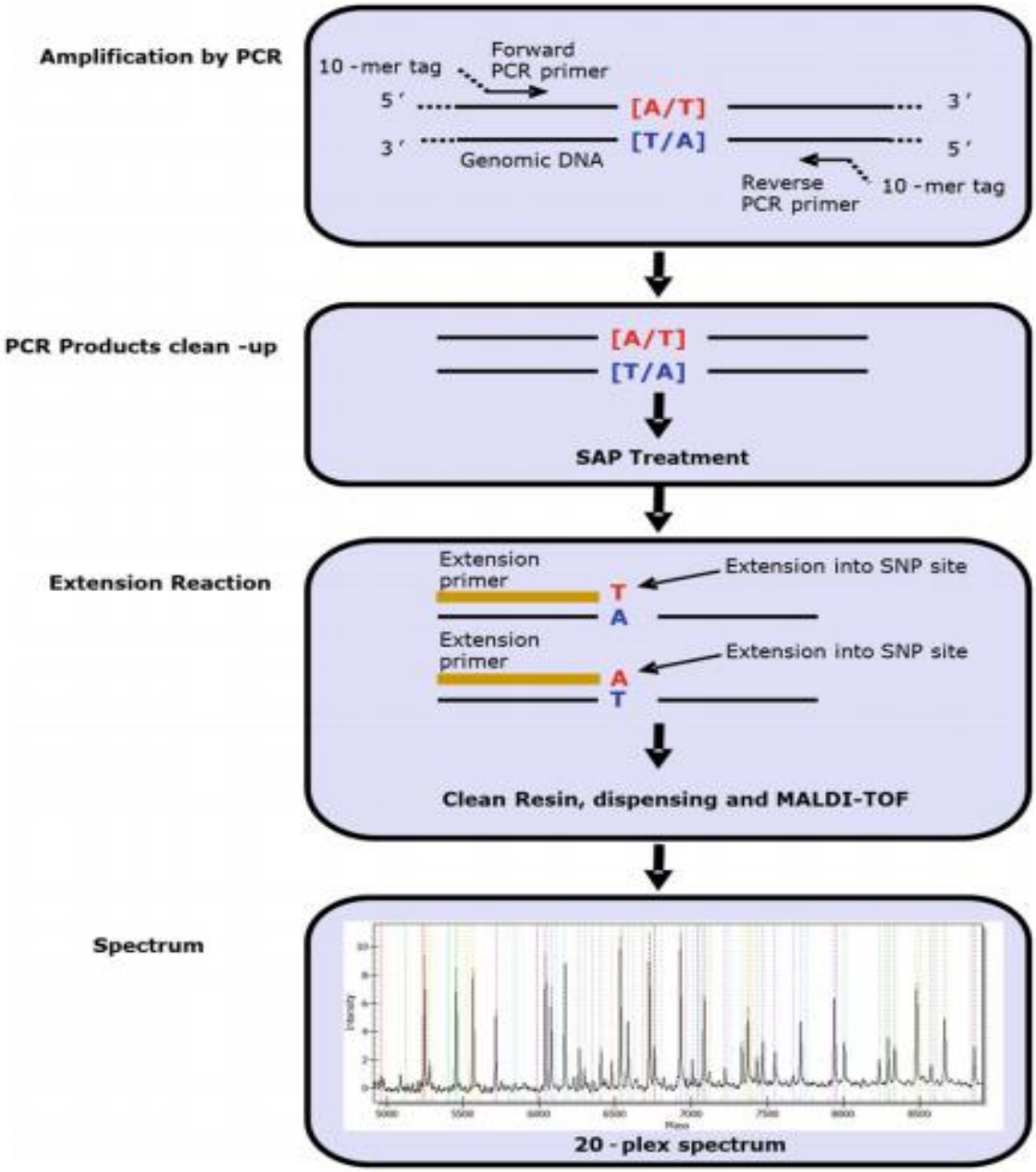

Figure 4: Steps involved in the generation of SNP genotypes using sequenom iPLEX massARRAY chemistry (Ellis & Ong, 2017).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study design and subjects

This was a retrospective and prospective study that leveraged on archival (2008-2018) and freshly collected samples (2019-2021), respectively. The study samples were collected under ongoing epidemiology of Malaria and drug sensitivity patterns study. These samples were assessed for *P. falciparum* susceptibility and putative mutations associated with piperaquine (PPQ) resistance using the growth inhibition and genomic assays, respectively.

Individuals aged ≥ 6 months, presenting at the health care facilities outpatient department with symptoms consistent with uncomplicated malaria and confirmed positive for *Plasmodium falciparum* by malaria Rapid Diagnostic Test (mRDT) (Parascreen Pan/Pf; Zephyr Biomedicals, Verna, Goa, India) or microscopy were enrolled into the study upon obtaining a written informed consent. Written informed assent for individuals under 18 years was obtained in accordance with the laws of the government of Kenya. Patients' information including age, gender, place of birth, place of residence, occupation, malaria treatment and travel history two months prior to the enrollment time were recorded in the case report forms. Further, artemether-lumefantrine (Coartem®) was administered to the patients after sample collection.

3.2 Study sites

This study was conducted at Malaria Drug Resistance Laboratory (MDR) of the USAMRD-A in Kisumu County. Study samples were collected from Kenya Ministry of Health (MOH) sentinel hospital sites located in four of Kenya's five different malaria ecological zones, namely; the Lake endemic region (Kisumu East County and Kisumu West sub-county hospitals), Coastal endemic region (Malindi sub-county hospital), highland epidemic prone areas (Kericho and Kisii county referral hospitals), and semi-arid/seasonal prone area (Marigat sub-county hospital) as shown in (Figure 5).

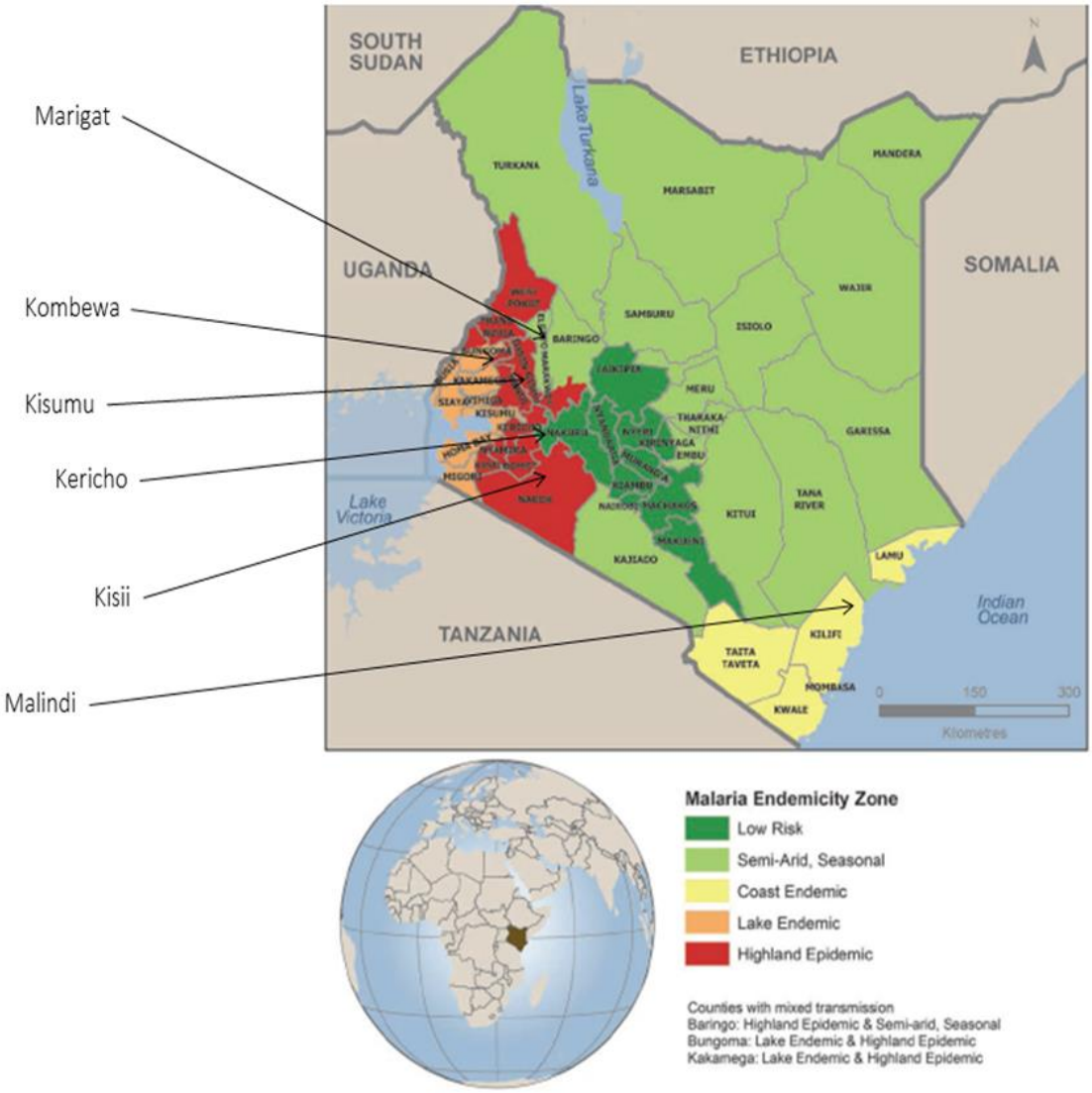

Figure 5: Map of Kenya depicting six Hospital surveillance sites (Malaria indicator survey, 2020)

3.3. Eligibility criteria

Parameters including type of infection, temperature, fever, anemia were considered for participation in the study

3.3.1 Inclusion criteria

An individual was eligible to participate in the study if he or she harbored *P. falciparum* mono-infection, has temperature ≥ 37.5°C and history of fever 24 hours before presentation. All gender including pregnant women after consenting were allowed to participate in the study.

3.3.2 Exclusion criteria

Individuals with any of the following characteristics were ruled out of the study. These included; unwillingness to give blood samples, children < 18 years without a parent or guardian to give a consent, severe anemia patients, infants > 6 months but weighing below 5 kg, anyone deemed unable to participate based on clinician's evaluation, or having mixed or non-*Plasmodium falciparum* infected patients.

3.4 Sample size determination

The minimum number of participants required in this study were determined using the Cochran formula (Chaokromthong & Sintao, 2021).

Cochran formula, $N= \frac{z^2 pq}{e^2}$

Where n = sample size

z = Standard error for mean at 1.96

p= estimated prevalence of PPQ resistance

q = 1-prevalence

e = margin of error at 5%

Prevalence of PPQ resistance is estimated at 50%. Substituting the values into the formula, the minimum number of persons required was 385 i.e.,

$n= \frac{z^2 pq}{e^2}$ ……. (1)

$n= \frac{1.96^2 x0.5x0.5}{0.05^2}$…… (2)

n=385

To cater for any limitation with sample integrity during collection, transportation, or processing, up to additional 5% persons were included.

3.5. Sample collection and preparation

About 2-3 mL venous blood sample was collected from each individual who tested positive for malaria prior to initiation of treatment in accordance with the Kenya Ministry of health guidelines on case management of uncomplicated malaria as previously reported (Akala *et al*., 2021). The venous blood sample was distributed in different tubes as follows, 0.5 mL in acid citrate dextrose

(ACD) tube (Becton-Dickinson, Franklin Lakes, NJ, USA) for immediate *ex vivo* growth inhibition assay and culture adaptation, 0.5 mL in sodium heparin tube (Becton-Dickinson, Franklin Lakes, NJ, USA) for pharmacokinetic studies, 1 mL in Ethylenediamininetetraacetic acid (EDTA) tube (Becton-Dickinson, Franklin Lakes, NJ, USA) for leukocyte depletion and genomic analysis, 2 drops on each of the two glass slides for microscopic parasite examination, and three blood spots of each 100 µL on FTA filter paper (Whatman Inc., Bound Brook, NJ) for DNA extraction and genomic analyses (Akala *et al*., 2011). Collected samples were transported to the central laboratory for processing, culture adaptation, genomic, growth inhibition assays, and storage.

3.6 Bioassays and reagent preparation

This section briefly describes reagents, blood samples and drug preparation procedures, drug and *P. falciparum* control strain sources and the *in vitro*/ *ex vivo* drug susceptibility assays.

3.6.1 Reference *Plasmodium falciparum* strains

The following parasite strains were obtained through BEI Resources, NIAID, NIH: *Plasmodium falciparum*, Strain 3D7, MRA-102, contributed by Daniel J. Carucci; Strain D6, MRA-285, and Strain W2, MRA-157 contributed by Dennis E. Kyle.

3.6.2 Reference antimalarial drugs

The reference antimalarials drugs piperaquine (PPQ), dihydroartemisinin (DHA), lumefantrine (LM), artemether (ART), and chloroquine (CQ) were donated by the WorldWide Antimalarial Resistance Network (WWARN) External Quality Assurance Programme, Bangkok, Thailand (Lourens *et al*., 2010).

3.6.3 Reagent preparation

a) Preparation of plain medium

To prepare 1 L, 5.94 g HEPES, 2 g glucose and 10.4 g 1640 Rosewell Park Memorial Institute 1640 (RPMI) were added to the beaker then topped up to 1 L with distilled water. Stirring to mix then filtration using 0.2 µl pore size filter unit was done followed by storage at 4°C for two weeks.

b) Preparation of 5% D-sorbitol

D-sorbitol (50 g) was dissolved in 1 L of distilled water, sterilized using 0.22 µm pore size filter unit and stored at 4°C.

c) Preparation of percoll solution

Stock solution of percoll was mixed with 10X PBS in the ratio of 9:10 to prepare 90% percoll. Further, 75% percoll was made by mixing RPMI and 90% percoll in the ratio of 1:5. It was stored at 4℃ up to two months.

d) Preparation of 10% giemsa stain

Giemsa stain was mixed thoroughly with working buffer (1x PBS) in the ratio of 1:9 and was used within 6 hours after preparation.

e) Preparation of PBS (Working buffer)

To prepare this, 36.38 g sodium phosphate monobasic and 59.29 g of sodium phosphate dibasic were added to a beaker then 1 L of distilled water was added followed by mixing thoroughly. The resulting solution was mixed with distilled water to make 1X PBS in the ratio of 1:99 following the manufacturer's protocol.

f) Preparation of complete medium with serum

This was prepared as described in Table 1. These contents were mixed thoroughly to dissolve in distilled water using a magnetic stirrer, and subsequently filtered using 0.2 µl pore size filter unit. This was stored at 4℃ for up to two weeks.

Table 1: Volumes of reagents for one Litre of culture medium

Reagent	Stock solution	Volume/weight
RPMI 1640+HEPES+ Glucose		42.4 Ml
Hypoxanthine	1.45 Mm	24 Ml
Gentamycin	10 mg/Ml	2 mL for 20% CMS
Sodium bicarbonate	75%	38.4 Ml
Heat inactivated ABO serum		120 mL and 240 mL for 10% and 20% respectively

g) Preparation of Lysis buffer containing SYBR green

About 15.76 g of TRIS HCL was dissolved in 700 mL distilled water. The solution pH was adjusted to 7.5 using concentrated HCL. Further, 160 mg saponin and 16.0 mL triton X-100 were added respectively, and the volume was adjusted to 1 L. Particulate matters were removed by filtration using 0.2 µl pore filter unit. The filtrate was stored at room temperature for up to 6

months. SYBR Green 1 (10 µL) was thawed and added to 11 mL of lysis buffer followed by thorough mixing in the dark. This was adequate for terminating one plate of 96 wells.

3.6.4 Parasite preparation

Parasite isolates from naturally infected malaria patients was collected and transported to MDR laboratory where parasitaemia was evaluated by microscopy. Acid citrate dextrose (ACD) vacutainer tube (Becton-Dickinson, Franklin Lakes, NJ, USA) samples were transferred to 15 mL falcon tubes then centrifuged at 2500 rpm for 3 minutes. The supernatant (plasma and buffy coat) was removed and then 5 mL of plain media was added to resuspend the pellet. The later step was repeated twice. The infected red blood cells pellet with a parasitaemia of 1% at 2% haematocrit was ready for immediate *ex vivo* drug testing. Uninfected red blood cells and 40 mL of 10% complete media with serum (CMS) was added to parasite isolates with >1% parasitaemia to lower the parasitaemia to 0.5-1% at 2% haematocrit before testing.

Archival parasite field isolates and laboratory reference strains, namely; 3D7- chloroquine sensitive, D6- chloroquine sensitive, and W2- chloroquine resistant were retrieved from the liquid nitrogen archive. They were further, thawed and maintained in 20% complete tissue culture media consisting of 10.4 g RPMI 1640 medium [Gibco BRL, United kingdom], 25 mM HEPES, 5.5 mM D-glucose, 10 mM Hypoxanthine, 7.5% sodium bicarbonate, 15% (vol/vol) heat-inactivated pooled ABO human sera and 0.1µl/mL; gentamycin) per liter of distilled water, adjusted at 2% hematocrit with uninfected human red blood cells and maintained at 37°C, 5% CO_2, 5% O_2, 90% N_2 and humidified environment as earlier described (Akala *et al*., 2011; Boonyalai *et al*., 2020; Duru *et al*., 2015). The cultures were monitored at 24 hour intervals till attaining 3-8% parasitaemia. Cultures that could not attain this parasitemia were considered unsuccessful (Duru *et al.,* 2017; Hao *et al*., 2013).

3.6.5 Drugs

Malaria SYBR Green I IC_{50} based *in-vitro* and *ex-vivo* drug sensitivity assay was used to test *P. falciparum* field isolates and reference clones sensitivity against five selected antimalarial drugs procured as piperaquine (PPQ), dihydroartemisinin (DHA), lumefantrine (LM), artemether (ART), and chloroquine diphosphate (CQ).

3.6.6 Drug reconstitution for malaria SYBR Green I IC_{50} based sensitivity assay

Stock drug solutions at 1 mg/mL were prepared by dissolving 5 mg of drug in 5 mL of 100% dimethyl sulfoxide (DMSO) for ART, LM, DHA and PPQ or 1.5 mL of deionized water plus 3.5 mL absolute ethanol for CQ. Moreover, concentrated drugs (1 mg/mL) were diluted further to desired starting concentrations in 10% CMS, followed by 2-fold serial dilutions to generate 12 concentrations for IC_{50} evaluation on 96 well plates (mother plates). Starting concentrations (ng/mL) from highest to lowest were as follows; ART (200-0.381), LM (200-0.381), DHA (200-0.381), PPQ (500-0.976) and CQ (2000-3.906). The drug plates were stored at -80°C up to 1 month or 12.5 µl from each well of the respective drugs were transferred to new plates to form daughter plates (pre-dosed plates) for immediate drug testing.

3.6.7 Piperaquine/Dimethyl sulfoxide control reconstitution for Piperaquine Survival Assay (PSA)

A PPQ salt of 5 mg was suspended in 1 mL 89.5% DMSO then vortexed to dissolve completely; a 5,000,000 nM stock solution was obtained. A stock solution of 20 µl was diluted further in 180 µl of 10% CMS to lower concentration to 5 000 nM. Aliquot of 20 µl of 5 000 nM PPQ solution was then diluted with 4.8 mL of 10% CMS to make 2 000 nM PPQ solution. Drug Aliquots of 400 µl in cryotubes were used immediately or stored at -80°C for up to 6 months.

Dimethyl sulfoxide (DMSO) [Sigma-Aldrich, ST. Louis, Germany] control vehicle was prepared by adding 20 µl of DMSO to 180 µl of 10% CMS then vortexed to form 5 000 nM solution. Aliquot of 20 µl of this solution was diluted further by adding it to 4.8 mL 10% CMS to obtain 89.5% of the solution. DMSO Aliquots of 400 µl in cryotubes were used immediately or stored at -80°C for up to 6 months.

3.6.8 Drug sensitivity testing by malaria SYBR Green I based assay

Archived *P. falciparum* reference clones 3D7- chloroquine sensitive and mefloquine resistant, D6-chloroquine sensitive and mefloquine resistant, and W2- chloroquine resistant and mefloquine sensitive alongside selected field isolates were retrieved from liquid nitrogen (LN2) for *in-vitro* assay. Samples were thawed as follows, they were opened to release pressure and then placed in warm water (56°C) to thaw and then transferred into 15 mL falcon tubes for volume determination. Slowly, 1 volume of 12% NaCl was added in drops to 5 volumes of cells while shaking the tube

gently. The tube was left to stand for 5 minutes at 25°C after which 10 mL of 0.9 NaCl was added slowly in a dropwise manner while gently shaking. Spinning was done at 1 500 RPM for 5 minutes followed by aspiration of the supernatant. 10 mL of 0.9 NaCl and 2% dextrose mixture was added to the pellet slowly while shaking gently then centrifugation at 1 500 RPM for 5 minutes was done after which the supernatant was aspirated. The revived pellet was resuspended in 4.5 mL of 20% culture media in a 25 cm^2 culture flask. The flask was gassed with 5% CO_2, 5% O_2 and 90% N_2 then incubated at 37°C for culture adaptation as earlier described (Akala *et al.*, 2011).

Immediate *ex-vivo* assay was carried out using the freshly collected samples reaching the laboratory within six hours of phlebotomy. Parasite isolates with 1% parasitemia at 2% hematocrit was tested directly while samples with > 1% parasitaemia were adjusted to 1% parasitemia and 2% haematocrit before testing using complete media and uninfected O+ RBCs.

In both *in vitro* and *ex vivo* assays, 100 µl of malaria infected erythrocytes was transferred into each well on a 96 well pre-dosed drug plate (catalog no: 167008 Nunc, Inc, Roskilde, Denmark) containing different concentration of drug aliquots, then it was incubated at 37 $^{\circ}C$ in 90% N_2, 5% O_2, and 5% CO_2 gas mixture in the humidified environment and terminated 72 hours later. Lysis buffer containing SYBR Green I dye was added and then incubated for 24 h in the dark. Readings were taken using Tecan Genios Plus® (Tecan US, Inc., Durham, NC) which gave the relative frequency units (RFUs). Using these readouts, the strength of inhibition of each drug, the 50% inhibition concentration (IC_{50}) was calculated using Graphpad prism® 8.1 windows software (Graphpad software, San Diego, CA, USA) using non-linear regression analysis of the dose-response curve.

3.6.9 Piperaquine survival assay (PSA)

PSA was performed as previously described by Duru and coworkers (Duru *et al*., 2015). *In-vitro* PSA was performed on culture adapted field isolates which were synchronized as follows; Parasite cultures with 50% proportion of rings were centrifuged to get the RBC pellet. Incubation of the pellet in 10 volumes of 5% D-sorbitol for 10 minutes and spinning was done. The supernatant was removed followed by transferring the pellet into 25 cm^2 culture flasks with 20% CMS. Gassing and incubation for 48 hours was done as described earlier (Duru *et al.*, 2015), then this step was repeated. When the proportion of schizonts was > 0.5% 30 hours later, 4 mL of 75% percoll was

added into 15 mL falcon tube then subsequently parasite pellet was added to percoll slowly. The schizont layer was collected after spinning at 1000 g for 15 minutes; a thin smear was prepared and examined by microscopy. When the proportion of schizonts had reached > 10%, the pellet was washed by RPMI and then transferred to 25 cm^2 culture flasks for culturing under the conditions mentioned earlier for 3 hours. The flask contents were mixed then a thin smear was made, methanol fixed and rapid giemsa stained for microscopy. When the proportions of rings were > 0.5%, the cultures were transferred to 15 mL tubes for centrifugation as earlier described (Witkowski & Menard, 2017). The supernatant was removed then synchronized using D-sorbitol as described earlier (Witkowski & Menard, 2017). The contents were mixed and centrifuged at 2500 rpm for 3 minutes. The supernatant was removed to obtain 0-3 hours post invasion rings for *in vitro* PSA.

Fresh parasite isolates collected from Kisumu West and Kisumu East hospital sites between 2019 and 2021 were analyzed using the immediate *ex vivo* PSA as described (Duru *et al*., 2015). Samples at equal or greater than 1% parasitemia, arriving at the central laboratory within 6 hours after phlebotomy at ambient temperature, were reconstituted to 1% parasitemia, and 2% hematocrit, and then subjected to the assay. Importantly, a blood smear was made from the reconstituted samples at 1% parasitemia for reference as initial parasitemia (INI). Briefly, aliquot of 900 µl of the reconstituted parasitized red blood cell sample were loaded to a 48-well culture plate (Nunc, Inc., Roskilde, Denmark) containing 100 µl of 2000 nM piperaquine tetraphosphate tetrahydrate giving a final concentration of 200 nM piperaquine (exposed culture) and 89.5% dimethyl sulfoxide (Sigma-Aldrich, ST. Louis, Germany) (non-exposed culture/control) in triplicates (Duru *et al*., 2015). The assay plates were incubated for 48 hours at 37°C in a mixture of 5% O_2, 5% CO_2, and 90% N_2 in a humidified environment as earlier described by Duru and co-workers. After 48 hrs incubation, contents of the assay wells were separately washed three times in plain media, then resuspended in drug-free 20% complete medium for a further 24 hours. Thin blood smears were prepared, methanol-fixed, and stained by 10% Giemsa (Sigma-Aldrich, ST. Louis, Germany) for 20 minutes (Duru *et al.,* 2015). The percentage of viable *P. falciparum* which developed into second-generation rings or trophozoites in the exposed and non-exposed cultures were determined by assessing parasites with normal morphology in 10 000 red blood cells by two independent microscopists blinded to the clinical data. Piperaquine (PPQ) sensitivity was expressed as survival rate and computed as described (Duru *et al*., 2015),

Percentage PSA survival rate $= \frac{\text{number of viable parasites in exposed culture}}{\text{number of parasites in non-exposed culture (NE)}}$ x 100%.

Clinical isolates with piperaquine survival rate ≥ 10% were considered PPQ resistant. Percentage survival rates were interpretable if the growth rate (NE/INI) ≥1 (*ex vivo* PSA) and ≥1.5 (*in vitro* PSA).

3.7 Extraction of DNA from *P. falciparum* parasites

Parasite nucleic acids were extracted from whole blood samples and dry blood spots using the QIAamp® DNA mini kit (QIAGEN, Inc., Germany) following the manufacturer's instructions. Qiagen protease (20 µL) was added into eppendorf tubes for protein digestion, 200 µL of whole blood samples were further added into the tubes containing the protease then mixed by vortexing. Lysis buffer (200 µL) was added and then mixed well by vortexing. The mixture was centrifuged and then incubated at 56°C for 10 minutes. Absolute ethanol (96-100%) (200 µL) was added into the de-proteinized DNA sample, mixing and centrifuged. The mixture was transferred to the QIAamp spin columns for DNA purification, the columns were centrifuged at 8000 rpm for 1 minute followed by discarding the filtrate tubes. Spin columns containing the DNA were transferred into another 2 mL collection tubes and 500 µL of wash buffer one (AW1) was carefully added to the columns and then subsequently centrifuged at 8000 rpm for 1 minute, the filtrate was discarded. The spin columns were transferred to clean 2 mL collection tubes followed by addition of 500 µL wash buffer two (AW2), and centrifuged at 14 000 rpm for 3 minutes. The old collection tubes with the filtrate were discarded and a dry spin at 8 000 rpm for one minute was done. Elution of purified DNA from the spin column was done by addition of 150 µL of elution buffer (AE) to the columns. Incubation at room temperature for 5 minutes was done to increase DNA yield. Spinning at 8 000 rpm for one minute was done after incubation and then stored at -20°C in elution buffer for molecular analyses. DNA extraction from dry blood spots required an extra step of tissue lysis using the ATL buffer and further incubation periods.

3.8 Estimation of *Pfmdr1, Pfpm2,* and *Pfpm3* copy number variation

Forward and reverse primers for Plasmepsin-2 (*Pfpm*2) [forward-5′-ATGGTGATGCAGAAGTTGGA-3′; reverse- 5′AACATCCTGCAGTTGTACATTTAAC-3′], Plasmepsin-3 (*Pfpm*3) [forward- 5′- CCACTTGTGGTAACACGAAATTA-3′; reverse- 5′-TGGTTCAAGGTATTGTTTAGGTTC-3′)], *Plasmodium falciparum* multi-drug resistance transporter-1 (*Pfmdr*1) [forward 5′- TGCATCTATAAAACGATCAGACAAA-3′, reverse 5′-

TCGTGTGTTCCATGTGACTGT-3′], and β-tubulin (forward 5′-TGATGTGCGCAAGTGATCC-3′; reverse 5′-TCCTTTGTGGACATTCTTCCTC-3′) for TaqMan real-time PCR (Applied Biosystems Inc., Foster City, CA) assay were designed using an online GeneScript tool as earlier described (Ansbro *et al*., 2020), and sent to Applied Biosystems (Foster City, CA) for synthesis. Copy number variation in the above genes was analyzed as described by Ansbro and others with some modification (Ansbro *et al*., 2020). The assay master mix for multiplex PCR (*Pfpm2*, *Pfmdr*1 and *Pf-tubulin*) and duplex PCR assays (*Pfpm*3 and *Pf-tubulin*) were prepared as described (Appendix 1).

Briefly, each 20 µL PCR reaction composed of 10 µL of TaqMman multiplex, 1 µM of forward and reverse primer for each gene, 5 µM of each probe, nuclease-free water, and 1-2 µL of template DNA. In each real-time PCR run, 3D7 and DD2 strains of *P. falciparum* were used as positive controls with single and multiple copies of *Pfmdr1*; respectively, nuclease-free water was also included as the negative control (Ansbro *et al*., 2015; Ngalah *et al*., 2015). A housekeeping gene β-tubulin, and 3D7 were used as internal control and calibrator for the assay as earlier described (Boonyalai *et al*., 2020; Inoue *et al*., 2018; Win *et al*., 2016). A master mix of 4 µL was loaded in each well of the 96 well plate (Applied Biosystems), this was sufficient for 29 field isolates, two positive and one negative controls, further 1 µl of the template field isolate and positive controls (3D7, DD2) DNA and nuclease free water (negative control) were added into each well in triplicates. The plate was sealed with a Micro-Amp seal (Applied Biosystems) and subsequently centrifuged at 4680 rpm to spin down samples. The plate was then loaded unto the QuantStudio 6 pro real-time PCR system (Applied Biosystems Inc., Foster City, CA) where samples were amplified at the following PCR cycling conditions: initial holding step of 95ºC for 5 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds. PCR cycle threshold (CT) values were generated for all the DNA amplicons. Only those with CT value ≤ 32 were considered for analysis by SDS software (version 2.0.6; Applied Biosystems Inc., Foster City, CA). The relative quantification method, $2^{-\Delta\Delta Ct}$ was used to estimate copy number variation for each test gene as earlier described (Ansbro *et al*., 2020; Boonyalai *et al*., 2020). ΔΔCt is [Ct *Pfmdr*1 or *Pfpm*2/*Pfpm*3 − Ct *Pf β-tubulin*] sample − [Ct *Pfmdr*1 or *Pfpm2*/*Pfpm*3 − Ct β-tubulin] 3D7. Field isolates with copy number ≥1.5 were interpreted as true multiple copies (Gupta *et al*., 2020).

3.9 Genotyping of piperaquine resistant genes on the MassARRAY platform

A Matrix-A Laser Desorption Ionization-Time of flight Mass Spectrometry (MALDI-TOF MS) coupled with a single-base extension PCR (iPLEX PCR) (Agena Biosciences, San Diego, CA) was used to analyze single nucleotide polymorphisms (SNPs) in *Pfdhps*, *Pfdhf*r, *Pfcrt* and *Pfmdr1* genes of *P. falciparum* following the manufacturer's protocol. Field isolates from all study sites collected between 2008-2013 and 2018-2021 study periods were analyzed. The primers for this assay were designed by the Agena Bioscience Assay Design Suite (ADS) version 2.0 (Agena Bioscience, San Diego, CA). Primer sequences for primary and secondary PCR are shown in (Appendix 2). The primary PCR reaction was run on a GeneAmp 9700 PCR system (Applied Biosystems, Foaster City, California, USA), using the Agena Bioscience PCR Reagent Set. Each reaction mixture comprised of 0.5 µL 10x PCR buffer, 1.8 µL HPLC-grade water, 0.40 µL $MgCl_2$, 0.10 µL dNTPs mix, 1.00 µL primer mix, 0.20 µl Taq polymerase enzyme, and 1 µL of DNA. This PCR was set at the following conditions; initial denaturation at 95ºC for 2 minutes, 44 cycles of 95ºC for 30 S, 56ºC for 30 S, and 72ºC for 60 S, and a final extension at 72ºC for 5 min. The unincorporated dNTPs from the primary PCR were dephosphorylated by the shrimp alkaline phosphatase enzyme (SAP). The iPLEX PCR was then performed on a GeneAmp 9700 PCR system with the iPLEX Gold Reaction Kit (Agena Bioscience), following the manufacturer's instructions. For PCR, 2 µL of the prepared extension primer cocktail was added to each well. Cycling conditions for this step were as follows; initial denaturation at 94ºC for 30 S, 40 cycles of one step at 94ºC for 5 S with five sub-cycles of 52ºC for 5 S, and 80ºC for 5 S, and final extension at 72ºC for 3 min. Extended products from the later step were conditioned using a resin (Agena Bioscience) and HPLC-grade water. Approximately 10 nL of the extended products was dispensed into a 96-well SpecroCHIP (Agena Bioscience) using the MassARRAY Nano-dispenser RS1000 (Agena Bioscience) followed by automatic data acquisition on the mass spectrometer using SpectroAcquire software.

3.10 Data analysis

In-vitro susceptibility and genotype alongside ex-vivo PSA data were expressed as median IC_{50}s with interquartile range (IQR) and proportions, respectively. The isolates were grouped into three periods (2008-2013, 2014-2017, and 2018-2021). Differences in IC_{50}s between study periods, and study sites were compared using the Kruskal-Wallis (H-test) with Dunn's multiple comparison tests post-hoc analyses. Proportions were examined using the Chi-square test (χ^2) and Fisher exact

test. Associations between genotype and drug susceptibilities by malaria SYBR Green I assay (IC_{50}s) were calculated using the Kruskal-Wallis H-test and Mann-Whitney U test, respectively. The drug *in-vitro* activity correlations were calculated using the Spearman correlation coefficient. All statistical tests were carried out in GraphPad version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). Two-sided *P* value < 0.05 was considered statistically significant.

3.11 Ethical statement

This study was approved by the Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit and the Walter Reed Army Institute of Research (WRAIR) institutional review boards. Archived (2008-2017) and freshly collected (2018-2021) samples were collected under protocol numbers: KEMRI #1330 and #3628, WRAIR #1384, and #2454 (Appendix 3).

CHAPTER FOUR

RESULTS

4.1 Analysis of polymorphisms in *Pfpm2, Pfpm3, Pfexo, PfK13, Pfcrt* and *Pfmdr1* genes that confer resistance to frontline antimalarial drugs.

4.1.1 Assessment of copy number variation for *Pfmdr1, Pfpm2,* and *Pfpm3* genes

A total of 280, 286, and 296 samples were successfully analyzed for copy number variation for the *Pfmdr1, Pfpm2,* and *Pfpm3* genes, respectively. The overall median copy number and IQR for all the genes were; 1.02 (0.9-1.2) for *Pfmdr*1; 0.99 (0.9-1.1) for *Pfpm*2 and 1.1 (0.9-1.2) for *Pfpm*3. Using a copy number threshold of 1.5 to define multiple gene copy isolates (Gupta *et al.*, 2020), the proportion of samples having multiple copies for *Pfmdr1* were shown to increase across the study periods from 1.7% in 2008 to 3.2% in 2021. *Pfpm2* and *Pfpm3* multiple copies declined from 3.0% to 2.0%, and 10.1 to 4.0 between 2008-2013, and 2018-202, ($P > 0.05$) study periods, respectively. However, statistical analysis of the temporal trends of copy number estimate across the study period revealed absence of statistically significant differences in the proportions of multiple copies of *Pfpm2* (P=0.8318), *Pfpm*3 (P =0.4522) and *Pfmdr*1 (P =0.7304) genes between the three study periods (2008-2013, 2014-2017 and 2018-2021) (Figure 6 and Table 2).

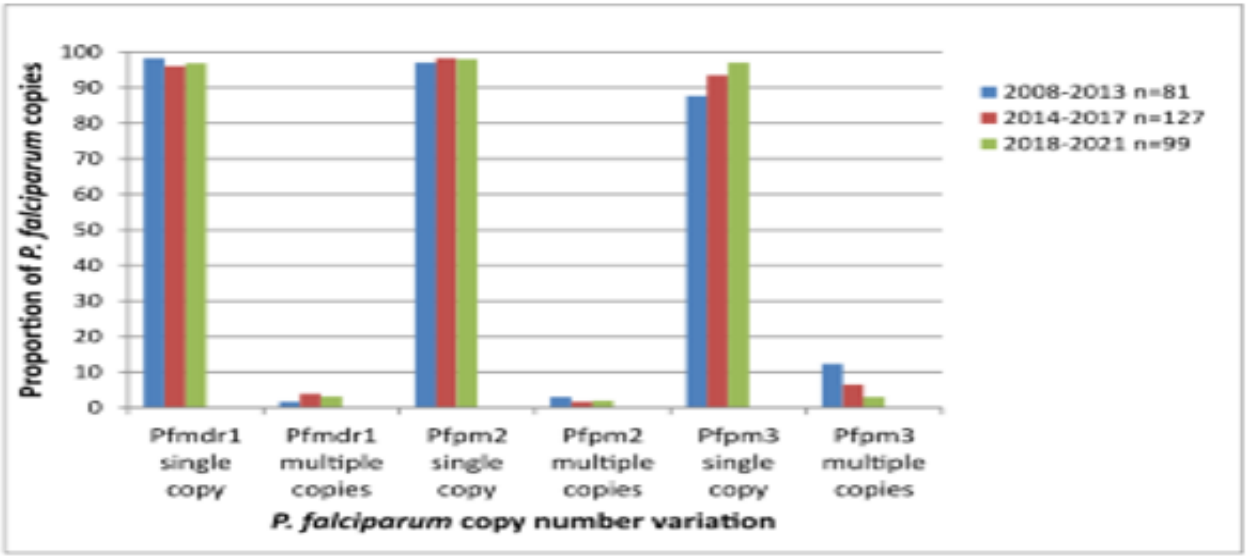

Figure 6: The frequency of *Pfmdr1*, *Pfpm2* and *Pfpm3* copy numbers in infections during the study period. *Pfmdr1*; *Plasmodium falciparum* multi-drug resistance 1 gene, *Pfpm2*; *Plasmodium falciparum plasmepsin*-2 gene, *Pfpm3*; *Plasmodium falciparum plasmepsin*-3 gene

Table 2: Frequency of Kenyan parasites harboring *Pfpm*2/3 and *Pfmdr*1 multiple copies between 2008 and 2021

		Study Period						
		2008-2013		2014-2017		2018-2021		*P*- value
Genotype		**N**	**Proportion**	**N**	**Proportion**	**N**	**Proportion**	
Pfmdr1	Single copy	57	98.3%	122	96.1%	92	96.8%	
	Multi-copy	1	1.7%	5	3.9%	3	3.2%	0.7304 NS
Pfpm2	Single copy	65	97.0%	118	98.3%	97	98.0%	
	Mult-copy	2	3.0%	2	1.7%	2	2.0%	0.4522 NS
Pfpm3	Single copy	80	89.9%	100	93.5%	96	96.0%	
	Multi-copy	9	10.1%	7	6.5%	4	4.0%	0.8318 NS

*Pfmdr*1; *Plasmodium falciparum* multi-drug resistance 1 gene, *Pfpm2*; *Plasmodium falciparum plasmepsin* 2; *Pfpm3*; *Plasmodium falciparum plasmepsin* 3, N-Number of samples, NS- Not statistically significant.

Further, genomic analyses of isolates with PSA rate ≥10 (resistant to piperaquine) did not detect any piperaquine resistance candidate marker. The *Pfpm2, Pfpm3* and *Pfmdr1* copy numbers did not differ among the isolates with PSA ≥ 10 (resistant to piperaquine) versus PSA < 10 (sensitive to piperaquine) ($P > 0.999$).

Multiple copies of these genes were reported in field isolates collected from the six hospital study sites at varying frequencies between 2008 and 2021 (Table 3). *Pfmdr1* multiple copies (mutants) were reported in isolates collected from all the study sites except Kericho County referral hospital. Marigat sub county hospitals recorded the highest number (4) of *Pfmdr1* multiple copies followed by Kisumu west sub county hospital (2). The remaining hospital sites except Kericho reported one multiple copy each (Table 3). *Pfpm*2 multiple copies were depicted in isolates collected from Kericho County, Kisumu West and Kisumu East hospitals. Kericho hospital site had the highest number (3) of *Pfpm2* multi copies followed by Kisumu west (2) and Kisumu East hospital (1). Moreover, *Pfpm*3 multiple copies were shown in isolates from all the six hospital sites (Table 3). Kisumu west hospital reported the highest number of *Pfpm3* multiple copies (7) followed by

Malindi hospital (4), Kisumu East and Kisii each reporting 3 multiple copies, Kericho (2), and Marigat (1) (Table 3).

Table 3: Frequencies of *Pfpm2/3* and *Pfmdr*1 copy number variations of Field isolates collected from different hospital sites between 2008 and 2021

		Hospital sites					
Gene	**Copy number variation**	**KDH**	**KOM**	**KSI**	**KCH**	**MGT**	**MDH**
Pfmdr1	Single copy	60	61	62	40	23	25
	Multiple copies	1	2	1	0	4	1
Pfpm2	Single copy	62	61	64	37	30	26
	Multiple copies	1	2	0	3	0	0
Pfpm3	Single copy	59	57	66	38	30	26
	Multiple copies	3	7	3	2	1	4

Pfmdr1; Plasmodium falciparum multidrug resistant 1 gene, *Pfpm2; Plasmodium falciparum plasmepsin 2*; *Pfpm3; Plasmodium falciparum plasmepsin* 3. KDH- Kisumu East County hospital, KOM- Kisumu West sub-county hospital, KSI- Kisii County hospital, KCH- Kericho County hospital, MGT- Marigat Sub-county hospital and MDH- Malindi Sub-county hospital

4.1.2 Prevalence of *P. falciparum* genetic polymorphisms associated with antimalarial resistance

Analysis of *P. falciparum* isolates for genetic polymorphisms that confer antimalarial resistance revealed a significant decrease in frequencies of *Pfcrt* K76T ($P = 0.0357$) and *Pfdhps* 437G ($P = 0.0218$) mutants from 39.3% (11/28) to 0% (0/9) and 50% (8/16) to 0% (0/9), respectively between the study periods, 2008-2013 and 2018-2021. Conversely, *Pfdhps* 436F/A mutant allele proportion increased significantly from 0% (0/15) to 75% (3/4), ($P = 0.0227$) between 2008-2013 and 2017-2021 (Table 4). Additionally, *Pfmdr1* 86Y allele proportions dropped from 25.7% (9/35) to 0% (0/9) across the study period but this was not statistically significant ($P = 0.1674$). On the contrary, no evidence of significant changes in proportions of *Pfdhfr* C59R, *Pfdhfr* I164L, and *Pfmdr1* Y184F polymorphisms were reported between the 2008-2013 and 2017-2021 study periods. (Table 4). Further, screening for polymorphisms implicated in sulphadoxine resistance did not

unravel any mutations in *Pfdhps* gene at loci A581G and A613S (Table 4).

Table 4: Proportions of antimalarial resistance polymorphisms in Kenyan *P. falciparum* isolates over time

Polymorphism	2008-2013	2018-2021	*P*-value **
	n (%)	n (%)	
Pfmdr1 N86Y	9 (25.7)	0 (0)	0.1674
Pfmdr1 Y184F	16 (43.2)	3 (42.9)	> 0.999
Pfcrt K76T	11 (39.3)	0 (0)	0.0357**
Pfdhps S436F/A	0 (0)	3 (75)	0.0227**
Pfdhps 437G	8 (50)	0 (0)	0.0218**
Pfdhps A581G	0 (0)	0 (0)	NA
Pfdhps A613S	0 (0)	0 (0)	NA
Pfdhfr C59R	18 (81.8)	13 (100)	0.2735
Pfdhfr I164L	2 (9.5)	0 (0)	0.5343

** $P < 0.05$ consistent with statistically significant differences in the proportions of polymorphisms across the study period by Fisher exact test. *Pfmdr1*; *Plasmodium falciparum* multi-drug resistance 1 gene, *Pfcrt*; *Plasmodium falciparum* chloroquine resistance transporter gene, *Pfdhps*; *Plasmodium falciparum dihydropteroate synthetase*, *Pfdhfr*; *Plasmodium falciparum dihydrofolate reductase*. NA-Not applicable (no mutations were detected for *Pfdhps* A613S and A581G).

4.2. Determination of *P. falciparum* isolates susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine using the malaria SYBR Green I based method and exploring *in vitro* antimalarial activities correlation.

4.2.1 Analysis of *P. falciparum in vitro* susceptibility to antimalarial drugs over time and by Hospital site

A total of 252 clinical field isolates were successfully collected from six different hospital sites in four of the five malaria epidemiological zones in the Country between 2008 and 2021. Majority 138/252 (54.8%) of the field isolates were collected from the Lake endemic region which comprised of Kisumu West and Kisumu East hospital sites. 75/252 (29.8%) of the isolates were

collected from the highland epidemic prone zone consisting of Kisii and Kericho County hospitals while 24/252 (9.5%) were obtained from the seasonal malaria epidemiological zone (Marigat sub county hospital). Malindi sub county hospital in the coastal endemic zone contributed the least number of field isolates 15/252 (5.9%) (Table 5).

Table 5: Number of field isolates collected from each study site the period spanning between 2008 and 2021

	KDH	KOM	KSI	KCH	MGT	MDH	TOTAL
2008-2013	11	11	17	11	1	7	58
2014-2017	21	37	22	13	12	2	107
2018-2021	22	36	8	4	11	6	87
TOTAL	54	84	47	28	24	15	252

KDH- Kisumu East County hospital, KOM- Kisumu West sub-county hospital, KSI- Kisii County hospital, KCH- Kericho County hospital, MGT- Marigat Sub-county hospital and MDH- Malindi Sub-county hospital.

Out of the 252 isolates 239 (94.8%) were successfully assayed for drug susceptibility against PPQ, DHA, LM, ART and CQ using the standard malaria SYBR Green I method. Chloroquine-sensitive 3D7 and D6 alongside chloroquine resistant W2 reference clones were analyzed in parallel as the assay controls. Assessment of the field isolates against lumefantrine and artemether, showed a significant increase in median fifty percent inhibitory concentration (IC_{50}) across the study periods (Figure 7C and D, Table 6). Post-hoc analysis revealed a significant temporal changes in susceptibility of the parasites to LM between 2014-2017 and 2018-2021 study periods, whereas to ART, between 2008-2013 and 2018-2021 alongside 2014-2017 and 2018-2021 study periods, respectively (Figure 7C and D). Conversely, there was a significant decrease in chloroquine median IC_{50} across the study period (Figure 7E and Table 6). Significant temporal changes in susceptibility of the parasites to CQ was observed between 2014-2017 and 2018-2021 study periods (Figure 7E). Additionally, the median IC_{50} of piperaquine against clinical isolates was 32.62 (20.1-45.6) nM for 2008-2013, 27.64 (12.4-47.6) nM for 2014-2017 and 27.32 (6.9-52.9) nM for 2018-2021 (Table 6). Assessment of the temporal trends of *in vitro* response to piperaquine revealed a marginal decline in IC_{50} between 2008 and 2021, although this was not statistically significant (P=0.1615) (Figure 7A). However, DHA median IC_{50} variation during the study period

was insignificant (Figure 7B). The median IC_{50} of DHA against the isolates was 2.8 (1.4-11.1) nM for 2008-2013, 5.2 (2.0-19.0) nM for 2014-2017 and 7.2 (2.5-14.1) nM for 2018-2021 (Table 6).

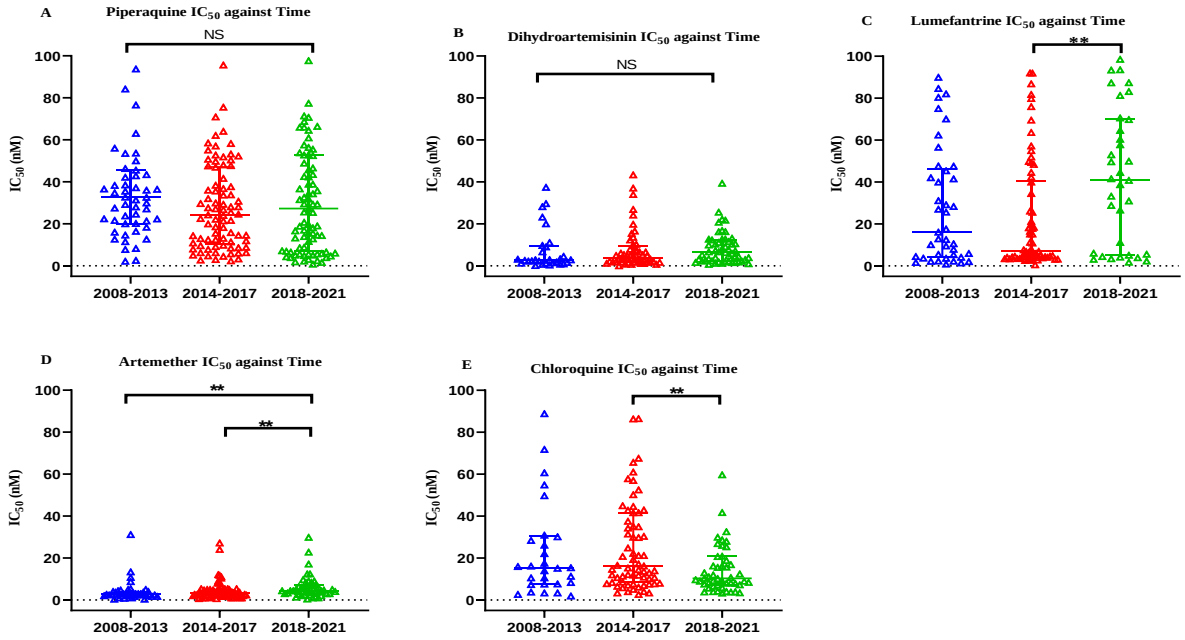

Figure 7: Scatter plots showing temporal changes of parasites response to A) Piperaquine, B) Dihydroartemisinin, C) Lumefantrine, D) Artemether, E) Chloroquine between 2008-2013, 2014-2017 and 2018-2021. Scatter plots horizontal bars and whiskers represent median IC_{50}s and interquartile range in nM. ** represent statistically significant change in drug median IC_{50}, $P < 0.05$.

Table 6: Temporal trends in *in vitro* susceptibility of field isolates to selected antimalarial drugs between 2008 and 2021

Study period	2008-2013		2014-2017		2018-2021		
Drugs	Median IC50s (IQR)	N	Median IC_{50}s (IQR)	N	Median IC_{50}s (IQR)	N	*P*-value
LM	26.3 (5.1-64.3)	46	17.2 (4.5-80.4)	82	62.4 (26.9-100.8)	48	0.0201**
CQ	15.3 (7.6-30.4)	29	16.5 (9.3-42.2)	65	10.4 (7.2-20.9)	51	0.0318**
ART	2.7 (0.3-4.3)	42	3.7 (2.3-5.7)	98	4.4 (3.0-7.4)	49	0.0021**
PPQ	32.7 (20.2-45.6)	51	27.6 (12.5-47.6)	105	27.3 (6.9-52.8)	83	0.1615
DHA	2.8 (1.4-11.1)	31	5.2 (2.0-19.0)	64	7.2 (2.5-14.1)	58	0.1265

IQR Interquartile range (nM), N sample size. ** represent statistically significant change in IC_{50} with $P < 0.05$. Significant changes in susceptibility for each drug across the three study periods were calculated by the Kruskal Wallis H-test. LM-Lumefantrine, CQ- Chloroquine, ART-Artemether, PPQ- Piperaquine, DHA- Dihydroartemisinin.

The overall field isolates susceptibility assay for the entire study period was conducted alongside the reference clones as the assay controls. Field isolates PPQ median IC_{50} was more than that of the reference clones. The field isolates and reference clones median IC_{50} and IQR to PPQ were as follows; Field isolates 32.62, n= 239 (IQR 20.1-45.6), D6 9.51, n=7 (IQR 3.76-45.71), W2: 22.87, n=8 (IQR 7.57-48.14) and 3D7: 8.78, n=19 (IQR 37.84-32.14) nM (Figure 8A, Table 7). The field isolates and reference clones' susceptibility profile (median IC_{50} and IQR) to ART was as follows; Field isolates 3.35, n= 167 (IQR 2.35-5.03), D6 7.62, n=6 (IQR 2.69-11.39), W2: 5.07, n=9 (IQR 4.73-6.68) and 3D7: 8.01, n=14 (IQR 5.97-13.57) nM (Figure 8B, Table 7). DHA median IC_{50} and IQR were; Field isolates 4.44, n= 135 (IQR 1.84-410.31), D6 9.12, n=6 (IQR 2.27-9.91), W2: 2.86, n=10 (IQR 2.14-5.72) and 3D7: 2.46, n=17 (IQR 1.93-4.36) nM (Figure 8C, Table 7). LM; Field isolates 26.80, n= 116 (IQR 4.60-52.18), D6 61.31, n=3 (IQR 10.88-83.70), W2: 9.37, n=9 (IQR 3.74-11.14) and 3D7: 13.46, n=18 (IQR 4.87-31.80) nM (Figure 8D, Table 7). CQ; Field isolates 13.30, n= 142 (IQR 7.67-28.96), D6 4.73, n=14 (IQR 3.32-6.30), W2: 74.45, n=9 (IQR 47.99-86.64) and 3D7: 16.39, n=14 (IQR 14.70-28.02) nM (Figure 8E, Table 7).

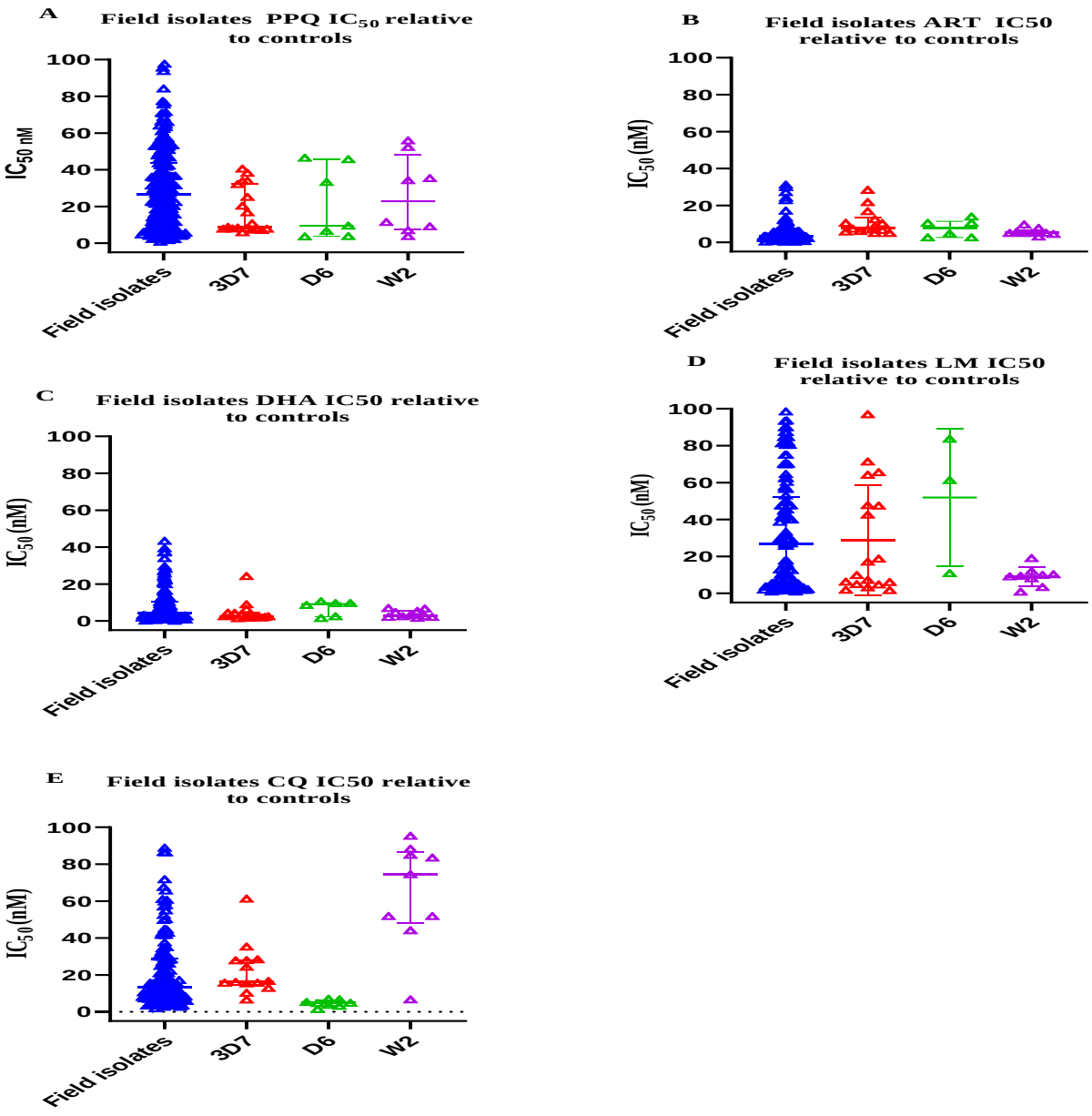

Figure 8: Scatter plots showing **s**usceptibility of *P. falciparum* field isolates and reference clones to antimalarial drugs; A) Piperaquine, B) Artemether, C) Dihydroartemisinin, D) Lumefantrine, E) Chloroquine. Reference clones D6 and 3D7 are chloroquine sensitive, W2 chloroquine resistant. Horizontal bars and whiskers represent median IC_{50}s and interquartile range in nM.

Table 7: *In vitro* susceptibility of field isolates and reference clones to selected antimalarial drugs

P. falciparum strain Drugs	Field isolates Median IC_{50}s (IQR), N	3D7 Median IC_{50}s (IQR), N	D6 Median IC_{50}s (IQR), N	W2 Median IC_{50}s (IQR), N
PPQ	32.6 (20.1-45.6), 239	8.8 (7.8-32.1), 19	9.5 (3.8-45.7), 7	22.9 (7.6-48.1), 8
ART	3.4 (2.3-5.0), 167	8.0 (6.0-13.8), 14	7.6 (2.7-11.4), 6	5.1 (4.7-6.7), 9
DHA	4.4 (1.8-10.3), 135	2.5 (1.9-4.5), 17	9.1 (2.3-9.9), 6	2.8 (2.1-5.7), 7
LM	26.8 (4.6-52.2), 116	61.3 (10.8-83.7), 3	4.7 (3.3-6.3), 8	74.5 (48.0-86.6), 9
CQ	13.3 (7.7-29.0), 142	16.4 (14.7-28.0), 14	4.7 (3.3-6.3), 8	74.5 (48.0-86.6), 9

Reference clones: D6 and 3D7 are chloroquine sensitive, W2- chloroquine resistant

Assessment of parasite susceptibility to PPQ between hospital sites from 2008 to 2021 was also done. The overall PPQ median IC_{50} and IQR for all the Hospital sites were as follows; 30.8 (16.2-47.1), 37.2 (19.0-53.2), 27.6 (12.7-47.2), 22.4 (10.8-58.3), 15.1 (6.3-55.5), and 18.1 (6.2-35.5) for Kisumu west, Kisumu East, Kisii, Kericho, Marigat, and Malindi hospitals, respectively (Figure 8, Table 8). Comparison of parasites susceptibility to piperaquine among the six hospital sites using the Kruskal Wallis H-test depicted a significant variation in parasite sensitivity between Kisumu East County hospital and Malindi sub-county hospitals, (P = 0.0451). However, no statistically significant variation was observed between the remaining hospital sites (P > 0.05) (Figure 8, Table 8).

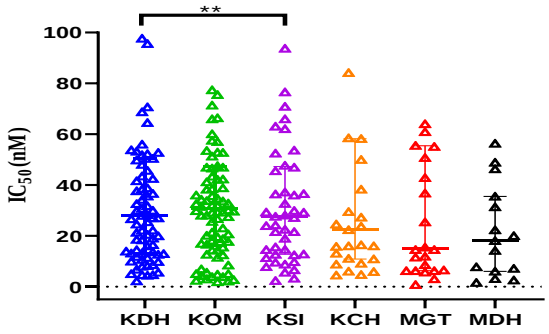

Figure 9: Scatter plot showing susceptibility of parasites from six different geographic locations in Kenya to piperaquine using malaria SYBR green I assay. Horizontal bars and whiskers represent the median and interquartile range of susceptibility.

Table 8: Piperaquine median IC_{50} and interquartile range of *P. falciparum* parasites collected from six different geographical zones between 2008 and 2021

	Kisumu West Sub-couty	Kisumu East Couty	Kisii Couty	Kericho Couty	Marigat Sub-couty	Malindi Sub-couty
PPQ median IC_{50} (nM)	30.8	37.2	27.6	22.4	15.1	18.1
Interquartile range (nM)	16.2-47.1	19.0-53.2	12.7-47.2	10.8-58.3	6.3 -55.5	6.2-35.5
N	82	52	45	27	24	15

***P* value** = 0.0451 **

Significant diffence was observed betweeen Kisumu West and Malindi hospitals (*P* = 0.0451).

** represent statistically significant change in parasite response to PPQ. N- Sample size.

We also investigated for temporal trends of piperaquine susceptibility across different study sites during the three study periods, categorised as, 2008-2013, 2014-2017, and 2018-2021. A significant decrease in the PPQ median IC_{50}s was observed between 2008-2013 and 2014-2017 study periods in field isolates collected from Kisii county hospital site (*P*=0.04) (Table 8). On the contrary there was no significant variation in parasite susceptibility to PPQ during the three study periods in the remaining five hospital sites, namely, Kisumu west, Kisumu East, Kericho, Marigat, and Malindi (*P* > 0.05) (Table 9).

Table 9: Temporal trends of piperaquine susceptibility across different study sites during the study period.

	Hospital sites Median IC_{50} (IQR), n				
Study period	KDH	KOM	KSI	KCH	MDH
2008-2013	24.6 (15.5-43.0), 9	32.6 (20.1-42.2), 11	36.5 (30.9-56.0), 14	38.3 (20.2-127.3), 5	22.3 (7.8-35.5), 7
2014-2017	37.7 (19.3-51.1), 21	32.4 (21.8-50.3), 36	23.2 (9.7-29.4), 22	18.9 (5.4-35.8), 9	8.7 (3.1-14.2), 2
2018-2021	36.7 (22.8-44.1), 9	27.5 (5.9-52.8), 36	15.6 (6.6-52.5), 7	18.1 (6.6-28.9), 4	12.6(4.9-50.8) ,6
P value	0.6670 NS	0.3085 NS	0.0485**	0.1271 NS	0.5647 NS

KDH- Kisumu East County hospital, KOM- Kisumu west sub-county hospital, KSI- Kisii County hospital, KCH- Kericho County hospital, MGT- Marigat Sub-county hospital and MDH- Malindi Sub-county hospital. ** represent statistically significant change in parasite response to piperaquine $P < 0.05$, NS-Not statistically significant.

4.2.2 *In-vitro* drug activity correlation between 2008-2021

Drug susceptibility association among PPQ, DHA, LM, ART alongside CQ was successfully assessed using the Spearman correlation method to explore the relationship in drug sensitivity against the parasites among the five drugs. The drugs were grouped into 10 pairs as follows; PPQ vs DHA, PPQ vs LM, PPQ vs ART, PPQ vs CQ, DHA vs LM, DHA vs ART, DHA vs CQ, LM vs ART, LM vs CQ, and ART vs CQ. A significant positive correlation was observed between the *in vitro* activity of one drug pair comprising of two artemisinin derivatives; DHA, and ART (rho=0.4, *P*=0.0003) (Table 9). Conversely, there was no correlation observed between the *in vitro* activity of LM or CQ and artemisinin derivatives (DHA and ART) ($P > 0.05$). Additionally, no

correlation was noted between the *in vitro* activity of PPQ and LM, DHA, ART drug pairs alongside CQ (Table 10).

Table 10: Correlation between *in vitro* responses of Kenyan *P. falciparum* isolates to piperaquine and other antimalarials

Drug pair	Number of Isolates	Correlation Coefficient (r)	*P*- value
PPQ vs. DHA	77	0.0840	0.4679
PPQ vs. LM	80	0.0579	0.6097
PPQ vs. ART	80	0.0254	0.8230
PPQ vs. CQ	80	0.0278	0.8068
DHA vs. LM	77	0.2105	0.0662
DHA vs. ART	77	0.4036	0.0003**
DHA vs. CQ	77	0.0589	0.6111
LM vs. ART	80	0.0602	0.5957
LM vs CQ	80	0.1328	0.2402
ART vs CQ	80	0.0092	0.9355

The correlation for each pair of drugs per isolate is based on IC_{50} values. PPQ, piperaquine; DHA, dihydroartemisinin; LM, lumefantrine; ART, artemether and CQ, chloroquine. ** $P < 0.05$ consistent with statistically significant difference assessed by Spearman correlation coefficient analyses

4.3 *Plasmodium falciparum* susceptibility to piperaquine by Piperaquine Survival Assay (PSA)

A total of 34 freshly collected field isolates characterized by the immediate *ex vivo* PSA had an overall piperaquine median survival rate and IQR of 0 (0-5.3) % (Table 11). Out of these, 29 (85.3%) had a piperaquine survival rate < 10% (below resistance threshold), and were characterized as susceptible to piperaquine. 14 (48.3%) of the susceptible isolates were from Kisumu West sub-county hospital and 15 (51.7%) from Kisumu East County referral hospital. The remaining 5/34 (14.7%) had piperaquine survival rate ≥10% (above resistance threshold), and were categorized as piperaquine resistant. Five of the resistant isolates, four were from Kisumu West

sub-county hospital, and one from Kisumu East County referral hospital (Table 11). There was no significant variation in median piperaquine survival rates for the isolates collected from the two hospital sites, Kisumu West Hospital 4.2 (0-28.21) % versus Kisumu East Hospital, 0 (0-3.12) % (Figure 10 and Table 11).

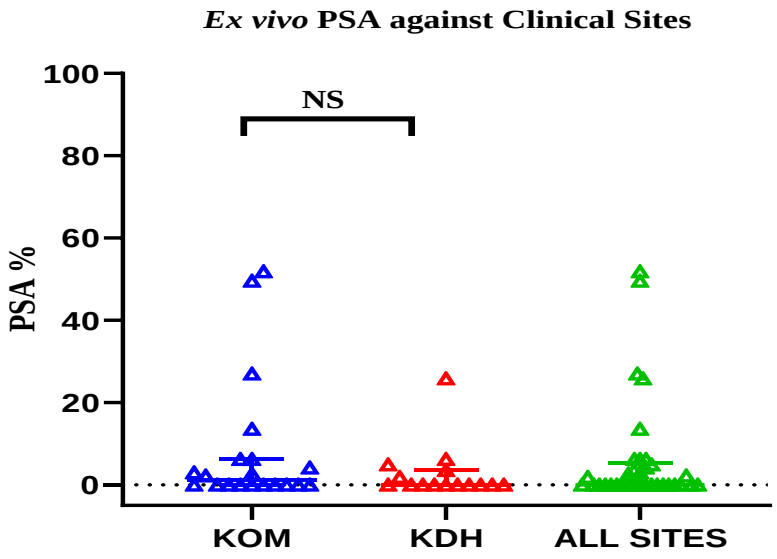

Figure 10: Scatter plot showing *Ex vivo* piperaquine survival rates of Kenyan clinical isolates from two locations. Horizontal bars and whiskers represent the median and interquartile range of susceptibility.

Table 11: Range of *ex vivo* PSA values for Kenyan parasite isolates

	Kisumu East	**Kisume West**	**All sites**
Number of samples (n)	16	18	34
Minmum	0	0	0
25% percentile	0	0	0
Median	0	4.2	0
75% percentile	3.12	28.21	3.0
Maximum	25.86	51.88	51.88

4.4 Association between *in vitro* piperaquine susceptibility and PPQ resistance molecular markers.

Assessment of association between PPQ IC_{50} values and mutations in *Pfcrt*, *Pfdhfr*, *Pfdhps*, *Pfpm2/3*, and *Pfmdr1* mutations using the Mann Whitney-U test and Kruskal Wallis H test showed a significant positive relationship between PPQ IC_{50}s and, *Pfcr*t K76T SNP (P = 0.0026) (Figure 11A, Table 12). Post-hoc analysis further revealed that parasites harboring the *Pfcrt* K76T mixed species genotype (both mutant and wild type) had higher median *in vitro* PPQ IC_{50} of 29.90 nM compared to the wild-type isolates 18.86 nM (P = 0.0026) (Figure 11A, Table 12). However, there was no significant association between PPQ IC_{50}s and the remaining *Pfcrt* SNPs, namely; T356C, and G371C (Figure 11A, Table 12). Moreover, no association was depicted between PPQ IC_{50}s and the *Pfmdr1* SNPs (N86Y, 86N/Y, Y184F, 184Y/F, A1034T, 1034A/T) which are known for conferring resistance to lumefantrine (Figure 11B, Table 12). Exploring for an association between PPQ IC_{50}s and the antifolates (sulphadoxine and pyramethamine) resistance markers, namely; *Pfdhfr* (C59R, I164L), and *Pfdhps* (S436F, A437G) did not reveal any significant relationship (Figure 11C, Table 12). Also an association was sought between PPQ IC_{50}s and the copy number variants of *Pfpm2*/3, and *Pfmdr1* genes that are known for conferring piperaquine resistance and no significant association was shown between them (Figure 11D, Table 12).

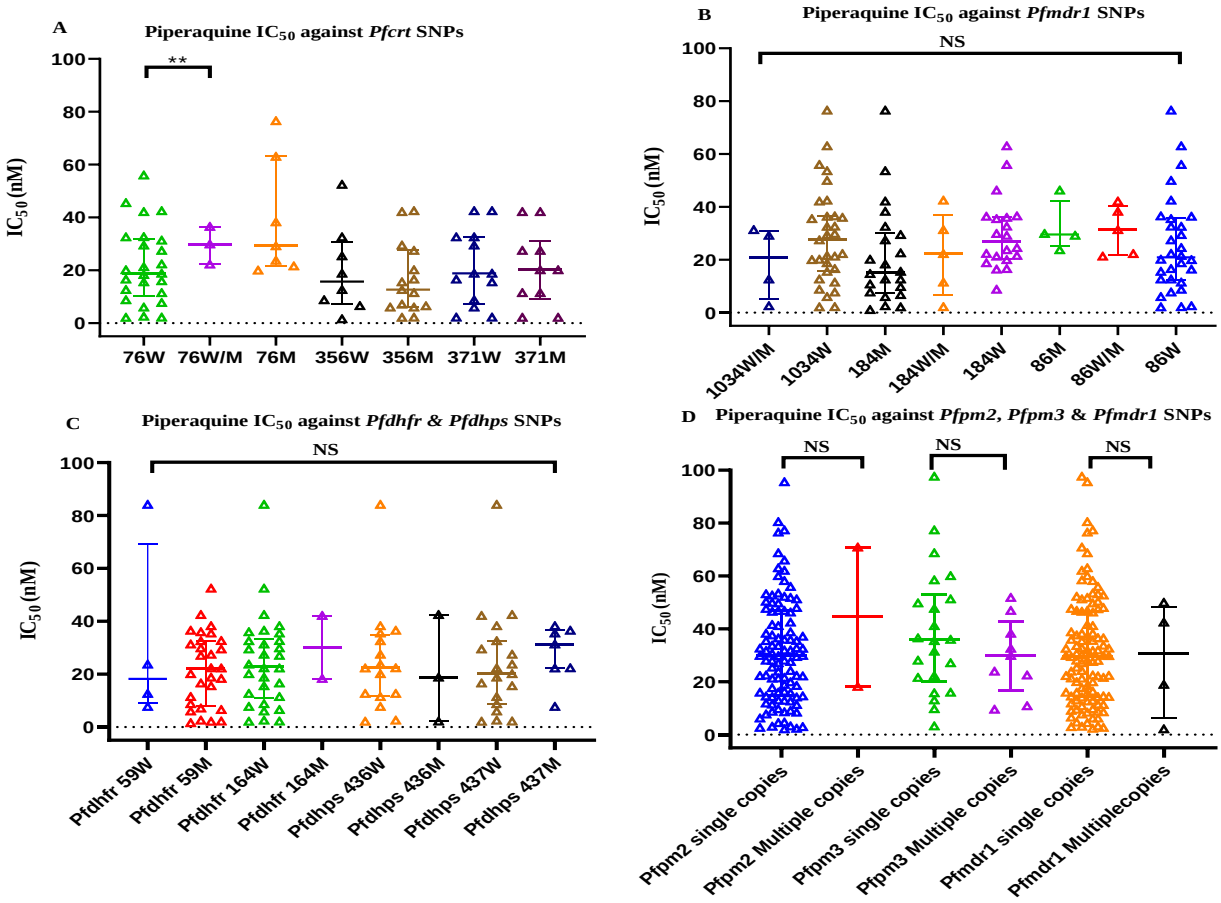

Figure 11: Scatter plots showing A) Association between *Pfcrt* polymorphisms and *in vitro* susceptibility to piperaquine (IC_{50}), B) Association between *Pfmdr1* polymorphisms and *in vitro* susceptibility to piperaquine (IC_{50}), C) Association between *Pfdhfr & Pfdhps* polymorphisms and *in vitro* susceptibility to piperaquine (IC_{50}), D) Association between *Pfmdr1*, *Pfpm2/3* copy number variation and *in vitro* susceptibility to piperaquine (IC_{50}). Scatter plots horizontal bars and whiskers represent median IC_{50}s and interquartile ranges for each locus. M-mutant genotype, W-wild-type genotype, W/M- mixture of mutant and wild-type genotype in an infection. *Pfcrt- Plasmodium falciparum chloroquine resistance gene*, *Pfmdr1- Plasmodium falciparum multidrug resistance gene 1*, *Pfdhps- Plasmodium falciparum dihydropteroate synthetase* gene, *Pfdhfr- Plasmodium falciparum dihydrofolate reductase* gene, *Pfpm2- Plasmodium falciparum plasmepsin* 2 gene and *Pfpm3- Plasmodium falciparum plasmepsin* 3 gene.

Table 12: Association between piperaquine (PPQ) resistance mutations (SNPs as well as Copy number variation (CNV) with *in vitro* PPQ IC_{50}s of Kenyan field isolates collected between 2008-2021.

				In vitro SYBR Green I assay	
Gene Polymorphisms		N	Proportions (%)	PPQ median IC_{50} (IQR) Nm	*P* value*
P. falciparum multidrug resistance 1 gene, *Pfmdr1* SNPs	N86	34	75.6	23.5 (12.7-44.4)	0.26
	86N/Y	6	13.3	34.9 (22.0-80.5)	
	86Y	5	11.1	29.9 (26.5-288.5)	
	Y184	22	47.8	29.5 (21.1-39.0)	0.55
	184Y/F	6	13.0	26.9 (9.2-80.7)	
	184F	18	39.1	23.9 (11.4-45.0)	0.81
	A1034	34	85.0	29.7 (21.1-39.0)	
	1034T	6	15.0	30.3 (10.3-279.4)	
P. falciparum chloroquine resistance transporter 1 gene, Pfcrt SNPs	K76	25	65.8	18.9 (10.1-31.9)	0.0026**
	76K/T	5	13.2	36.6 (26.1-249.9)	
	76T	8	21.0	33.8 (22.2-73.2)	
	T356	9	37.5	18.9 (7.7-42.6)	0.73
	356C	15	62.5	15.7 (6.5-29.5)	
	G371	10	64.3	16.6 (8.0-30.3)	
	371T	18	35.7	20.2 (9.2-31.2)	0.96

Continuation Table 12: Association between piperaquine (PPQ) resistance mutations (SNPs as well as Copy number variation (CNV) with *in vitro* PPQ IC50s of Kenyan field isolates collected between 2008-2021.

P. falciparum dihydrofolate reductase gene, Pfdhfr SNPs	C59	4	11.8	18.3 (7.8-69.1)	0.98
	59R	30	88.2	22.4 (1.7-33.4)	
	I164	30	92.3	23.2 (10.8-33.4)	0.58
	164L	2	7.7	30.3 (18.3-42.2)	
	S436	16	84.2	22.4 (11.8-34.7)	0.88
P. falciparum dihydropteroate synthetase gene, Pfdhps SNPs	436F	3	15.8	18.9(2.2-416.1)	
	A437	19	73.1	20.2 (8.8-32.7)	0.33
	437G	7	26.9	31.4 (22.4-36.6)	
*P. falciparum multidrug resistance 1 gene, Pfmdr*1 CNV	Single copy	271	96.8	29.9 (14.4-47.4)	0.48
	Multiple copies	9	3.2	46.1 (12.1-99.9)	
P. falciparum plasmepsin 2 gene, *Pfpm*2 CNV	Single copy	280	97.9	29.9 (14.4-47.2)	0.63
	Multiple copies	6	2.1	44.6 (18.3 -71.0)	
P. falciparum plasmepsin 3 gene, *Pfpm*3 CNV	Single copy	276	92.9	30.0 (14.5-47.6)	0.72
	Multiple copies	20	7.1	31.3 (19.6-48.2)	

** show $P < 0.05$ consistent with statistically significant differences in frequency over time using Kruskal Wallis (H) tests for three groups and Mann Whitney (U) test for two groups.

CHAPTER FIVE

DISCUSSION

The WHO recommends continuous monitoring of the performance of recommended drugs for early detection of resistance (Chenet *et al*., 2019; Nsanzabana *et al*., 2018; WHO, 2021), and growth inhibition assays and genomic analyses are the most scalable and cost-effective drug resistance surveillance methods hence mostly deployed globally (Chenet *et al*., 2019). Our study successfully described polymorphisms in *Pfpm2, Pfpm3*, *Pfcrt* and *Pfmdr*1 genes that confer resistance to frontline antimalarial drugs in circulating *Plasmodium falciparum* parasites in Kenya. Mutations in *Pfpm*2/3, *Pfmdr*1, *Pfdhps*, *Pfdhfr* and *Pfcrt* have been associated with antimalarial resistance leading to a change of dosing regimen in the Thai-Cambodian border (Amato *et al*., 2017; Duru *et al.*, 2015; Imwong *et al.*, 2017). Polymorphisms in *Plasmepsin*-2/3 (*Pfpm*2/3) and, *Pfmdr1* genes have been implicated in PPQ resistance. Our assessment of these resistance markers in Kenyan isolates between 2008 and 2021 revealed a low frequency of *Pfpm*2/3 and *Pfmdr*1 genes copy number variation mutations. This finding agrees with other studies in Africa (Gupta *et al*., 2020; Leroy *et al*., 2019), suggesting that circulating Kenyan parasites are susceptible to drugs containing PPQ (Gupta *et al*., 2020; Ogutu *et al*., 2014; Rasmussen *et al*., 2017; Tumwebaze *et al*., 2021). Studies have shown that parasites with impaired response to antimalarial drugs linger in longer in an infection and are prone to transitioning to gametocytes as the drug dose diminishes (Chawl *et al*., 2021). It is therefore noteworthy that the presence of the polymorphisms in *Plasmepsin* 2/3 (*Pfpm2/3*) and *Pfmdr*1 genes in Kenyan parasites presents a potential risk of rapid selection of resistance in Kenya as drug pressure builds due to the continued use of DHA-PPQ. This emphasizes the need for sustained genomic surveillance as is evocative of rapid selection as PPQ use becomes more widespread. Additionally, all the low frequency multiple copies of *Pfpm*3 and *Pfpm*2 genes were observed in isolates collected from the endemic Lake region of Western Kenya, suggesting that resistance to PPQ may emerge from this region with time as DHA-PPQ use becomes widespread.

In this study we also reported a significant increase in the frequency of isolates with *Pfcrt* K76T mutation. Increasing prevalence of chloroquine resistance *Pfcrt* K76 wild type allele in Kenyan parasites is in line with findings by our earlier study (Eyase *et al*., 2013) and other studies in Kenya and elsewhere (Balikagala *et al*., 2020; Chebore *et al*., 2020; Lu *et al*., 2017). This finding has been attributed to the withdrawal of chloroquine and adoption of AL as a first-line drug which

selects for *Pfcrt* K76 allele and lumefantrine tolerance favoring return of chloroquine sensitive parasites (Eyase *et al*., 2013).

The frequencies of *Pfmdr*1 N86 allele in the analyzed samples remained largely unchanged over time which is contrary to what was reported in our earlier study (Eyase *et al*., 2013), and other studies in Western Kenya (Chebore *et al*., 2020), Angola, and Uganda (Dimbu *et al*., 2021; Ebong *et al*., 2021). Though our growth inhibition findings suggest decreasing sensitivity of parasites to lumefantrine, continuous surveillance of the *Pfmdr*1 N86 allele should be done using a large sample size to investigate their role in modulating lumefantrine resistance.

Plasmodium falciparum isolates susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine was established in this study using the malaria SYBR Green I based method. Despite reports of low PPQ resistance markers in the analyzed isolates, our *in vitro*/*ex vivo* growth inhibition assays (phenotype) data and findings from other studies in Uganda and Tanzania, have demonstrated high piperaquine treatment efficacy in Africa (Asua *et al.,* 2021; Gupta *et al*., 2020; Mwai *et al.*, 2009; Robert *et al*., 2018; Tumwebaze *et al*., 2021), supporting adoption of DHA-PPQ as alternative first-line drug for malaria treatment in Kenya and the East African region (Assefa *et al*., 2021).

The median piperaquine 50% inhibitory concentration (IC50) discerned by this study was 29.40 nM, which is below the resistance cut-of points of 135.00 nM reported in Mali (Traora *et al.,* 2019), and France (Pascual *et al*., 2013). Similarly, median IC_{50}s below the resistance cut-off have previously been reported in Uganda, Senegal, and Sierra Leone (Rasmussen *et al.,* 2017; Robert *et al*., 2018; Smith *et al*., 2018; Tumwebaze *et al.,* 2021), suggesting continued sensitivity of parasites from most regions of Africa to PPQ. Additionally, the per-period piperaquine IC_{50} between 2008 and 2021 remained low below resistance threshold suggesting sustained parasite susceptibility to PPQ (P=0.1615). This observation was consistent with previous studies across Africa (Rasmussen *et al*., 2017; Tumwebaze *et al*., 2021). In 2021, Tumwebaze and coworkers reported increasing PPQ sensitivity in isolates collected from Tororo and Busia Districts in Eastern Uganda (Tumwebaze *et al*., 2021). Moreover, studies done in the coastal site of Kilifi between 2005-2008 and 1995-2013, reported median IC_{50}s of 31.70 nM and 54.00 nM respectively suggesting marginally higher PPQ sensitivity (Mwai *et al*., 2009; Okombo *et al*., 2014). These

marginally higher IC_{50}s in Kilifi coincided with a period when ACT use had just been rolled out and could be associated with the presence of residual CQ resistant parasites that could harbor impaired PPQ response given the inherent molecular similarities between the latter, a bis-quinoline of the former. This could be evocative of genetic variations in drug resistance molecular markers between *P. falciparum* across transmission regions of Kenya, as previously reported elsewhere during the same period (Okombo *et al*., 2014). Continuous parasite susceptibility to PPQ in Kenya could be due to the low PPQ drug pressure, given that it had never been deployed in Kenya and the increasing frequency of chloroquine-sensitive isolates (Rasmussen *et al*., 2017). PPQ parasite response variation between Kisumu East and Malindi study sites could be attributed to the difference in disease burden geographical zones as suggested earlier by a study in Uganda (Francis *et al*., 2006).

The artemether-lumefantrine ACTs regimen was rolled out in sub-Saharan Africa more than a decade ago as a first-line regimen for the treatment of uncomplicated and has demonstrated good efficacy over time despite reports of artemisinin resistance in SEA and recently in East Africa (Balikagala *et al.,* 2021; Denis *et al*., 2006; Uwimana *et al*., 2021). However, reports of impaired sensitivity to this drug have been reported in the Cambodia-Thailand border and in Africa, thus raising concerns about the continued use of artemether-lumefantrine (Coartem®) (Bormann *et al*., 2011; Dimbu *et al*., 2021; Ebong *et al*., 2020). Chloroquine was previously used as a first-line drug and was withdrawn in 1998 due to widespread resistance. Since its withdrawal, chloroquine-sensitive parasites have emerged in Africa (Balikagala *et al*., 2020; Lu *et al*., 2017). Our findings on susceptibility analyses of Artemether and lumefantrine that are presently in use, in comparison with findings on CQ show a significant; increase in LM and ART alongside a reciprocal decrease in CQ median IC_{50} during the study period stretching between 2008 and 2021. These trends suggest decreasing *in vitro* susceptibility of parasites to LM and ART, although it remains below the WWARN resistance cut-off point of 115.00 nM and 12.00 nM, respectively (Pascual *et al*., 2013; Staehli *et al*., 2013), and increasing parasite's susceptibility to CQ as reported elsewhere in Africa (Balikagala *et al*., 2020; Lu *et al*., 2017; Tumwebaze *et al*., 2021). The observed sensitivity profile trend is due to the withdrawal of CQ drug pressure and increased use of ART and LM in Africa, which acts as selective pressure for parasites resistant to LM, and sensitive to piperaquine, and chloroquine in the region (Balikagala *et al*., 2020; Diakite *et al*., 2019; Lu *et al*., 2017). Consistent with findings from Uganda, no significant change in DHA susceptibility was depicted during the

study period (Rasmussen *et al.*, 2017; Tumwebaze *et al*., 2021). This was in agreement with reports of sustained artemisinin derivatives efficacy in Africa except Rwanda, suggesting that Kenyan circulating parasite populations are sensitive to artemisinin derivatives (Lu *et al*., 2017; Uwimana *et al*., 2021). However, recent reports of artemisinin-resistant parasite emergence in Uganda (Balikagala *et al*., 2021), warrants continued surveillance of artemisinin derivatives resistance in sub-Saharan Africa.

The development of resistance to a drug has been shown to affect the effectiveness of other drugs with similar chemical structure, and target proteins within the parasite (Francis *et al.*, 2006; Mungthin *et al.*, 2017). This study assessed correlation among the selected frontline antimalarial drugs and expectedly, a significant positive correlation in activities of DHA-ART drug pair was observed, suggesting that treatment failure of DHA will lead to ineffectiveness of ART and vice-versa (cross-resistance); these could be due to the similar mechanism of action (Francis *et al*., 2006; Mungthin *et al*., 2017). This was in line with earlier findings by Briolant and coworkers in a study that showed *in vitro* response correlation between ART and DHA (Briolant *et al.*, 2018; Denis *et al*., 2006). There was no significant association between the response of field isolates to piperaquine and the rest of the drugs suggesting that the emergence of resistance to any of these currently used partner drugs and artemisinin derivatives will not affect the efficacy of PPQ. This was in agreement with reports from earlier studies (Muangthin *et al*., 2017).

Piperaquine survival assay (PSA), described by Duru and colleagues was successfully adapted by our laboratory and it was used to assess PPQ susceptibility of field isolates collected from the Lake endemic sites of Kisumu, Kenya. This assay has been successfully used for earlier detection of piperaquine resistance in Cambodia (Duru *et al*., 2015). The accuracy of PSA in the determination of PPQ susceptibility makes the method feasible and deployable tool for sustainable surveillance of PPQ resistance. Studies have shown that PSA ≥ 10% corresponds to the PPQ-resistant phenotype (Duru *et al*., 2015). To the best of our knowledge, this is the first report on PSA assay analysis of clinical field isolates from sub-Saharan Africa. We showed that Kenyan field isolates have a median piperaquine survival rate of 0.0% which was below the established PPQ resistance cut-off mark of ≥10% (Duru *et al*., 2015). Up to 85% of the isolates tested by PSA had < 10% consistent with sensitivity to PPQ. However, isolates having PSA rate of ≥10% (PPQ resistance cut-off mark) remained susceptible to PPQ when analyzed using the *ex vivo/in vitro* malaria SYBR

Green I method, and did not harbor candidate PPQ resistant markers in their genome. These findings are contrary to the already reported ones in SEA, a region prone to PPQ-resistant parasites (Duru *et al.*, 2015; Hao *et al.*, 2013), suggesting that parasites from Kenya are sensitive to PPQ. Our analyses of IC_{50}s of those isolates with PSA ≥ 10% versus those with PSA < 10% showed that they were comparable, suggesting that sustained exposure of the parasites to drug in the *ex vivo* or *in vitro* assay, for the 72 h could be masking the stage-specific response to PPQ that is well assessed by the PSA.

Association between *ex vivo/in vitro* phenotype (PPQ sensitivity), and *Pfcrt* single nucleotide polymorphisms in the parasite genome was established by this study using the Kruskal-Wallis H-test and Mann-Whitney U test. *P. falciparum* transporters; *Pfcrt*, and *Pfmdr1* SNPs have been linked to amino quinolines resistance globally (Smith *et al*., 2018), while *Pfdhps* and *Pfdhfr* are associated with sulphadoxine-pyrimethamine resistance (Chenet *et al*., 2017). Notably, *Pfcrt* 76T SNP that is associated with CQ susceptibility correlated with PPQ sensitivity. *Pfcrt* SNPs were shown to affect parasite response to PPQ, K76T mixed genotype conferred higher PPQ IC_{50}s than the wild type contrary to earlier findings (Koleala *et al*., 2015; Rasmussen *et al.*, 2017). However, a significant association between *Pfcr*t 76T SNP, and *in vitro* PPQ response (IC50s) appear to argue for the role of *Pfcrt* SNPs in modulating parasite response to PPQ. Importantly, this study did not find an association between PPQ resistance mutations (*Pfpm*2/3, and *Pfmdr*1) and neither *in vitro/ex vivo* IC_{50}s further suggesting sensitivity of Kenyan field isolates to piperaquine. Moreover, there was no association between PPQ sensitivity and *Pfmdr*1 N86, 184F, *Pfdhps*, and *Pfdhfr* polymorphisms that confer resistance to LM, MQ and SP, respectively as previously reported in Africa and SEA (Chenet *et al*., 2017; Koleala *et al*., 2015; Smith *et al*., 2018), therefore suggesting PPQ susceptibility in malaria-endemic countries prone to LM, and CQ resistance. Absence of a significant association between PPQ and SP resistance mutations (*Pfdhps* and *Pfdhfr*), suggests that SP resistance parasites are sensitive to PPQ, and this further supports the adoption of DHA-PPQ as the next drug for seasonal malaria chemoprevention in sub-Saharan Africa areas prone to SP resistance (Gupta *et al*., 2020).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

All the four objectives of this study were achieved, and from its findings we conclude that;

i. Absence of polymorphisms in *Pfcrt, Pfpm2/3* and *Pfmdr1* that confer resistance to frontline antimalarial drugs suggests continued susceptibility to frontline drugs although this warrants sustained antimalarial drug resistance surveillance using *in vitro/ex vivo* and genomic analyses

ii. The currently used antimalarials in Kenya namely; lumefantrine, artemether, dihydroartemisinin, and piperaquine alongside chloroquine remain efficacious against *P. falciparum* uncomplicated malaria.

iii. Circulating Kenyan *P. falciparum* parasites are sensitive to piperaquine using PSA assay suggesting the benefits of its use as an alternative first-line drug and PSA is applicable and deployable in Kenya for monitoring PPQ sensitivity.

iv. Positive association between piperaquine *in vitro* sensitivity (IC_{50}) and *Pfcrt* K76T polymorphism suggests the role of this mutation in piperaquine resistance. However, this should be investigated further using gene editing tools.

6.2 Recommendations

i. Screening for genetic polymorphisms in other genes namely; *Pfcrt, Pfexo,* and *Pfk*13 that are associated with piperaquine resistance should be conducted using molecular and growth inhibition assays optimized by this study that are less costly and sustainable for real-time tracking of PPQ resistance.

ii. Current dihydroartemisinin-piperaquine (Duo-cotecxin®) implementation in the Country as an alternative first-line regimen should continue, and chloroquine should be reintroduced in combination with another drug in sub-Saharan Africa for treatment of uncomplicated malaria as evidenced by the return of chloroquine sensitive parasites in the region.

iii. Continued surveillance of the efficacy of currently used antimalarials namely; artemether, lumefantrine, dihydroartemisinin and piperaquine, in Africa using our established genotypic and phenotypic tools should be embraced for early detection of parasite resistance to frontline antimalarial drugs, and piperaquine survival assay (PSA) a more accurate technique than malaria SYBR Green I assay should be adopted for surveillance of piperaquine resistance in sub-Saharan Africa alongside genomic analyses.

iv. Associations between *Pfcrt* K76T allele and piperaquine sensitivity alongside the role of *Pfmdr*1 N86 allele in modulating lumefantrine resistance should be investigated further using a large sample size.

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APPENDICES

Appendix I: Master Mix for PCR reactions

Master Mix for one multiplex PCR reaction

Reagents	Volume for one reaction (µl)
Taqman multiplex	10
Pfmdr1 forward primer (5′- TGCATCTATAAAACGATCAGACAAA-3′)	1
Pfmdr1 reverse primer (5′-TGGTTCAAGGTATTGTTTAGGTTC-3′)	1
Pfmdr1 probe (NED-NMFQ) [5′ NED-TTTAATAACCCTGATCGAAATGGAACCTTTG-3′-NMFQ]	1
Pfpm2 forward primer (5′-ATGGTGATGCAGAAGTTGGA-3′)	1
Pfpm2 reverse primer (5′AACATCCTGCAGTTGTACATTTAAC-3′)	1
Pfpm2 probe (FAM-NMFQ) [5′FAM-CAGGATCTGCTAATTTATGGGTCCCA-3′-NMFQ]	1
Pfβ-tubulin forward primer (5′-TGATGTGCGCAAGTGATCC-3′)	1
Pfβ-tubulin reverse primer (5′-TCCTTTGTGGACATTCTTCCTC-3′)	1
Pfβ-tubulin probe (VIC-NMFQ) [5′VIC-TAGCACATGCCGTTAAATATCTTCCATGTCT-3′-NMFQ]	1
Deionized water	2

Master Mix for one duplex PCR reaction

Reagents	**Volume for one reaction (µl)**
Taqman multiplex	10
Pfpm3 forward primer (5′- CCACTTGTGGTAACACGAAATTA-3′)	1
Pfpm3 reverse primer (5′-TGGTTCAAGGTATTGTTTAGGTTC-3′)	1
Pfpm3 probe (FAM-NMFQ) [5′Fam-CCAACACTCGAATATCGTTCACCAA-3′NMFQ]	1
Pfβ-tubulin forward primer (5′-TGATGTGCGCAAGTGATCC-3′)	1
Pfβ-tubulin reverse primer (5′-TCCTTTGTGGACATTCTTCCTC-3′)	1
Pfβ-tubulin probe (VIC-NMFQ) [5′VIC-TAGCACATGCCGTTAAATATCTTCCATGTCT-3′NMFQ]	1
Deionized water	2

Appendix 11: IPLEX MassARRAY PCR primer sequences

Primary amplification primer sequences for various SNPs

SNP ID	Forward primer sequence (5′−3′)	Reverse primer sequence (5′−3′)
Pfcrt 76	ACGTTGGATGTTACCTGTATACACCC	ACGTTGGATGGTTTCGGATGTTACAAA
Pfcrt 356	ACGTTGGATGCTTACGGCTAAGAATT	ACGTTGGATGCGACAAATTTTCTACCAT
Pfcrt 371	ACGTTGGATGTCACACTTACCAAAGT	ACGTTGGATGCTTTTTAATTTTATAGGG
Pfmdr1 86	ACGTTGGATGTTATCAGGAGGAACA	ACGTTGGATGTATAGGATTAATATCAT
Pfmdr1	ACGTTGGATGCGGAAAAACGCAAGT	ACGTTGGATGCATATGCCAGTTCCTTTT
Pfmdr1	ACGTTGGATGTGTAAATGCAGCTTTA	ACGTTGGATGGAAGGATCCAAACCAAT
Pfdhps 436	ACGTTGGATGTGAAGGTGCTAGTGTT	ACGTTGGATGGGTACTACTAAATCTCTT
Pfdhps 437	ACGTTGGATGCTTTATTTGAGTAACA	ACGTTGGATGCCACTTCAACTATATCA
Pfdhps 581	ACGTTGGATGGGATACTCATCATATA	ACGTTGGATGCTCGTTATAGGATACTAT
Pfdhps 613	ACGTTGGATGGTATATGATGAGTATC	ACGTTGGATGCAACATTTTGATCATTCA
Pfdhfr 59	ACGTTGGATGGGAGTATTACCATGG	ACGTTGGATGGATTCATTCACATATGTT
Pfdhfr 164	ACGTTGGATGGATCTAATAGTTTTAC	ACGTTGGATGTCTTGATAAACAACGGA

Allele-specific extension primer sequences for various SNP alleles

SNP ID	Alleles	Unextended primer (UEP) sequence (5′−3′)
Pfcrt 76	A/C	GTTTAAAGTTCTTTTAGCAAAAATT
Pfcrt 356	T/C	CCGTATACAAGGTCCAGCAA
Pfcrt 371	G/T	ATTTTATAGGGTGATGTTGTAA
Pfmdr1 86	A/T	TAGGATTAATATCATCACCTAAAT
Pfmdr1 184	A/T	CCAGTTCCTTTTTAGGTTTAT
Pfmdr1 1034	A/T	TAATTGAGCGCTTTGAC
Pfdhps 436	C/T	GATATAGGTGGAGAATCCT
Pfdhps 437	G/C	TATAGGTGGAGAATCCTCTG
Pfdhps 581	C/G	ACTATTTGATATTGGATTAGGATTTG
Pfdhps 613	G/A/T	TTTTGATCATTCATGCAATGGG
Pfdhfr 59	T/C	ATTCATTCACATATGTTGTAACTGCAC
Pfdhfr 164	A/T	CCCTAAACAACGGAACCTCCTA

APPENDIX III: **Ethical approval**

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 April 25, 2018

TO: DR. BEN ANDAGALU,
PRINCIPAL INVESTIGATOR

THROUGH: THE DIRECTOR, CCR,
NAIROBI

02/05/2018

Dear Sir,

RE: SERU PROTOCOL NO. 3628 *(RESUBMISSION OF INITIAL SUBMISSION):* EPIDEMIOLOGY OF MALARIA AND DRUG SENSITIVITY PATTERNS IN KENYA

Reference is made to your letter dated March 28, 2018 The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised application on April 6, 2018.

The SERU Secretariat acknowledges receipt of the following documents:

a. WR2454_MDR_protocol_v1.7 dated 12th March 2018, clean and tracked
b. WR2454_MDR_AdultParent_consent v1.5 MoH_12Mar2018 clean and tracked
c. WR2454_MDR_AdultParent_consent_KDF_v 1.0 dated_12Mar2018 clean
d. Removal letter of Maj Jacob Johnson
e. Appendix 1_attempts to find Maj Jacob Johnson
f. Local trabslations of the ICFs, Luo, Kiswahili, Kisii, Kipsigis
g. Certificates of translations for the ICFs.

This is to inform you that the Committee notes that the issues raised during the 272nd Committee B meeting of the SERU held on **February 21, 2018** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **April 25, 2018** for a period of one year. Please note that authorization to conduct this study will automatically expire on **April 24, 2019.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **March 13, 2019**.

You are required to submit any proposed changes to this study to the SERU for review and the changes should not be initiated until written approval from the SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise the SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

FOR: THE HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

APPENDIX IV: Publication

Wakoli et al. BMC Medicine (2022) 20:448
https://doi.org/10.1186/s12916-022-02652-2

BMC Medicine

RESEARCH ARTICLE Open Access

Impact of parasite genomic dynamics on the sensitivity of *Plasmodium falciparum* isolates to piperaquine and other antimalarial drugs

Dancan M. Wakoli[1,2*], Bartholomew N. Ondigo[1,3], Douglas O. Ochora[4], Joseph G. Amwoma[2,5], Winnie Okore[2,6], Edwin W. Mwakio[2], Gladys Chemwor[2], Jackeline Juma[2], Raphael Okoth[2], Charles Okudo[2], Redemptah Yeda[2], Benjamin H. Opot[2], Agnes C. Cheruiyot[2], Dennis Juma[2], Amanda Roth[2], Benhards R. Ogutu[2], Daniel Boudreaux[2], Ben Andagalu[2] and Hoseah M. Akala[2*]

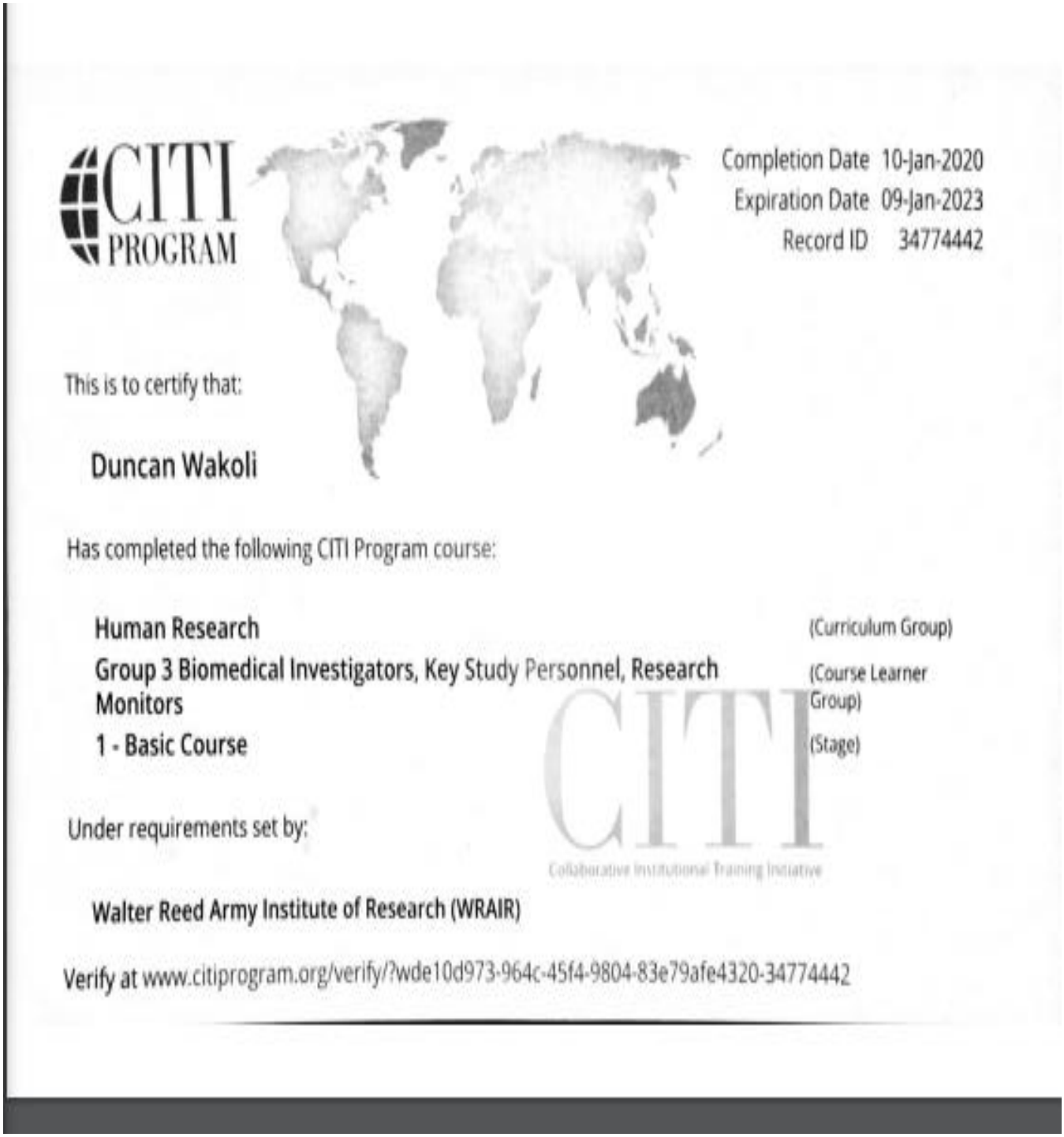
Abstract

Background: Dihydroartemisinin-piperaquine (DHA-PPQ) is an alternative first-line antimalarial to artemether-lumefantrine in Kenya. However, recent reports on the emergence of PPQ resistance in Southeast Asia threaten its continued use in Kenya and Africa. In line with the policy on continued deployment of DHA-PPQ, it is imperative to monitor the susceptibility of Kenyan parasites to PPQ and other antimalarials.

Methods: Parasite isolates collected between 2008 and 2021 from individuals with naturally acquired *P. falciparum* infections presenting with uncomplicated malaria were tested for in vitro susceptibility to piperaquine, dihydroartemisinin, lumefantrine, artemether, and chloroquine using the malaria SYBR Green I method. A subset of the 2019–2021 samples was further tested for ex vivo susceptibility to PPQ using piperaquine survival assay (PSA). Each isolate was also characterized for mutations associated with antimalarial resistance in *Pfcrt*, *Pfmdr1*, *Pfpm2/3*, *Pfdhfr*, and *Pfdhps* genes using real-time PCR and Agena MassARRAY platform. Associations between phenotype and genotype were also determined.

Results: The PPQ median IC_{50} interquartile range (IQR) remained stable during the study period, 32.70 nM (IQR 20.2–45.6) in 2008 and 27.30 nM (IQR 6.9–52.8) in 2021 (P=0.1615). The median ex vivo piperaquine survival rate (IQR) was 0% (0–5.27) at 95% CI. Five isolates had a PSA survival rate of ≥10%, consistent with the range of PPQ-resistant parasites, though they lacked polymorphisms in *Pfmdr1* and *Plasmepsin* genes. Lumefantrine and artemether median IC_{50}s rose significantly to 62.40 nM (IQR 26.9–100.8) ($P = 0.0201$); 7.00 nM (IQR 2.4–13.4) ($P = 0.0021$) in 2021 from 26.30 nM (IQR 5.1–64.3); and 2.70 nM (IQR 1.3–10.4) in 2008, respectively. Conversely, chloroquine median IC_{50}s decreased significantly to 10.30 nM (IQR 7.2–20.9) in 2021 from 15.30 nM (IQR 7.6–30.4) in 2008, coinciding with a

APPENDIX V: Collaborative Institutional Training Initiative Certificate



CITI PROGRAM

Completion Date 10-Jan-2020
Expiration Date 09-Jan-2023
Record ID 34774442

This is to certify that:

Duncan Wakoli

Has completed the following CITI Program course:

Human Research (Curriculum Group)
Group 3 Biomedical Investigators, Key Study Personnel, Research Monitors (Course Learner Group)
1 - Basic Course (Stage)

Under requirements set by:

Walter Reed Army Institute of Research (WRAIR)

Verify at www.citiprogram.org/verify/?wde10d973-964c-45f4-9804-83e79afe4320-34774442

APPENDIX VI: National Commission for Science, Technology and Innovation Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **600571**

Date of Issue: **31/March/2023**

RESEARCH LICENSE

This is to Certify that Mr.. Duncan Musamali Wakoli of Egerton University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Baringo, Kericho, Kisii, Kisumu on the topic: ASSESSMENT OF Plasmodium falciparum PIPERAQUINE RESISTANCE IN KENYA USING MOLECULAR MARKER ANALYSES AND GROWTH INHIBITION ASSAYS for the period ending : 31/March/2024.

License No: **NACOSTI/P/23/23988**

600571

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY &
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