PREVALENCE OF *Plasmodium falciparum* KELCH 13-PROPELLER REGION, *PF*AP2-MU, *PF*CRT, AND *PF*MDR1 MUTATIONS ASSOCIATED WITH RESISTANCE TO ARTEMISININ-BASED COMBINATION THERAPIES AND CHLOROQUINE IN NYANDO, KISUMU COUNTY

KELVIN BRIAN MUSYOKA

MASTER OF SCIENCE

(Molecular Biology and Bioinformatics)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2021

Prevalence of *Plasmodium falciparum* kelch 13-propeller region, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 mutations associated with resistance to artemisinin-based combination therapies and chloroquine in Nyando, Kisumu County

Kelvin Brian Musyoka

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Bioinformatics of the Jomo Kenyatta University of Agriculture and Technology

2021

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature ……..……. Date ……..

Kelvin Brian Musyoka

This thesis has been submitted for examination with our approval as university supervisors.

Signature ……. Date…….……...

Dr. Joseph K. Nganga, PhD

Department of Biochemistry

JKUAT, Kenya

Signature…….. Date…….…..…….....

Dr. Daniel Kiboi, PhD

Department of Biochemistry

JKUAT, Kenya

Signature…… Date…….....

Mr. Francis Kimani T.

Centre for Biotechnology Research and Development

Kenya Medical Research Institute, Kenya

DEDICATION

This thesis is dedicated to my mother and father for their unlimited support and inspiration.

ACKNOWLEDGEMENT

First and foremost, I thank the Almighty God for His guidance, wisdom, patience, and grace that saw me through this course.

Second, the work presented herein would not be possible without the help and support from many people I would like to thank. I would like to thank my project supervisors Dr. Joseph Nganga and Mr. Francis Kimani, for their immense guidance, patience, and support throughout my project.

Special thanks to Dr. John Kiiru and Dr. Daniel Kiboi for your kind understanding and guidance throughout my project.

I would like to thank Protus Omondi, John Lukoye, Kelvin Thiongo, Terry Judah, and Susan Kiiru for Laboratory work assistance.

Thank you to all children from Nyando in Kisumu County who took part in the drug clinical trial, thus providing samples for this study.

Thank you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin Combination Therapies
AL	Artemether-Lumefantrine
AQ	Amodiaquine
CQ	Chloroquine
CQR	Chloroquine Resistance
CNV	Copy Number Variation
DEAQ	Desethylamodiaquine
DNA	Deoxyribonucleic Acid
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteorate Synthetase
HCl	Hydrochloric acid
INDELS	Insertions-Deletions
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Mosquito Net
IQR	Inter-Quartile Range
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
NaOH	Sodium hydroxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
***Pf*ap2mu**	*Plasmodium falciparum* clathrin adaptor protein 2 encoding the mu sub-unit
***Pf*crt**	*Plasmodium falciparum* chloroquine transporter gene
***Pf*mdr1**	*Plasmodium falciparum* multi-drug resistant gene
ROS	Reactive Oxygen Species
SERU	Scientific Ethics Review Unit
SNP	Single Nucleotide Polymorphism
SP	Sulphadoxine-Pyrimethamine

WHO World Health Organisation

ABSTRACT

The treatment and eradication of malaria disease is still a major challenge due to the acquisition and spread of resistant parasites to the antimalarial drug in use. Resistance to the mainstay antimalarial drugs is a significant concern in the control of malaria. Delayed *Plasmodium falciparum* parasite clearance following artemisinin-based combination therapy (ACT) administration has been associated with Single Nucleotide Polymorphisms (SNPs) in the kelch 13 propeller region (k13). However, SNPs in the *Pf*-adaptor protein complex two mu subunit (*Pf*ap2-mu), *Pf*crt, and *Pf*mdr1 are possible markers associated with multi-drug resistance. This study aimed at establishing the prevalence of mutations in the kelch 13 propeller region, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 gene(s) associated with resistance to ACT and chloroquine (CQ) antimalarial drugs in Nyando, Kisumu County. Moreover, the study determined the functional implications of mutation present in the k13 gene of *P. falciparum* based on the structural analysis. A total of 94 dried blood spot field isolates collected from children aged below 12 years infected with *P. falciparum* during a cross-sectional study were utilized in this study. The samples were collected in 2015 during the peak malaria transmission season in the Nyando region of Western Kenya before treatment with Artemether-lumefantrine (AL), the first-line artemisinin-based combination therapy (ACT) in Kenya. However, 47 of the 94 samples had recurrent parasitemia and were interrogated for the SNPs' presence in k13 and *Pf*ap2-mu. The PCR amplification and sequencing were used to evaluate specific regions of k13 (codons 432–702), *Pf*ap2-mu (codons 1–350), *Pf*mdr1 (codons 86, 1034–1246), and *Pf*crt (codons 72–76) gene(s). The majority of parasites harbored the wild-type k13 sequence. However, one unique non-synonymous W611S change was detected. *In silico* studies on the impact of the W611S predicted structural changes in the overall topology of the k13 protein. Of the 47 samples analyzed for SNPs in the *Pf*ap2-mu gene, 14 (29%) had S160N mutation. The CVIET haplotype associated with CQ resistance in the *Pf*crt yielded a 7.44% (7/94), while the CVMNK haplotype was at 92.56%. Mutations in the *Pf*mdr1 region were detected only in three samples (3/94; 3.19%) at codon D1246Y. This study suggests that parasites in the western part of Kenya harbor wild-type strains and might be susceptible to AL and CQ antimalarial drugs. However, detecting the unique SNP in k13 and *Pf*ap2-mu linked with ACT delayed parasite clearance may suggest slow filtering of AL resistant parasites. In this view, the study recommends continued surveillance to monitor the possibility of unprecedented ACT resistance in the future.

Keywords: *Plasmodium falciparum,* ACT, Delayed Clearance, SNPs, *Pf*ap2mu, *Pf*K13

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Globally, malaria disease caused by the genus *Plasmodium's* apicomplexan parasite remains among the most significant and communicable parasitic tropical diseases. The most predominant malaria parasites that infect humans are *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale*, *Plasmodium malariae,* and the *Plasmodium knowlesi* (WHO, 2019). Other new species that infect humans are *Plasmodium cynomolgi* and *Plasmodium simium* (Brasil *et al.,* 2017; Hartmeyer *et al.,* 2019). *Plasmodium falciparum* is the most lethal species out of these malaria parasites, which causes most malaria cases in sub-Saharan Africa. In Kenya, 3,605,498 malaria cases and 12,416 associated deaths have been reported and contribute to a total of 228 million cases and 405,000 deaths reported globally (WHO, 2019). The treatment, elimination, and projected eradication of malaria disease is still a growing challenge primarily due to the quick emergence and spread of resistant parasites against the mainstay antimalarial drugs (Haldar *et al.,* 2018). To date, treatment for uncomplicated malaria infection relies on artemisinin-based combination therapies (ACT). The rationale behind ACT usage is that it contains a fast-acting artemisinin moiety and a slow-acting partner drug with a longer half-life specifically targeted to eliminate the residue parasite load (Nosten & White, 2007). Over the last decade, many countries in Africa, including Kenya, adopted Artemether-Lumefantrine (AL) as the first-line ACT while dihydroartemisinin-piperaquine (DHA/PQ) as the second-line combination (WHO, 2019).

Historically, low malaria transmission areas of the Thai-Cambodia border in the South-East Asia region have been the epicenter of antimalarial drug-resistant parasites and subsequently spread to sub-Saharan Africa (Haldar *et al.,* 2018). Resistance to previously mainstay drugs such as chloroquine and

sulfadoxine/pyrimethamine originated from the South-East Asia region and spread to sub-Saharan Africa (Anderson & Roper, 2005). Although most artemisinin derivatives are effective against multidrug-resistant *P. falciparum*, treatment failures of DHA/PQ, the first-line ACTs in Cambodia (Amato *et al.*, 2017), present a worrying trend for elimination and eradication of malaria globally. Also, a rapid spread of the multi-drug resistant parasites and dominance of the mutant parasite lineage from Cambodia to Laos and Vietnam (Hamilton *et al.*, 2019). The expansion of these resistant lineages underscores the need for continuous surveillance of the emergence and potential spread of drug-resistant *P. falciparum*, especially in Africa, which has inherited drug-resistant parasites over the years from the South East Asia belt.

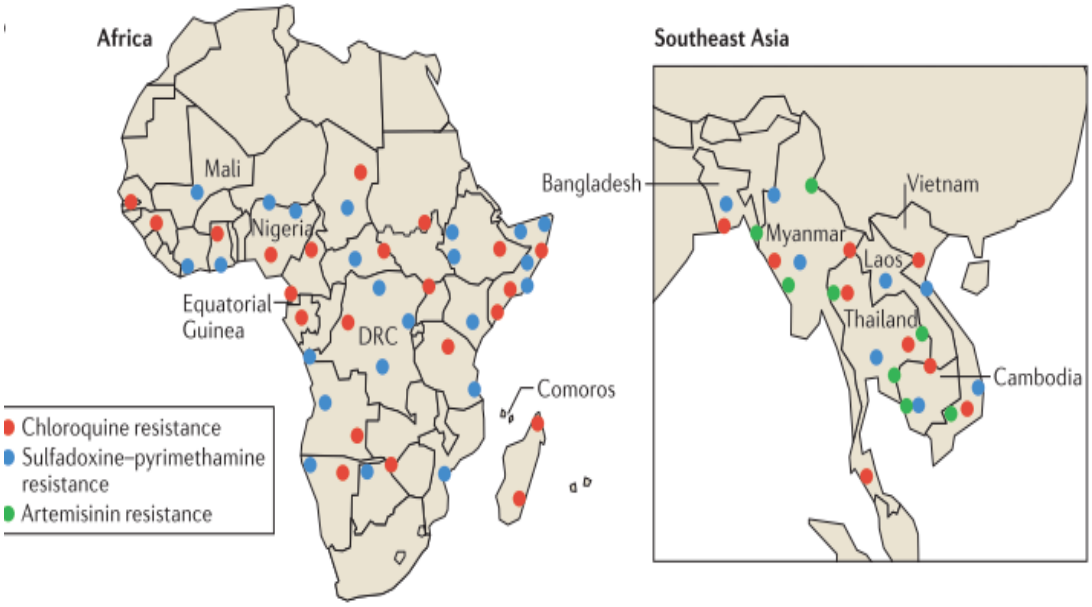


Figure 1.1: Detailed maps showing the distribution of P. falciparum resistance to different antimalarial drugs. Each dot represents a region of the emergence of drug resistance (Haldar et al., 2018).

Mutations exist on essential parasite transporters and other proteins in the *P. falciparum*, such as chloroquine resistance transporter (CRT), multidrug resistance protein 1 (mdr1), and *kelch13 (K13)*, are associated with mediating multi-drug

resistance (Miotto *et al.,* 2015). Studies in the Greater Mekong Sub-region of Cambodia, Myanmar, Thailand, and Vietnam associated mutations in the kelch13 gene of the *P. falciparum* with artemisinin resistance (Ariey *et al.,* 2014; Miotto *et al.,* 2015). Recent studies in Kenya revealed that residual *P. falciparum* parasites following ACT treatment are associated with delayed parasite clearance (Beshir *et al.,* 2013), suggesting a possible selection of drug-resistant parasite lines. Molecular analysis of the residual *P. falciparum* parasite population linked polymorphisms in at least four genes; *Pf*crt, *Pf*mdr1, the *P. falciparum* mμ-subunit of clathrin-associated adaptor protein 2 *(Pf*ap2mu*),* and *P. falciparum* ubiquitin carboxyl-terminal hydrolase 1 (*Pf*ubp1*)* with the slow filtering of the less susceptible parasites to the ACTs (Henriques *et al.,* 2014a).

To date, at least five SNPs, namely, M476I, Y493H, R539T, I543T, and C580Y within the K13 gene propeller region, are known to mediate artemisinin resistance (Straimer *et al.,* 2015). Mutations in ap2-mu, a gene involved in clathrin-mediated endocytosis, and hemoglobin transport in malaria parasites were initially mapped using artemisinin-pressured *Plasmodium chabaudi* clones (Henriques *et al.,* 2013). Subsequent analysis of the *P. falciparum* field isolates surviving ACT treatment showed a directional selection of a Ser160Asn/Thr mutation in the *Pf*ap2-mu (Henriques *et al.,* 2014a). Mutations in the *Pf*crt protein are associated with reduced chloroquine susceptibility and background control of artemisinin resistance (Fidock *et al.,* 2000; Miotto *et al.,* 2015). There seems to be a reciprocal resistance between CQ and lumefantrine (LM) at codon 76 of the *Pf*crt protein (Mwai *et al.,* 2009). Thus, the introduction of LM through the AL combination may select CQ wild-type parasites at codon 76 of the *Pf*crt protein.

Different studies carried out between 2011 -2015 in Kenya using *Pf*crt (CVMNK haplotype) and *Pf*mdr1 (N86 and D1246) markers associated with reduced susceptibility to lumefantrine in the AL showed an increasing trend (Venkatesan *et*

al., 2014; Achieng *et al.*,2015), suggesting a subtle emergence of AL resistant parasites. The Western part of Kenya was the initial focal point for CQ resistance in Africa (Fogh *et al.*,1979; Achieng *et al.*, 2015). Therefore, monitoring old and newly discovered molecular markers associated with *P. falciparum* delayed clearance after AL or other ACT treatment would indicate the emergence of resistance in Kenya. In this study, besides assessing the prevalence of known SNPs in the *Pf*crt and *Pf*mdr1 gene, the research has also established the SNPs' prevalence level in the kelch propeller region *Pf*ap2-mu in *P. falciparum* parasite isolates from Nyando, Kisumu County, a malaria-endemic area of the Western part of Kenya. The functional implication of the mutation mapped in the K13 propeller region was analyzed using *in silico* structural approach.

1.2 Statement of the Problem

Malaria treatment relies on antimalarial drugs since they are the most effective interventions in preventing and controlling *Plasmodium* parasites. The available vaccine, Mosquirix (RTS, S), has proved to be effective among children under the age of five years in clinical trials, while other vaccines are still under development (Palacpac & Horii, 2020). Increasing rates of transmission of parasite strains resistant to antimalarial drugs have prompted drug adoption changes in the country. Artemisinin, a rapidly acting antimalarial agent, has been the foundation for the treatment of malaria globally. However, clinical resistance to artemisinin and its derivatives has been reported in Southeast Asia (Dondorp *et al*., 2009; Ariey *et al*., 2014). Southeast Asia has long been considered the epicenter of antimalarial drug resistance; resistance to sulphadoxine-pyrimethamine, mefloquine chloroquine, proguanil, and piperaquine has surfaced there and spread globally. This is devastating as there is presently no further well-established treatment options alternative to ACTs.

Interestingly, there has been reduced ACT responsiveness in the Kenyan population (Kamau *et al.,* 2015; Beshir *et al.,*2017). Different studies show that the *Pf*ap2-mu variant alleles are more prevalent following AL post-treatment in Africa and could be a novel candidate gene for tracking artemisinin resistance (Henriques *et al.,* 2014b; Sutherland *et al.,* 2017). If artemisinin resistance were to spread to sub-Saharan Africa, it could have a tremendous impact on malaria-related morbidity and mortality. Thus, this study determined the prevalence of crucial SNPs in the *Pf*crt, *Pf*mdr, *Pf*ap2-mu, and screen for the presence or absence of mutations in the *Pf*k13 gene in conjunction with structural analysis of *Pf*k13 protein to evaluate the functional implications of mutations present.

1.3 Justification of the Study

Malaria remains amongst the infectious disease of public health concern in Kenya and even globally. *Plasmodium falciparum* is the most leading cause of the associated malaria cases. This cause might be attributed to many factors, but the key is the parasite's ability to develop resistance to antimalarial drugs (WHO, 2019). Treatment success with ACT largely depends on the current level of parasite tolerance to the recommended antimalarial drug. Therefore, molecular markers are used in population surveillance to monitor drug efficacy. The resistance markers for CQ and AL have been validated through various studies. Therefore, since these markers' prevalence is synonymous with malaria spread, the determination of these markers' prevalence would be a good measure of malaria treatment epidemiology and would go a long way in informing the necessary malaria treatment policies.

1.4 Null Hypothesis

Plasmodium falciparum parasite isolated from patients residing in Nyando, Kisumu County does not harbor mutations at kelch 13 propeller regions, *Pf*ap2-mu, *Pf*crt *Pf*mdr1 gene(s) associated with resistance to different antimalarial drugs.

1.5 Research Questions

i) What is the prevalence of the mutations in Kelch 13 and *Pf*ap2mu (S160N/T) genes *Plasmodium falciparum* associated with ACTs antimalarial drug resistance in Nyando?

ii) What is the prevalence of the mutation in *Pf*crt (K76T) and *Pf*mdr (N86Y, N1042D, and D1246Y) genes of *Plasmodium falciparum* associated with chloroquine and possible ACTs antimalarial drug resistance in Nyando?

iii) What are the functional implications of mutation present in the k13 gene based on In silico structural analysis?

1.6 Objectives

1.6.1 General Objective

To establish the prevalence and functional implications of mutations in the kelch 13 propeller region, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 gene(s) associated with resistance to ACTs and chloroquine Nyando, Kisumu County.

1.6.2 Specific Objectives

1. To determine the prevalence of mutations in Kelch 13 and *Pf*ap2mu (S160N/T) genes associated with ACTs antimalarial drug resistance in Nyando.

2. To determine the prevalence of mutations in *Pf*crt (K76T) and *Pf*mdr (N86Y, N1042D, and D1246Y) genes associated with chloroquine and possible ACTs antimalarial drug resistance in Nyando.

3. To structurally analyze, In silico, the functional implication of mutation present in the k13 gene.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

2.1.1 Geographical distribution

Malaria is among the most infectious disease of public health worry in countries where transmission repeatedly occurs and in areas where transmission has mainly been controlled or eliminated (Bates *et al.,* 2004). According to World Health Organisation (WHO) statistics, the number of malaria cases fell from an estimated 262 million cases in 2000 to 216 million in 2017, while the number of malaria-associated deaths fell to 445,000 from 839,000 in 2000. However, malaria-associated deaths and cases remain >80% African region. In Kenya, 3,605,498 million malaria cases and 12,416 deaths were reported in Kenya (WHO, 2019).

Malaria transmission occurs in tropical and subtropical regions in sub-Saharan Africa, Central and South America, the Caribbean island, the Indian, and South-East Asia (Bloland, 2001). The heterogeneity in malaria transmission in Kenya is a consequence of the sizeable Spatio-temporal multiplicity in Kenya's climate and ecology (Depinay *et al.*,2004; Kelly-hope *et al.,* 2009). Low temperatures delay parasite development within the mosquito, i.e., Extrinsic Incubation Period, contributing to reduction or no transmission of malaria.

Another factor observed in regions with no malaria transmission is the reduced moisture index. For instance, the areas of Lokichokio, which is on the extreme North-western part of Kenya, experience a low degree of moisture index. Human activities and the atypical climatic condition related to arid and semi-arid areas comprise a significant factor contributing to unpredictable and unstable malaria transmission in Garissa, Marsabit, Moyale, and Lodwar areas (Bayoh *et al.,* 2011).

The high malaria transmission regions in Kenya frequently occur around the Lake Victoria basin in Nyanza, Western, and coastal areas. Their main characteristics are high moisture index and high temperatures, which reduce the parasite's extrinsic incubation period and boost the vector reproduction rate (Imbahale *et al.*, 2012).

Some of the persistent challenges in malaria elimination include insecticide resistance, antimalarial drug resistance, the economic burden of malaria on health systems, improver infrastructure in health systems in countries with the most significant malaria burden, and gaps in intervention coverage (WHO, 2019). Among these factors, antimalarial drug resistance has also been associated with increased risk of anemia, low birth weight, and increased malaria transmission/reappearance. This is due to the development and spread of malaria parasites' drug-resistant strains, making it difficult to control malaria transmission.

2.1.2 Malaria

Mosquitoes are vectors of medical significance to spread arboviruses, protozoans, nematodes, and bacteria. Human malaria is a hematoprotozoan parasitic infection transmitted by female mosquitoes of the genus *Anopheles*, several species (Sinka *et al.*, 2010; White, 2014). Four genus *Plasmodium* species cause human malaria: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, while the simian malaria is caused by *P. knowlesi.* Among the causing human species, the *P. falciparum* is the most lethal species due to its aptitude to sequestrate in blood vessels and dodging the immune system. The fight against malaria relies mostly on chemotherapy and chemoprophylaxis. However, resistance to currently available antimalarial drugs has hugely reduced these drugs' efficiency (Aminake & Pradel, 2013).

Malaria infection can be diagnosed using numerous methods with different levels of specificity and sensitivity. The standard techniques are antigen detection (“dipstick test”), molecular tests (Polymerase Chain Reaction), clinical diagnosis (not preferred

since it can lead to misdiagnosis), and microscopic examinations (Bloland, 2001). Since microscopy has the highest sensitivity and specificity, it has become the gold standard for diagnosing malaria in infected individuals.

2.1.3 Parasite Life Cycle

The life-cycle of malaria parasites involves a cyclo-propagative transmission cycle, which requires two hosts to complete. The female *Anopheles* mosquitoes are the definitive host for the parasite (Aminake & Pradel, 2013). The infection with *P. falciparum* starts when infective sporozoites are injected into the human host by the female *Anopheles* mosquito while ingesting a blood meal. The sporozoites invade the host liver (hepatocytes) following transportation via the peripheral circulation system. In the liver, sporozoites undergo asexual development and result in the production of approximately 30,000 infective merozoites. The merozoites will then recognize and invade red blood cells (Biamonte, Wanner, & Le, 2013). The parasite develops into ring stages and subsequently develops into proliferative and morphologically distinct trophozoites and schizonts. After two days, the schizonts rupture and release 32 daughter merozoites to initiate a new infection to red blood cells. The fever experienced after every two days coincides with schizonts' rapturing (Haldar *et al.*, 2018).

After several cycles of asexual reproduction, some parasites' ring stages further differentiate into male and female gametocytes, containing only a half set of chromosomes. When ingested by mosquitoes, the male and female gametocytes fuse in the midgut to form a zygote. The zygote develops into an invasive ookinete that now penetrates the gut epithelium and develops into an oocyst (Biamonte, Wanner, & Le, 2013). The oocyst sexual replication results in the production of approximately 10,000 infective sporozoites. The sporozoites are then released into the hemocoel and migrate to the salivary glands of the mosquito. The sporozoites mature and wait until the mosquito bites a new host for transmission to occur (Aminake & Pradel, 2013;

Biamonte, Wanner, & Le, 2013). Suppose the parasites inoculated into the human host are mutants. In that case, the resistance of those parasites increases in the number and threatens the available drugs used in malaria treatment, thus posing a public health concern.

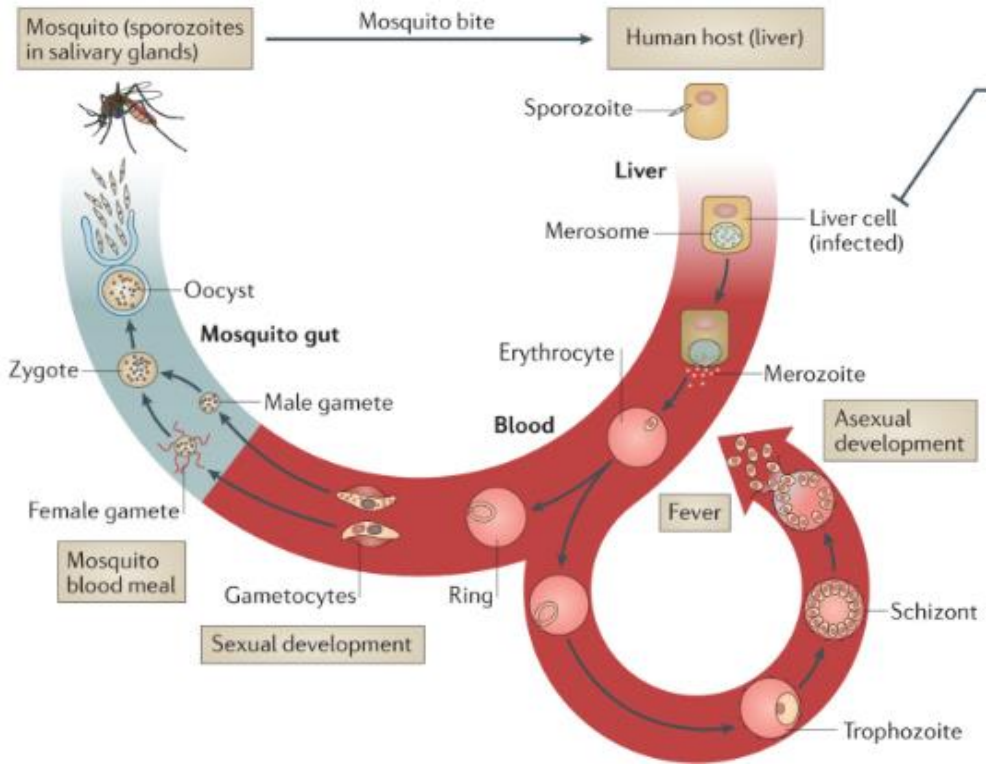


Figure 2.1: Plasmodium falciparum life cycle: liver, erythrocytic, and mosquito stages (Haldar et al., 2018).

2.2 Malaria Diagnosis

2.2.1 Clinical diagnosis

The clinical diagnosis basis solely depends on the patient's signs and symptoms, which are often non-specific. They commonly include fever, headache, weakness, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, and pruritus (Chandramohan *et al.*, 2002). This diagnosis method's specificity is compromised

due to the overlap of malaria signs and symptoms with tropical viral or bacterial infections. It can lead to over-diagnosis and over the treatment of malaria. This method is best suited in regions where laboratory support is limited.

2.2.2 Microscopy

The detection of malaria infection can be precisely determined through the microscopic technique, the gold standard for malaria diagnosis. This technique is carried out by applying Giemsa stain to the prepared thick or thin blood smears of patients since it helps in the visualization of blood stages of parasites. The patients can be accurately diagnosed as asymptomatic depending on a microscopist's expertise (Kitchen & Chiodini, 2006). This method is used in both qualitative to identify the species and stage of the parasite growth and quantitative analysis because it shows the degree of parasitemia during diagnosis and treatment (Tangpukdee *et al.,* 2009).

2.2.3 Immunochromatographic Rapid Diagnostic Tests (RDTs)

The RDTs rely on the principle of the impregnation of monoclonal antibodies specifically designed to target two of the three *Plasmodium* antigens for their diagnosis and for distinguishing *P. falciparum* infections (Jain *et al.,* 2014). The three utilized plasmodial antigens include the *P.falciparum* histidine-rich protein II (*Pf*HRPII), *Plasmodium* lactate dehydrogenase (pLDH), and aldolase. This malaria diagnosis method is used in many resource-poor areas where there is no access to the laboratory. However, the rheumatoid factor's presence has been shown to give false-negative results due to cross-reactivity between the factor and HRPII.

2.2.4 Polymerase chain reaction (PCR)

As the PCR allows amplification of specific conserved genes in all *Plasmodium* species, it is the most accurate and sensitive diagnostic method for malaria. This technique (PCR) can detect drug-resistant parasites by genotyping key mutations in

the parasite genes (Imwong *et al.*, 2001). The PCR technique can detect parasitemia of 0.005 iRBC/µL, making it the most specific and sensitive method (Zimmerman & Howes, 2015). They mostly come in handy in detecting sub-microscopic infections, which significantly threaten the elimination strategies that abound.

2.3 Prevention and control of Malaria

2.3.1 Chemotherapy for the treatment

Chemotherapy remains the central intervention for the treatment and control of malaria. Chemical groups of antimalarial drugs define their mechanisms of action and their classifications. Based on chemical grouping, there are six categories: quinolone alcohols, 4-aminoquinolines, aryl-alcohols, 8-aminoquinolines, and anti-folates artemisinins derivatives (Schlitzer, 2008). The different types target different parasite development stages, with some targeting the sporozoites, schizonts, and gametocytes stages. The gametocidal drugs such as primaquine help block gametocytes' transmission to mosquitoes and help minimize continued malaria transmission.

2.3.1.1 Quinoline alcohols

Quinine (QN) and Mefloquine are the main antimalarial drugs in this category, as shown in figure 2.2. The QN is most abundant in the cinchona tree's bark and has a rapid schizonticidal activity against intra-erythrocytic malaria parasites (Schlitzer, 2008). Quinine has been shown to inhibit the detoxification of heme, an essential process within the parasite (Fitch, 2004). On the other hand, Mefloquine is thought to exert its antimalarial activity via inhibition of heme detoxification (Eastman and Fidock, 2009). This drug is also thought to exert morphological changes in the digestive vacuole of the *Plasmodium* through high-affinity membrane

binding.

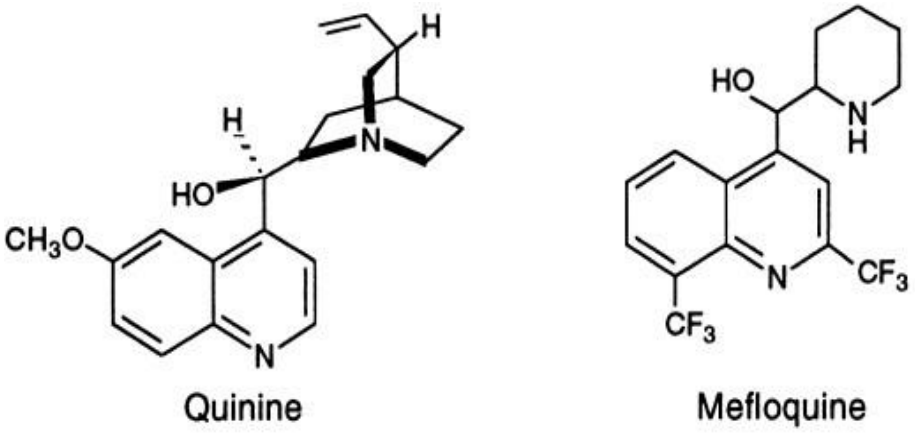


Figure 2.2: Structure of quinolone alcohols: Quinine and mefloquine (Schlitzer, 2008).

2.3.1.2 Aminoquinolines

There are two classes of aminoquinolines, namely the 4- and 8-aminoquinolines. The 4-aminoquinolines include chloroquine (CQ), amodiaquine (AQ), and piperaquine (PQ). The 8-Aminoquinolines, on the other hand, are comprised of tafenoquine and primaquine drugs (Schlitzer, 2008). The CQ and AQ are the main antimalarial drugs in the 4-aminoquinolines group and active against the parasite's intra-erythrocytic stage. As shown in figure 2.3, the CQ binds to the heme, preventing it from polymerization into a non-toxic malaria pigment called hemozoin, which enables the parasite to survive in the digestive vacuole of the parasite. The resultant chloroquine-heme complex then accumulates in the parasite's digestive vacuole to threshold levels capable of killing the parasite (Schlitzer, 2008). A study carried out by Shreekant & Bhimanna 2016 showed that the CQ drug combines with ferriprotoporphyrin IX (FPIX) that prevents hemozoin formation by inhibiting the formation of hematin. As shown in Figure 2.3, Amodiaquine is thought to have the same CQ mode of action since it is analogous to chloroquine. Among the various 8-

aminoquinolines drugs available, the tafenoquine and primaquine are the primary drugs of interest, given their significance in clearing gametocytes against all human malaria parasite species (Schlitzer, 2008). The primaquine is used in preventing malaria relapse resulting from *P. vivax* and *P. ovale* infection by eradicating hypnozoites associated with the malaria relapse (Baird,
2005).

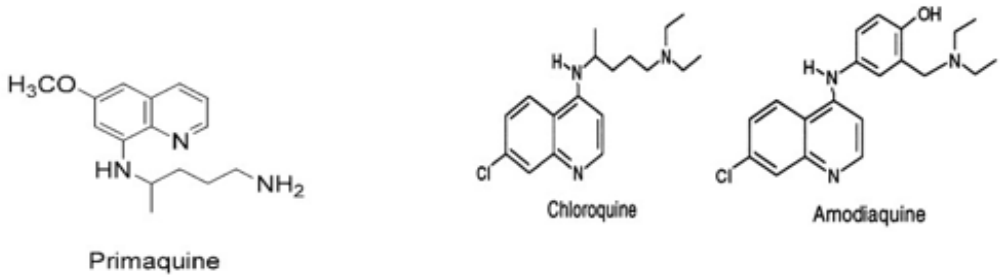


Figure 2.3: Structures of Primaquine, Chloroquine, and Amodiaquine (Shreekant & Bhimanna, 2016).

2.3.1.3 Folate Antagonists

There are two types in this category of antimalarial drugs, namely, Class 1 and II antifolate. Class one is comprised of dihydropteroate synthase (DHPS) inhibitors, e.g., sulfadoxine. In contrast, type II forms the dihydrofolate reductase (DHFR) inhibitors and includes the pyrimethamine and proguanil antimalarial drugs (Bloland, 2001). The antifolates in both classes are involved in preventing the formation of pyrimidine deoxythymidylate by inhibiting tetrahydrofolate co-factors essential in the synthesis of parasite DNA pyrimidine deoxythymidylate (Gregson & Plowe, 2005). Due to the marked synergistic effect, the DHFR and DHPS are used in tandem to treat malaria. The Sulfadoxine-pyrimethamine combination (Fansidar) was introduced in response to the emergence of CQ resistance (Gregson

&Plowe, 2005). Proguanil, a pro-drug, has also been widely used for malaria prevention.

2.3.1.4 Arly alcohols

The lumefantrine, halofantrine, and pyronaridine are antimalarial drugs in this category of aryl-alcohols (Ezzet *et al*., 2000). Lumefantrine, also known as benflumetol, is a synthetic fluorene derivative, which relates chemically to mefloquine. Benflumetol was discovered in the 1970s by the Academy of Military Medical Sciences in Beijing, China. Additionally, Benflumetol had undergone preliminary clinical studies in China in-conjunction with several Artemisinin derivatives before its introduction into the market (Basco *et al*., 1998). The co-administration of lumefantrine moiety with a fatty meal enhances its absorption.

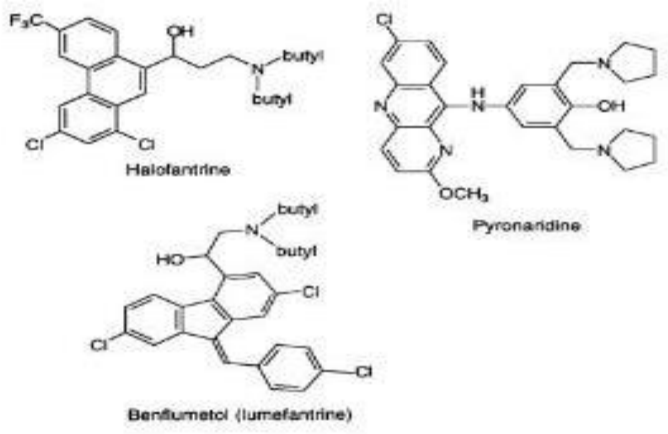


Figure 2.4: Structure of aryl-alcohols: halofantrine, pyronaridine, and lumefantrine (Schlitzer,

A fixed-dose of artemether-lumefantrine (AL) combination containing 20 mg of artemether and 120 mg of lumefantrine in each tablet has been introduced as the first or second-line treatment for malaria in several Africa countries (Sisowath *et al*., 2007). The artemether molecule in AL dosage ensures faster clearance of the mass of malaria parasites since it has a short half-life. At the same time, the partner drug, lumefantrine, clears the remaining parasites for several days. This helps against the selection and fixation of any potential antimalarial drug resistance phenotype. Single nucleotide polymorphism in the *Pf*mdr1, particularly the variant N86Y, and amplification of the encoding gene *Pf*mdr1 have been associated with reduced susceptibility lumefantrine in Africa and Asia (Sisowath *et al*., 2007).

Halofantrine (**Figure 2.4**) is effective against chloroquine-resistant *P.falciparum* strains. However, the drug has adverse effects on the body's cardio-system, expensive, and does not have parenteral formulation, limiting its usefulness as a therapeutic agent (Schlitzer, 2008).

Pyronaridine (**Figure 2.4**) and acridine derivative are a synthetic drug administered as an oral formulation is widely used in China (Schlitzer, 2008). Pyronaridine has been shown via *in vitro* studies that it interferes with glutathione-dependent heme degradation through inhibiting beta-hematin formation (Auparakkitanon *et al.*, 2006). The drug is structurally related to chloroquine and has utility for multidrug-resistant *P. falciparum* malaria since it is useful and well-tolerated in the body. However, pyronaridine's oral bioavailability is low, thus making the cost of treatment unacceptable (WHO, 2009).

2.3.1.5 Artemisinin Combination Therapy (ACT)

The world health organization has recommended using ACTs as the preferred first-line antimalarials against *falciparum* malaria to treat uncomplicated malaria (WHO, 2019). Artemisinin combination therapy comprises artemisinin derivatives with very short elimination half-life and prolonged elimination half-life drugs (Djimdé *et al.*, 2008). Artemisinin derivatives are the most potent antimalarial drugs due to artemisinin moiety's ability to reduce parasite biomass and gametocyte carriage rapidly. In contrast, the long-acting partner drug, lumefantrine or piperaquine (PQ), effectively clears the residual parasites, thus achieving the clinical and parasitological cure. The principle underlying the use of the drug in combination is based on the assumption that drug resistance essentially depends on DNA mutations. Therefore, if two drugs used have different modes of action, then the probability of parasites developing resistance to both drugs would be reduced compared to developing resistance to one drug (White, 2014). Moreover, ACTs would have similar pharmacokinetic properties so that no drug is left unprotected by the other.

More than 60 countries have adopted artemisinin combination therapy as first-line therapy for treating uncomplicated malaria. Kenya adopted the AL drugs in 2006

as the first-line antimalarial drug (WHO, 2019). However, an increasing body of evidence has continually shown that DHA-PQ is efficacious and well-tolerated. *In vivo* studies done in Asia have reported DHA-PQ efficacy of about 90% over 28-63 days. Other studies in Africa have also shown DHA-PQ to be as efficacious as artemether-lumefantrine (Bassat *et al*., 2009).

2.3.2 Vector control

The female *Anopheles* mosquito involved as a definitive host during malaria transmission can be controlled through indoor residual spraying of insecticides (IRS) and long-lasting insecticidal nets (LLINs) (WHO, 2019). The practice of indoor spraying of long-acting chemical insecticides on the houses' interior walls and roofs and domestic animal shelters in a given area to kill the adult vector mosquitoes that land on these surfaces is referred to as IRS. This method has led to the control of major vector *Anopheles gambiae S. S.*, which rests and bites mostly indoors (WHO,2019). The ITNs, on the other hand, provide a physical barrier between the human and mosquito vector while repelling and killing the mosquitoes because they are embedded with insecticides. There is evidence that wide-scale use of LLINs provides some extended level of protection to non- LLINs users, as it helps reduce the overall malaria transmission (Killeen *et al.*, 2014). However, due to the emergence and spread of insecticide resistance mosquitoes, there has been a concerted effort to develop novel vector control measures. For instance, insecticide-resistant mosquitoes may be released into the wild to breed with the local populations, spreading the genes that cause them to be refractory towards malaria parasites (Marshall & Taylor, 2009). The implementation of vector control strategies alongside the improved quality of health between the years 2000 and 2015 saw a reduction of mortality rates by 60% in Africa (WHO,2019).

2.3.3 Vaccines

The development of an effective vaccine against *Plasmodium falciparum* and *vivax* malaria is still on the search. The development of a vaccine is challenging because it entails the elucidation of antigens that serve critical functions in patients showing stable natural acquired immunity towards malaria. The currently developed malaria vaccines target only the parasite's pre-erythrocytic stages and help prevent hepatocyte invasion by sporozoites (Greenwood *et al.,* 2013). Currently, the RTS, S/AS02A, a hybrid molecule of the circumsporozoite protein of *P. falciparum* co-expressed with hepatitis-B surface antigen (HBsAg) in yeast, is the most advanced pre-erythrocytic vaccine (Girard *et al.,* 2007). The vaccine works by inducing a humoral and cellular immune response to the circumsporozoite protein present on the surface of sporozoites and liver-stage schizonts. RTS, S vaccine is given with an adjuvant system (AS01 or ASO2), and it has consistently shown protection against clinical episodes of malaria in the range of 30-60% (Abdulla *et al.,* 2008).

2.4 Mechanism of parasite drug resistance

Antimalarial resistance or prophylaxis failure is well-defined as a parasite strain's ability to stay alive and multiply, despite the administered drug's access to the infected red blood cell (Bloland, 2001). The fight against malaria depends on chemotherapy and chemoprophylaxis. However, the acquisition and spread of parasite strains resistant to antimalarial drugs have seriously reduced these drugs' effectiveness. They have led to changes in the adoption of treatment policies.

Resistance to antimalarial occurs as a result of several factors, namely: the number of parasites in the human host that are exposed to the administered drug, the concentrations of administered drug to which these parasites are exposed, i.e., both pharmacodynamics and pharmacokinetic properties of the drug, the host's immune system, fitness cost to the parasite, the presence of other substance in the blood that

will still kill the parasite if it develops resistance to one drug (i.e., the use of combinations), poor treatment practices, poor patient adherence to prescribed antimalarial regimens, and the widespread availability of artemisinin-based monotherapies and substandard forms of the drug (White, 2004).

Reduced sensitivity to a given drug is conferred by random single point mutation while others by multiple point mutations at the parasite's essential transporters. For instance, chloroquine resistance is associated with mutations in the *Pf*crt-codon K76T and *Pf*mdr- codons N86Y, N1042D, and D1246Y. These mutations result in the increased efflux of chloroquine from the acidic digestive vacuole to the cytosol. The parasite continues to form the hemozoin (a by-product of heme degradation) that enables it to survive. The chloroquine efflux occurs at 40 to 50 times faster among resistant parasites than sensitive ones (Bloland, 2001; White, 2014).

The ACT resistance recently reported in Greater Mekong sub-region: Cambodia, Myanmar, Thailand, and Viet Nam, identified kelch13 propeller region as the key molecular marker for ACT resistance (Wongsrichanalai & Meshnick, 2008). Other critical genes involved in modulating artemisinin resistance include *Pf*crt, *Pf*mdr, and Pcap2mu. Polymorphism in the *Pf*ap2mu/pcap2-mu has also been shown to modulate artemisinin resistance. The Pcap2-mu encodes the mu chain of the AP2 adaptor complex involved in cell surface endocytosis and recruits both cargo and structural components, including clathrin, to the vesicle (Henriques *et al.,* 2014b). It is also thought to be involved in hemoglobin trafficking in malaria parasites. The subsequent post ubiquitination of the synthesized proteins aids in modulating endocytosis and proteasomal recycling in-conjugation with known substrates such as P-glycoproteins, e.g., Pgh1, thus enabling parasite survival (Henriques *et al.,* 2015).

2.5 Factors contributing to antimalarial drug resistance

2.5.1 Genetic basis for drug resistance

The fountain for drug resistance in malaria parasites is associated with genetic variations in specified genes. These changes may result in poor expression and folding of the target protein involved in protein-drug interaction. When a poor modification is formed, it will affect the drug's antiparasitic concentrations. Thus, it will not clear the parasite and can lead to the resistance phenotype's appearance (Aminake & Pradel, 2013). In *Plasmodium*, drug resistance is mediated by two processes: 1) the rate at which de novo mutations conferring resistance are selected; and 2) the spread of those resistant alleles (Bloland, 2001). The parasite's phenotype change can be mediated by single point mutations, alterations to multiple loci, or gene duplication, which modify the parasite's phenotype.

2.5.1.1 The Role of Molecular markers

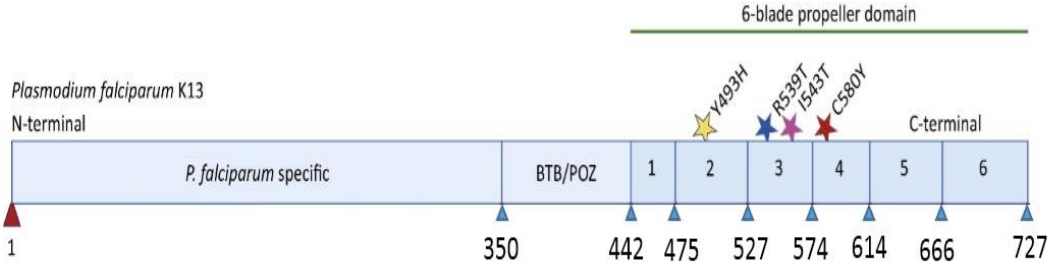
Early detection of antimalarial drug resistance is greatly enhanced by the identification of molecular markers of resistance. The markers have been utilized to monitor the origins and spread of antimalarial drug resistance, providing a better understanding of drug-resistant genotypes' population dynamics (White *et al.*, 2014). The sequencing and annotation of the *P. falciparum* genome have also provided a platform for identifying gene candidates linked to drug resistance phenotypes. This has helped in rapidly screening drug efficacy by exploiting the already elucidated drug resistance polymorphism. Such polymorphisms consist of microsatellites, single nucleotide polymorphisms (SNP), and small insertions or deletions (indels). This study utilized molecular methods in detecting the presence or absence of crucial SNPs associated with antimalarial drug resistance in the kelch 13, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 genes.

2.5.1.1.1. Kelch 13 propeller region

The kelch 13 propeller domain on the Kelch 13 gene in chromosome 13 of *Plasmodium falciparum.* The gene has only one exon and encodes the 726 amino acid K13 protein with a molecular weight of 83.66kDa. There are 6 Kelch motifs at the C-terminus end of the K13 protein. Each kelch motif consists of 50 amino acids involved in the secondary structured beta-sheets formation, as shown in figure 2.5. The six Kelch motifs are essential since they harbor protein-protein interaction sites in the resultant 3D-protein structure (Adams & Cooley 2000). Mutations in the propeller domain of the *k13* gene are the genetic correlates of *in vivo* and *in vitro* resistance to artemisinin in Southeast Asia (Ariey *et al.,* 2014).

The first *in vivo* and *in vitro* case studies on Artemisinin resistance was detected in Thailand-Cambodian border in 2008 after administration of artesunate monotherapy in which there was a prolonged parasite clearance times of >90 hours as compared to the median of 52 hours for patients who were cured (Wongsrichanalai & Meshnick, 2008). Further studies that employed a whole-genome sequencing strategy to understand African ART-resistant and clinical isolates from Cambodia led to a significant breakthrough in understanding the genetic architecture of artemisinin-resistant parasites. These studies found out that the k13- propeller domain mutations were associated with artemisinin resistance *in vitro* and *in vitro* (Ariey *et al.,* 2014). They elucidated that parasites carrying non-synonymous mutations at codons Y493H, R539H, I543T, and C580Y (key SNP) in the K13 propeller domain were associated with higher ring-stage parasite survival ($RSA_{0\text{-}3h}$ survival assay) rates as compared to the wild type. Additional gene-editing studies using CRISPR-Cas9 were then carried out to validate the role of the C580Y mutation and found out that the C580Y mutation was associated with increased ring-stage parasite survival of ~13.5% (Ghorbal *et al.,* 2014), almost similar to the rate previously reported for the Cambodian resistant parasite isolate (Ariey *et al.,* 2014).

Several epidemiological studies performed in Southeast Asia have identified multiple occurrences of mutations in the k13 propeller domain that result in drug resistance (Takala-Harrison *et al.,* 2015; Miotto *et al.,* 2015; Phyo *et al.,* 2016; Imwong *et al.,* 2017). Various studies in Africa have also identified low frequencies of non-synonymous in kelch 13 propeller, which have no significant impact on the ART efficacy (Neher, 2016). However, a recent study conducted in China described a migrant worker with *P. falciparum* K13-variant infection from Equatorial Guinea who displayed the delayed parasite clearance phenotype following several ACT rounds of treatment rounds (Lu *et al.,* 2017).



***F*igure 2.5: The Kelch 13 propeller structure showing the six propeller domains and the four non-synonymous SNPs associated with higher ring-stage parasite survival rates (Ariey et al., 2014).**

2.5.1.1.2 *Pf*ap2

The *Pf*ap2-mu gene is found on chromosome 12, encoding 621 amino acids with a molecular mass of 72.9kDa (Henriques *et al.,* 2014). The *Pf*ap2-mu is involved in drug trafficking through the endocytic process. Specific mutations in this gene, specifically S160N/T, have been shown to mediate delayed parasite clearance following ACT treatment among Kenya children (Henriques *et al.,* 2014; Beshir *et al.,* 2017). This mutation was elucidated by Henriques *et al.,* 2015 through the generation of transgenic *P. falciparum* parasites that expressed an extra copy of

either the wild-type (WT) *Pf*ap2-mu gene or the 160Asn form, that was compelled by a heterologous promoter, in addition to the endogenous WT *Pf*ap2-mu gene. The generated parasites were then subjected to a susceptibility test using various antimalarial drugs: (dihydroartemisinin [DHA], quinine, chloroquine, lumefantrine, mefloquine, and atovaquone) with a parental $Dd2^{attB}$ strain as the control in a classical 48-h growth inhibition assay.

2.5.1.1.3 *Pf*crt

The *Pf*crt gene is localized on chromosome 7, encoding 424 amino acids with a molecular mass of 48.6kDa (Fidock *et al.,* 2000). The *Pf*crt protein comprises ten putative transmembrane domains that span around the food vacuole of the parasites' membrane, as shown in figure 2.6 below, and belongs to the drug transporter superfamily (Martin & Kirk, 2004). A genetic cross-study by Wellens & Panton in 1991 between the CQ-sensitive HB3 isolate and the CQ-resistant Dd2 isolate provided conclusive evidence that *Pfcrt* is the primary determinant of CQR. Recent *in-vivo* and *in-vitro* studies by Fidock *et al.* (2000) and Djimdé *et al.* (2001) have also associated mutations in the *Pfcrt* gene as the key determinant of CQ resistance (CQR). Several studies comparing the wild-type and mutant *Pf*crt allele have shown less CQ accumulation inside the parasite vacuole of the mutant *Pf*crt (Yayon, Cabantchik, & Ginsburg, 1984; Sanchez *et al.,* 2005). A total of 15 different SNPs in the *Pf*crt gene are used in determining resistance to CQ. However, SNPs at codons 72-76 of the *Pf*crt gene have been used in distinguishing the CVIET (South-East Asia and Africa) and SVMNT (South America and Southeast Asia) geographically distinct haplotypes determination (Fidock *et al.,* 2000). The causal mutation in *Pf*crt, a switch from Lysine (K) to Threonine (T) at codon 76, is used as the molecular marker for monitoring CQ-resistance (Fidock *et al.,* 2000).

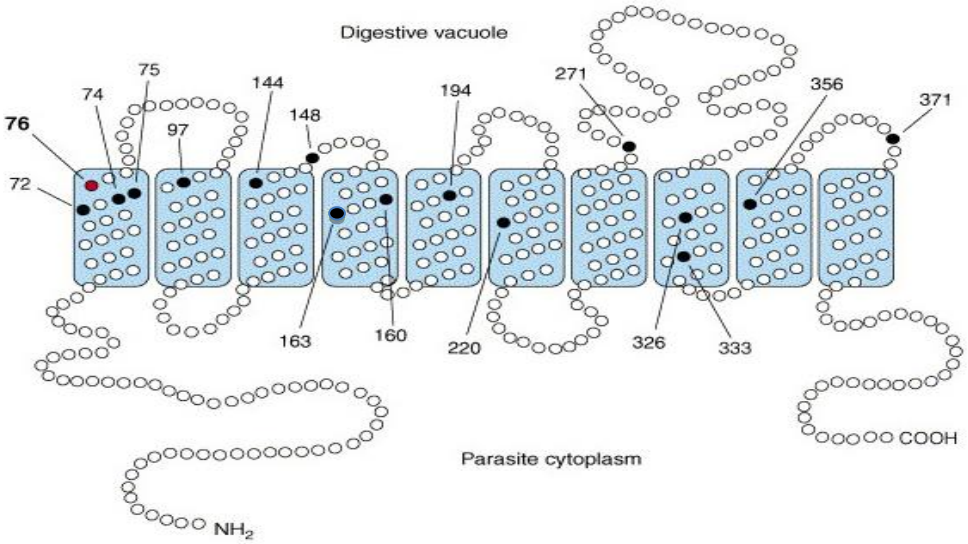


Figure 2.6: The structure of *Pf*CRT showing the ten transmembrane domains (shaded blue) and the known point mutations (Valderramos & Fidock, 2006).

2.5.1.1.4 *Pf*mdr1

The *Pf*mdr1 gene on chromosome 5 is comprised of one exon that encodes for 1419 amino acid P-glycoprotein homolog 1 (Pgh-1) with a molecular mass of 62.25kDa, as shown in Figure 2.7 below. This gene encodes a digestive vacuole membrane-bound ATP-binding cassette (ABC) transporter with two domains, consisting of 6 helical transmembrane domains. Pgh-1 regulates intracellular drug concentrations on the parasite food vacuole throughout the parasite's asexual cycle (Cowman *et al.*,1991). Studies using fluorescein derivatives (Fluo-4) provide validating evidence that *Pf*mdr1 imports solutes, including antimalarial drugs, into the parasite's food vacuole (Rohrbach *et al.*, 2006). P-glycoprotein polymorphisms have been linked to resistance to cancer drugs (Valderramos & Fidock, 2006). The SNP's and copy number variations (CNVs) of the *Pf*mdr1 gene are a major determinant of parasite resistance or susceptibility to several antimalarials (Foote *et al.*, 1989). These SNPs are located on both the amino-terminal (N86Y & Y184F) and carboxyl end

(D1246Y). The *Pf*mdr1 polymorphisms have been associated with a differential *in vivo* and *in vitro* parasite responses to a considerable range of key ACT antimalarial partner drugs, including amodiaquine (Holmgren *et al.*, 2006), mefloquine (Reed *et al.*, 2000), lumefantrine (Sisowath *et al.*, 2005) and artemisinin (Reed *et al.*, 2000).

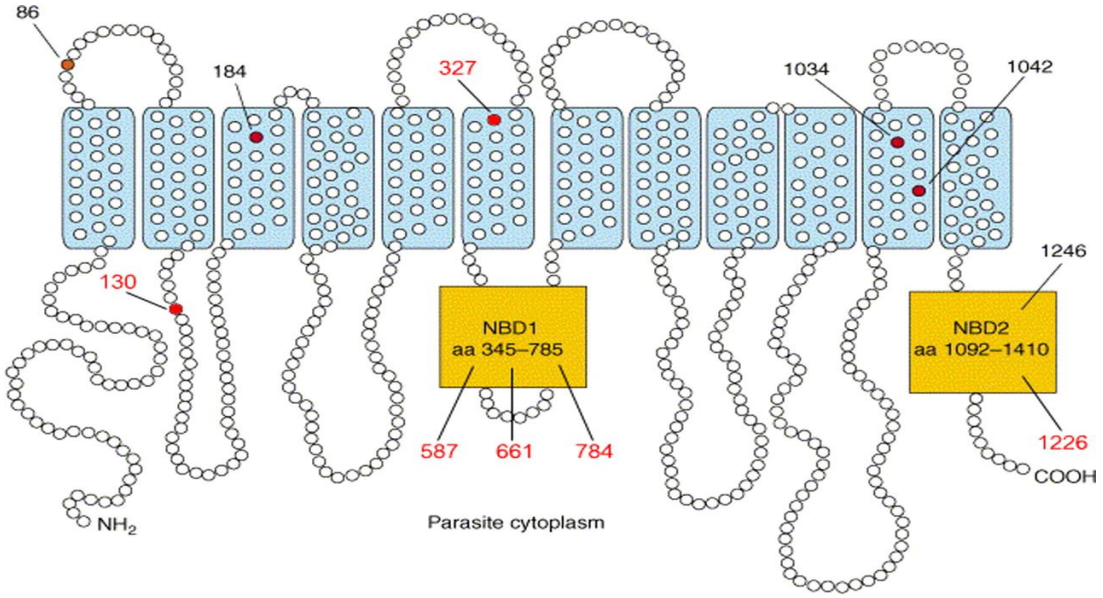


Figure 2.7: The structure of PfMDR1 showing the 12 transmembrane domains (shaded blue), the known point mutations indicated as shaded circles, and large cytoplasmic domains called nucleotide-binding domains (NBD). Adapted from (Valderramos & Fidock, 2006).

2.5.2 Poverty

In sub-Saharan Africa, 53% of the total 834 million people are at risk of mosquito bites due to inadequate/lack of access to ITN or the IRS. In a study done in 18 households in sub-Saharan African, many febrile children do not access healthcare services (median 35%: IQR 24–41%). The study further discloses that even if the children are taken to a formal health facility, they might not receive a diagnostic test or appropriate antimalarial treatment. This is because the healthcare provider might have inadequate stocks, or the patient may not afford the antimalarial drug charges. Again, there are a high proportion of poor people who have insufficient funds to treat the disease. In most of these countries, the purchase of antimalarial drugs is

unrestricted in many local pharmacies or drug stores without a prescription (WHO, 2019). Misguided self-prescription exposes the patient to counterfeit medications containing inappropriate quantities of active elements or none because of mislabelling. When the parasites are exposed to these drugs' sub-therapeutic doses, they might favor the selection of resistant parasites (Aminake & Pradel, 2013)**.**

2.5.3 Immune system

Antimalarial immunity is developed slowly and imperfectly. In less prone malaria regions, the infections are symptomatic, and resistance is contented with the antimalarial drug in use. On the other hand, in high malaria-prone areas, i.e., where transmission is high, asymptomatic infections are acquired throughout an individual life. Once a mutant parasite is generated, the host defense is put into action to fight the parasite, and thus the parasite levels are minimized and cleared (White, 2004). However, if the resistant mutant does survive drug treatment, the chance that it will produce sufficient gametocyte for transmission is minimized. Since the infection occurs throughout, there is endless competition for red blood cells. As a result, there is an increased likelihood of possible outbreeding of multigenic resistance mechanisms or competition in the feeding *Anopheles* mosquito. Pregnant women are even extra attractive to mosquitoes and are an essential contributor to antimalarial drug resistance. Also, in the Hiv-positive immuno-suppressed individuals, the antimalarial immunity is compromised, and thus the selection and spread of resistant parasites are enhanced (White, 2004)**.**

2.5.4 Asymptomatic infections prevalence

A more significant number of people living in malaria-endemic areas harbor undetectable malaria parasites by microscopy. The asymptomatic persons have parasites present in their circulatory system. Such parasites will then form mutant gametocytes in the presence of drug pressure, and over a while, they became tolerant

to drugs in use (White, 2004). Antimalarial drug resistance is selected by administering drug concentrations sufficient to inhibit the multiplication of sensitive parasites but not resistant ones. Subsequently, resistant parasites multiply selectively and ultimately develop resistance to the antimalarial drug in use. Generated gametocytes by resistant parasites in humans are then passed on to the next persons when an infected female *Anopheles* mosquito bites and inoculates the resistant strains during a blood meal (White, 2004; Aminake & Pradel, 2013).

2.6 Monitoring the emergence of resistance

2.6.1 *In vivo*/ therapeutic efficacy testing

Therapeutic efficacy that happens *in vivo* settings is done to monitor the emergence of resistance against antimalarial drugs. For accurate and valid surveillance, the World Health Organization has created standards over Time for monitoring antimalarial resistance and transmission status (WHO, 2009). Drugs with longer half-life and regions with high malaria transmission rates require an extended follow-up period to monitor the emergence of antimalarial resistance (Nsanzabana *et al.*, 2018). The current standard of determining the emergence of malaria resistance involves the treatment of symptomatic patients with known doses of antimalarial drugs followed by a fixed period of follow-up 7 to 14 days for drugs with a short half-life or more days of 21/28/42/63 with a long half-life to detect any reappearance of symptoms and signs of clinical malaria or parasites in the blood (Nsanzabana *et al.*, 2018). This would indicate reduced parasite sensitivity for the particular drug being used (Aminake & Pradel, 2013). The *in vivo* protocol, according to WHO, is the gold standard for monitoring resistance through surveillance and is usually conducted on children less than five years of age since they have less favorable therapeutic responses due to partial or no immunity against malaria. However, these studies' complexity, financial constraints, reinfection, immunity, and pharmacokinetics factors make it challenging (Bloland, 2001; Aminake, & Pradel, 2013).

2.6.2 *In vitro* test

This technique involves the determination of the susceptibility of malaria parasites to various concentrations of antimalarial drugs. The source of parasite strains can either be from cultured isolates (*in vitro*) or infected patients (*ex vivo*) (Nsanzabana *et al.,* 2018). One category of the collected samples is subjected to drug/crude product under test, while the other sample acts as a control. The inhibitory concentration that eliminates 50% of the cultured isolates (IC_{50}) is determined and compared with those obtained from reference strains whose genotype and phenotype are known (Apinjoh *et al.,* 2019). However, this method is relatively expensive given the equipment's sensitivity (Aminake & Pradel, 2013)**.**

2.6.3 Molecular techniques

Molecular tests use PCR to determine genetic mutations, such as point mutations or duplications, which confer resistance to antimalarial drugs. This method involves the extraction of genetic material from infected blood and sequencing to identify mutations (Nsanzabana *et al.,* 2018). The DNA is extracted, sequenced, and SNPs or mutations associated with drug resistance are determined/processed (Aminake & Pradel, 2013). CQ resistance's genetic determinants are point mutations at *Pf*crt-codon K76T and *Pf*mdr1- codons N86Y, N1042D, and D1246Y (White, 2004; Apinjoh *et al.,* 2019). Moreover, the AL resistance is associated with genotypes encompassing the mutation in *Pf*crt-codon K76T and *Pf*mdr1- codons N86Y, N1042D and D1246Y codons, *Pf*ap2-mu (S160N/T), and k13 propeller region (Wongsrichanalai & Meshnick, 2008; Henriques *et al.,* 2014b; Sutherland *et al.,* 2017). The rate of occurrence of specific gene mutations associated with particular drug resistance would serve as a supplement to *in vitro* studies and consequently provide information on the health policymakers on the level of drug resistance (Aminake & Pradel, 2013). However, the PCR method is quite expensive compared

to the *in vivo* and *in vitro* tests. Other methods include malaria case reports or passive surveillance (Bloland, 2001).

2.7 Prevalence of drug resistance markers

ACTs are the recommended current first-line treatment of malaria globally by WHO. They are three days dosing and are very safe and effective (Aminake & Pradel, 2013). Kelch 13 is a molecular marker associated with ACT's resistance**.** The kelch 13 does not work in isolation but in combination with other genetic or non-genetic factors that differ among parasite populations (Kamau *et al.*, 2015). Various studies have shown that AL selects for single nucleotide polymorphisms (SNPs) in *Pf*crt (K76), *Pf*ap2-mu (S160N/T), and *Pf*mdr1 (N86, 184F, and D1246) in parasite re-infection (Aminake & Pradel, 2013; Henriques *et al.*, 2014b; Henriques, Schalkwyk *et al.*, 2015; Achieng *et al.*, 2015).

A study carried out by Achieng *et al.*, 2015 showed a substantial rise in polymorphisms at key codons in the *Pf*crt and *Pf*mdr1 genes selected for by AL, with the prevalence of *Pf*crt K76 + NFD alleles well above 90%. This could be an indication of increased susceptibility of *Plasmodium* parasites to CQ many years after its withdrawal. Another study carried out in Msabweni, Kenya, showed a significant decline in CQR parasites. The 76T codon prevalence for CQR is 41% from 63%, five years after the retrospective study was carried out in the same area (Kiarie *et al.*, 2015).

Studies carried out in sub-Saharan Africa, Kenya, have recently shown that the K3-propeller region' is the most probable molecular marker for tracking artemisinin resistance. For instance, two African non-synonymous SNPs, A578S and V566I, might be key markers for monitoring artemisinin resistance in Africa since they were found in more than one location. Also, they are close to C580Y, a key SNP determinant for artemisinin resistance in Southeast Asia. However, A578S SNP was

most prevalent at 2.7% in Kenya (Kamau *et al.,* 2015). The A578S SNP mutant was also found in studies carried out in Uganda and Kenya (Muwanguzi *et al.,* 2016). Thus, this study accessed the prevalence of mutation at the kelch 13-propeller regions, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 genes associated with resistance artemisinin-combination therapies and chloroquine in Nyando, Western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

The study employed a retrospective cross-sectional design to examine the prevalence of mutations in the kelch 13-propeller region, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 genes associated with resistance artemether-lumefantrine and chloroquine drugs in Nyando, Kisumu County. The study was part of the main study carried out in Nyando in 2015 to evaluate the efficacy of ACT.

3.2 Study site

This study was carried out in Nyando, a region in the western part of Kenya. The area is malaria-endemic and sits at 1212m above sea level. The area experiences heavy and frequent rainfall throughout the year, making it a suitable breeding region for Kenya's malaria vector species; *Anopheles gambiae*, *Anopheles funestus*, and *Anopheles arabiensis* (Okara *et al.*, 2010; Amek *et al.*, 2012). According to the 2015 Kenya malaria indicator survey, malaria prevalence in western regions was 27%.

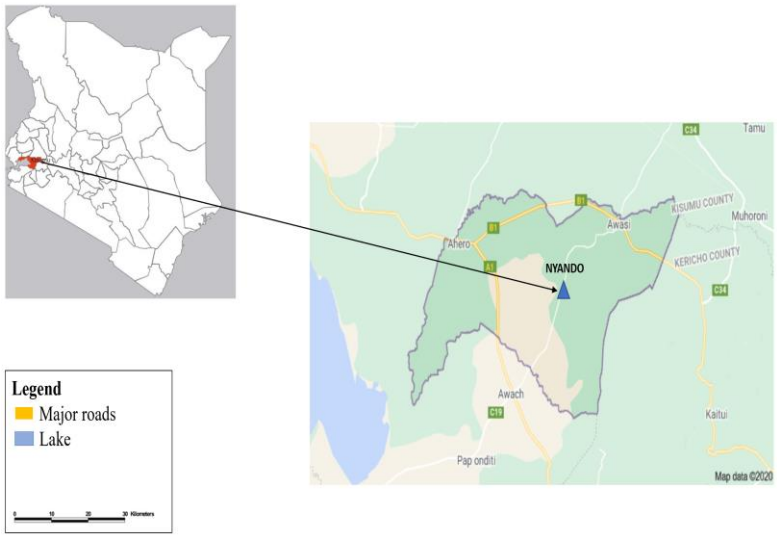


Figure 3.1: Map of Kenya showing Kisumu County and Nyando alongside other bordering constituencies.

3.3 Sample collection

The current study used ninety-four (94) archived dried blood spot samples collected under approved protocols SSC 2276/3236 (Efficacy of ACTs in Kenya) by the Scientific Ethical Review Unit (SERU) of the Kenya Medical Research Institute. In the study protocols SSC 2276/3236, the eligibility criteria included informed consent, history of fever, the measured body temperature of ≥37.5 °C, *P. falciparum* mono-infection, and parasitemia between 1000 - 200,000 parasites/µL of blood. Patients treated for malaria within the preceding fourteen days or voluntarily withdrawals from the study were excluded. The presence of malaria was done using a rapid diagnostic test (RDT; Parascreen®, Zephyr Biomedicals, Verna Goa, India) and confirmed by microscopy, the gold standard for malaria parasite test.

3.4 Ethical considerations

The ethical approval to conduct this study was approved by the Kenya Medical Research Institute (KEMRI) Nairobi, Scientific Ethics Review Unit (SERU), SSC 3726, as shown in appendix 2.

3.5 Inclusion and exclusion criteria

The inclusion criteria used for archived samples in this study were done by investigating the sample's integrity and looking into the sample's labeling, whether contaminated or not and how the archived samples were stored. Samples were excluded if the parent/guardian did not consent for future use of the sample, contaminated or poorly labeled, and poorly kept.

3.6 Sampling procedure

3.6.1 Sample size determination

The sample size was determined using Fisher's formula. The estimated prevalence of these target mutations in western Kenya at the start of this study was 96% (Achieng *et al.,* 2015). The sample size was calculated as follows,

$n = \frac{Z^2 Xpq}{d^2}$ where: n = desired sample size,

Z = Standard normal deviation at required confidence interval (95%),

p = proportion of the target mutations which is 96%, q = 1-p and

d = level of statistical significance at 95% confidence interval.

$n = 1.96^2 * 0.96 * 0.04/0.05^2 = 87$ samples (at least)

Therefore, the study selected 94 samples out of the 315 samples collected during the parent study through a systematic random sampling to determine the target mutations' prevalence.

3.7 DNA extraction

The DNA extraction was carried out according to Fischer *et al.,* (2004) protocol. Briefly, the Harris uni-core punch, a pair of forceps, was sterilized by immersing in 5M HCl and Subsequent immersion in 5M NaOH (neutralization). The filter paper containing the dried blood spot was removed from the ziplock using a pair of forceps and excised about 2mm area of the dried blood spot using Harris uni-core punch into a sterile 1.5ml microfuge tube. The Harris uni-core punch and a pair of forceps were decontaminated before excising the next sample in the zip lock bag. One milliliter of 0.5% saponin of 1PBS was added to each tube containing the excised DBS, inverted several times, and left overnight at four degrees, after which the brown solution was

discarded in a beaker with 10% bleach and replaced with 1ml of PBS, inverted several times and placed at 4^{o}C for 30 minutes. The brown solution formed was then discarded, and 50 µL of 20% chelex plus 150ul of DNAse water was added. Each tube was vortexed actively for 30 seconds and placed in a heating block for 2minutes at 100^{o}C, vortexing vigorously after heating for 2minutes and placing in a heating block for a period of 10minutes. The tubes were spun at 10,000 rpm for 2 minutes, after which the supernatant was transferred into a fresh, sterilized microfuge tube and the pellet discarded. The supernatant was spun again at 10,000 rpm for 2 minutes, and the resulting supernatant was transferred to a new tube. The process was carried out three times, after which the last supernatant/DNA containing portion was stored at -20^{o}C.

3.8 Genotyping for drug resistance markers

3.8.1. PCR amplification of the target Kelch 13 propeller region

To interrogate for mutations in the target region of the K13 gene (located in chromosome 13), nested PCR using primers shown in Table 3.1 below amplified the k13 propeller region. In a total volume of 30 µl, the outer PCR consisted of 1 × of PCR buffer (Thermo Scientific™, USA), 1U unit of Taq polymerase (Thermo Scientific™, USA), and 1.0 µl of individual isolate DNA extract. The other reagents $MgCl_2$, dNTPs, forward and reverse primers, and cycling conditions were optimized, as shown in Table 3.2. The Nested PCR master mix was performed in a total volume of 30 µl. It consisted of 17.35µl nuclease-free water, 1 × of PCR buffer, 1U unit of Taq polymerase (Thermo Scientific™, USA), and 0.5 µl of DNA alongside other reagents under optimized cycling conditions as shown in Table 3.2.

Table 3.1: Primer sequences of the four genes alongside their respective target regions.

References	Primer Name	Primer sequence (5′ to 3′)	Position on the gene
Ariey *et al.*, 2014	*Pf*k13_K1_F outer	CGGAGTGACCAAATCTGGGA	**65-84**
	*Pf*k13_K4_R outer	GGGAATCTGGTGGTAACAGC	**2161-2142**
	*Pf*k13_K2_F nested	GCCTTGTTGAAAGAAGCAGA	**1279-1298**
	*Pf*k13_K3_R nested	GCCAAGCTGCCATTCATTTG	**2127-2108**
Henriques *et al.*, 2015	*Pf*ap2_F_outer	AAGACTGTCAAATGTAAAAGACCC	**26 - 9**
	*Pf*ap2_R_outer	CTCATGTAAAACAAAAAGTGAGG	**560 - 542**
	*Pf*ap2_F_nested	GATATCCACAAACATTAGAAGTG	**359 - 381**
	*Pf*ap2_R_nested	CCATCTGGTGGTGTGAAGG	**1199 - 1181**
Kiarie *et al.*, 2015	*Pf*crt_P1_F outer	GCGCGCGCATGGCTCACGTTTAGGTGGAG	**92-112**
	*Pf*crt_P2_R outer	GGGCCCGGCGGATGTTACAAAACTAT AGTTACC	**283-259**
	*Pf*crt_D1_F nested	TGTGCTCATGTGTTTAAACTT	**130-150**
	*Pf*crt_D2_R nested	CAAAACTATAGTTACCAATTTTG	**271-249**
Sabah *et al.*, 2007	*Pf*mdr1_A1_F outer	TGTTGAAAGATGGGTAAAGAGCAGAAAGAG	**1-21**
	*Pf*mdr1_A3_R outer	TACTTTCTTATTACATATGACACCACAAAC	**648-619**
	*Pf*mdr1_A2_F nested	GTCAAACGTGCATTTTTTATTAATGACCATTTA	**25-54**
	*Pf*mdr1_A4_F nested	AAAGATGGTAACCTCAGTATCAAAGAAGAG	**584-556**
Humphreys *et al.*, 2007	*Pf*mdr1_NewF_outer	GTGTATTTGCTGTAAGAGCT	**2834- 2853**
	*Pf*mdr1_NewRev_outer	GACATATTAAATAACATGGGTTC	**3791-3769**
	*Pf*mdr1_N1_nested	CAGATGATGAAATGTTTAAAGATC	**2921-2944**

*Pf*mdr1_N2_nest	TAAATAACATGGGTTCTTGACT	**3784-3763**

The name of the primer is derived from their respective genes. The genes were *Plasmodium falciparum* kelch 13 *(*Pfk13*),* adaptor protein 2-mu (*Pf*ap2-mu) subunit, chloroquine resistance transporter *(Pf*crt*),* and multidrug resistance *1(Pf*mdr1*).*
F – Forward primer and R – Reverse primer.

Table 3.2: Optimized Polymerase Chain Reaction (PCR) cycling conditions for *P. falciparum* kelch 13-propeller region gene amplification

PCR Conditions	**Temperature (°C), Time**	
	Outer	**Nested**
Initial denaturation	94°C, 3 min	**94°C, 3 min**
Denaturation	94°C, 30 sec	**95°C, 30 sec**
Annealing temperature	56°C, 2 min	**56°C, 2 min**
Elongation	68°C, 2 min	**68°C, 2 min**
Cycles	30	**35**
Final elongation	72 °C, 10 min	**68 °C, 10 min**
Primer (forward & reverse)	300nM/µl each	**300nM/µl each**
Mgcl2 (mM)	2.0	**2.0**
dNTPs(mM)	0.2	**0.2**

3.8.1.1 Analysis of PCR products and sequencing

The amplified PCR products were analyzed in 2% agarose gel. Briefly, the 2% w/v agarose gel was prepared by mixing 2g of agarose with 100ml of 1x Tris base/Acetic acid/ EDTA (TAE) buffer. The mixture was heated in a microwave till boiling and

let to cool. Ethidium Bromide dye and swirled to mix for easy visualization of DNA. The gel was then emptied into an electrophoresis casting tank and allowed to solidify. Subsequently, 5µl of each PCR product was mixed with 2µl loading dye and loaded on individual gel well, and ran at 80Vs for 35 minutes. The gel was visualized in a UV trans-illuminator (Bio-Rad machine). A single band in the individual sample was considered a positive upon comparison with the control.

The positive sample amplicons that remained in the PCR tube after gel electrophoresis were then purified using ExoSAP-IT® (Affymetrix, Santa Clara, CA). ExoSAP-IT consists of two hydrolytic enzymes, exonuclease 1 and Shrimp Alkaline Phosphatase (SAP), designed to remove unwanted primers and dNTPs from the PCR product mixture with the advantage of 100% recovery of PCR products. The enzymatic purification was carried out in a final volume of 7 µL containing 5 µL of the PCR product, 2 µL Exosap-it® and then incubated for 15 min at 37°C followed by 15 min at 80°C for enzyme denaturation (according to the manufacturer's instructions).

The purified product was then sequenced based on the BigDye® Terminator v3.1 Cycle Sequencing Kit reaction mix (Applied Biosystems, USA) using a 3500xL sequencer. Both Forward and Reverse primer (s) were used to sequence regions of interest. Cycle sequencing was conducted using ABI BigDye Terminator v3.1 chemistry (Life Technologies, Carlsbad, CA, USA). The sequencing mix of the big dye was prepared separately for both forward and reverse primers, as shown in Table 3.3 below.

Table 3.3: The big dye cycle sequencing reaction.

Reagent	Volume per reaction (µl)
5X Sequencing buffer	2.0
Sequencing primer (5pmol/µl) - Forward/Reverse	1.0
BigDye® Terminator v3.1	1.0
Template	4.0

The plate was then loaded to the PCR machine. The cycling conditions were set as follows: one denaturation hold at 94°C for 5 min followed by 30 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 2.30min and a final extension for 10 minutes. The sequenced reaction fragments were purified using prepared Sephadex G-50(Sigma Aldrich Co., USA) on HV plates (Millipore Corporation, USA). Briefly, 8 µL of the sequenced reaction fragments were placed on each well's center (Sephadex separation Matrix). The plates were then centrifuged at 2144 rpm for 5 min and collected the filtrate (DNA) in an optical plate placed under the HV multiscreen plate. 10 µl of Hi-Di formamide (Applied Biosystems, USA) was added to the wells and mixed samples. The sample plate was covered with septa, placed into a plate base, and then loaded into the 3500xL ABI Genetic analyzer to resolve the extension products.

3.8.1.2 Sequence alignment and protein structure prediction of the 611S SNP of the K13 gene

Multiple sequence analysis (MSA) and structure prediction of the K13 gene was performed using web-based programs. MSA of sequenced isolates alongside the

selected strains of *P. falciparum* was carried out using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira *et al.*, 2019). The selected strains included *Plasmodium, falciparum, Plasmodium vivax, Plasmodium berghei, and Plasmodium knowlesi.* Next, Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2), an online protein structure prediction tool that employs a homology technique (Kelley et al., 2015), was used for *in silico* modeling using amino acid sequences of wild-type and mutant parasites isolated from this study.

3.8.2 PCR amplification of the target region of the *Pf*ap2-mu gene

Nested PCR was used to amplify the target region, as shown in Table 3.4 of the *Pf*ap2mu (*PF*3D7_1218300) gene. In the PCR reaction mix of 25 µl, the outer PCR consisted of 1 × of PCR buffer (Thermo Scientific™, USA), 1U unit Taq polymerase (Thermo Scientific™, USA), and 5.0 µl of DNA. Other reagents such as $MgCl_2$, dNTPs, forward and reverse primers, and cycling conditions were optimized, as shown in Table 3.4. Nested PCR mixture amounting to 25 µl consisted of 15.8 µl nuclease-free water, 1 × of PCR buffer, 1U units Taq polymerase (Thermo Scientific™, USA), 1µl of DNA alongside other reagents under optimized cycling conditions as shown in Table 3.4 The primers used and the position on the gene targeted are shown in Table 3.4. Using a 2% agarose gel, the amplified PCR products were analyzed, purified, sequenced, and analyzed as described in section **3.8.1.1**.

Table 3.4: Optimized Polymerase Chain Reaction (PCR) cycling conditions for P. falciparum associated adaptor protein 2-mu subunit (Pfap2-mu) gene amplification.

PCR Conditions	Temperature (°C), Time	
	Outer	**Nested**
Initial denaturation	94°C, 3 min	**94°C, 3 min**
Denaturation	94°C, 30 sec	**95°C, 30 sec**
Annealing temperature	57°C, 30sec	**57°C, 30 sec**
Elongation	68°C, 1 min	**68°C, 45 sec**
Cycles	30	**35**
Final elongation	68 °C, 15 min	**68 °C, 10 min**
Primer (forward & reverse)	200 nM/µl each	**200 nM/µl each**
Mgcl2 (mM)	4.0	**4.0**
dNTPs(mM)	0.4	**0.4**

3.8.3 PCR amplification of the *Pf*crt target region

The *Pf*crt gene target fragment, as shown in Table 1B on chromosome 7 of the 3D7 isolate (*PF*3D7_0709000), was amplified using primers and PCR cycling conditions shown in Table 3.1 and Table 3.5, respectively. Briefly, in a total volume of 40 µl, the outer PCR consisted of 1 × of PCR buffer (Thermo Scientific™, USA), 0.0075 units of Taq polymerase (Thermo Scientific™, USA), and 5.0 µl of DNA. The other reagents $MgCl_2$, dNTPs, forward and reverse primers, and cycling conditions were optimized, as shown in Table 3.5. In Nested PCR reaction mix of 40 µl, 17.35 µl nuclease-free water, 1 × of PCR buffer, 0.0075 units of Taq polymerase (Thermo Scientific™, USA), and 3.0 µl of DNA alongside other reagents were mixed under optimized cycling conditions as shown in Table 3.5. Using a 2% agarose gel, the

amplified PCR products were analyzed, purified, sequenced, and analyzed as described in section **3.6.1.1**.

Table 3.5: Optimized Polymerase Chain Reaction (PCR) cycling conditions for Plasmodium falciparum chloroquine resistance transporter (Pfcrt) gene amplification.

PCR conditions	Temperature (°C), Time	
	Outer	**Nested**
Initial denaturation	95°C, 5 min	**95°C, 5 min**
Denaturation	92°C, 2 min	**92°C, 30 sec**
Annealing temperature	45°C, 30sec	**50°C, 30 sec**
Elongation	65°C, 1 min	**65°C, 45 sec**
No of cycles	45	**35**
Final elongation	72 °C, 15 min	**72 °C, 10 min**
Primer (forward & reverse)	200 nM/µl each	**200 nM/µl each**
Mgcl2 (mM)	1.5	**1.5**
dNTPs(mM)	0.4	**0.4**

3.8.4 PCR amplification of the target regions of the *Pfmdr1* gene

The *Pf*mdr1 target region on chromosome 5 was amplified by utilizing primers shown in Table 3.1 using nested PCR. In a total volume of 40 µl, the outer PCR consisted of 1 × of PCR buffer (Thermo Scientific™, USA), 1U unit Taq polymerase (Thermo Scientific™, USA), and 1.0 µl of DNA. The other reagents $MgCl_2$, dNTPs, forward and reverse primers, and cycling conditions were optimized as shown in Table 3.6. The PCR was done in a total volume of 40 µl and consisted of 17.35 µl nuclease-free water, 1 × of PCR buffer, 1U unit of Taq polymerase (Thermo Scientific™, USA), and 1.0 µl of DNA alongside other reagents under optimized cycling conditions as shown in Table 3.6. The amplified PCR products were

evaluated in 2.0% agarose gel, sequenced, and analyzed as described in section **3.8.1.1**.

Table 3.6: Optimized Polymerase Chain Reaction (PCR) cycling conditions for Plasmodium falciparum multidrug resistance 1 (Pfmdr1) gene amplification.

PCR conditions	Temperature (°C), Time	
	Outer	**Nested**
Initial denaturation	94°C, 3 min	94°C, 3 min
Denaturation	94°C, 30 sec	94°C, 30 sec
Annealing temperature	55°C, 30sec	60°C, 30 sec
Elongation	65°C, 1 min	65°C, 1 min
No of cycles	30	30
Final elongation	65 °C, 5 min	65 °C, 5 min
Primer (forward & reverse)	100 nM/µl each	100 nM/µl each
Mgcl2 (mM)	1.5	1.5
dNTPs(mM)	0.2	0.2

3.9 Data analysis

The raw sequencing data (ab1 files) of both forward and reverse sequences generated by the Sanger sequencing technique were uploaded into ChromasPro software (version 2.1.8). The extreme regions with low-quality reads were then trimmed, and ambiguous reads were visually analyzed (Technelysium Pty Ltd, 2020). Successively, forward and reverse sequences were assembled to generate contiguous sequences for each sample isolate and exported in the multifasta file format. The multiple sequence alignment (MUSCLE) program in MEGAX software was used to perform multiple sequence alignment to detect polymorphisms (Kumar *et al.*, 2018). By interrogating both the nucleotide and protein sequence translated using the standard code to that of the reference 3D7 strain genome obtained from PlasmoDB

(PlasmoDB, 2020), SNPs' positions were determined using jalview software (Waterhousr *et al.,* 2009).

CHAPTER FOUR

RESULTS

4.1 Novel single nucleotide polymorphism in the kelch propeller region mapped

The PCR amplification of K13 yielded the expected band size of 630bp, Figure 4.1

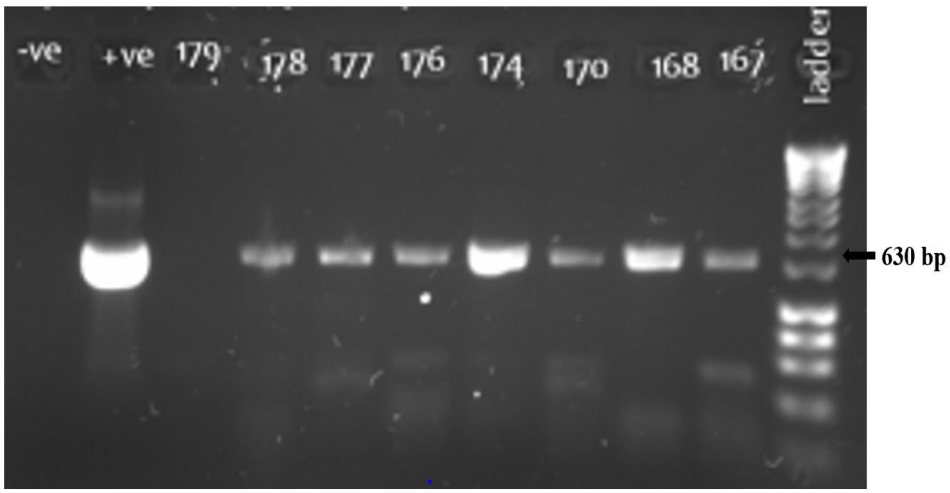


Figure 4.1: PCR products of kelch 13-propeller region resolved on 2% agarose gel. Lane1 – Negative control (nuclease-free water), Lane2 – positive control (3D7 strain of P. falciparum), Lane 3-10 (selected sample isolates) and Lane 11 – 100bp DNA ladder.

After interrogating the contiguous sequences through Multiple sequence alignment of the isolates for the traditional SNPs in the propeller domain associated with ACT resistance at positions M476I, Y493T, R539T, I543T, this study did not find any of those changes. Interestingly, one unique non-synonymous mutation was mapped, a change from tryptophan to serine at position 611 of the K13 protein (W611S) **figure 4.3,** representing 2% of the samples analyzed, **Figure 4.4**.

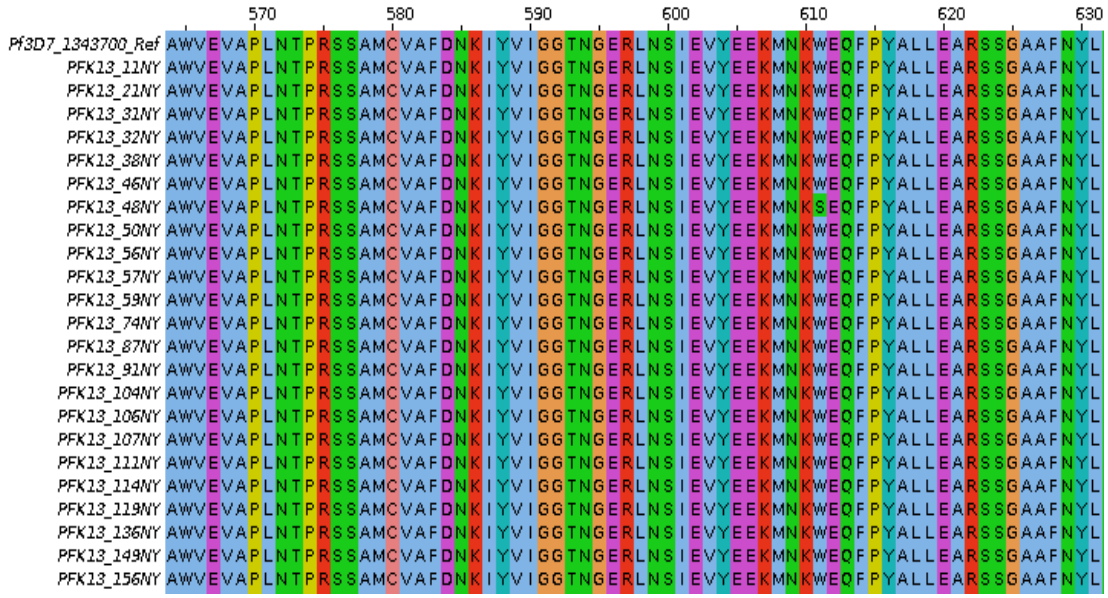


Figure 4.2: Multiple sequence alignment of P. falciparum kelch 13-propeller nucleotide sequences showing novel SNP (G1833T)

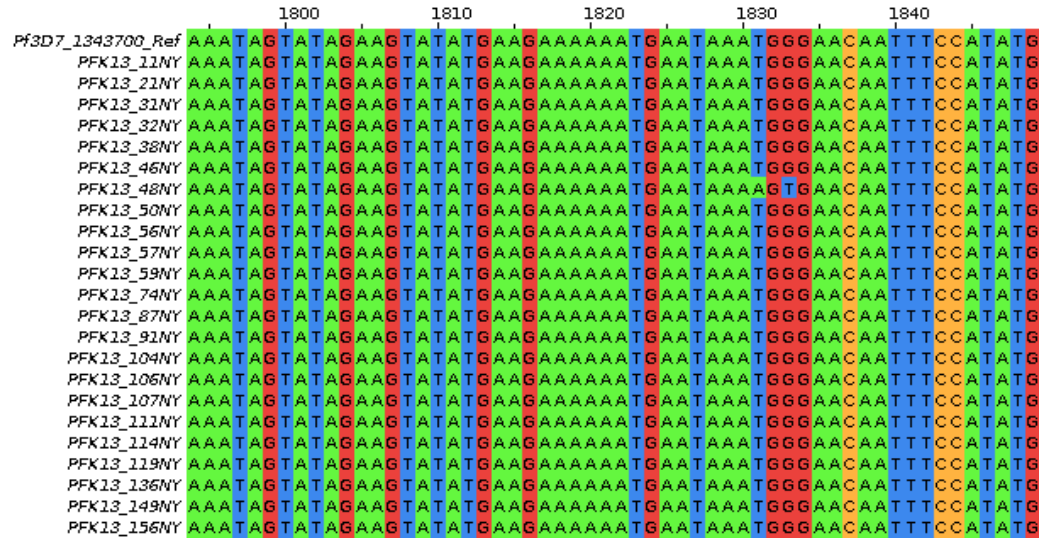


Figure 4.3: Multiple sequence alignment of P. falciparum kelch 13-propeller protein sequences showing novel nsSNP (W611S)

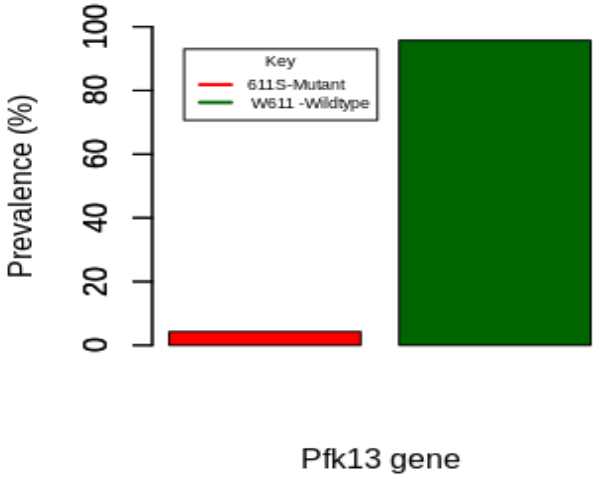


Figure 4.*4*: The prevalence of the W611S SNP in the *P. falciparum* kelch 13-propeller region from samples collected in Nyando 2015.

On further scrutiny of the position of nsSNP, it was established out the amino acid Try611 within the K13 protein is located on a conserved motif across the different malaria parasite species utilized for analysis, as shown in **figure 4.5 (A). T**he change in W611S position had an overall significant impact on the protein structure due to differences in amino acids, tryptophan, and serine functional groups (Wu, 2009; Newsholme *et al.*, 2011). The impact of the W611S mutation on the protein structure was predicted using Phyre 2, an online protein prediction tool (Kelley *et al.*, 2015). The protein structure of *P. falciparum* was determined through 3D modeling. Based on *in silico* modeling, the model is a 6-bladed propeller structure generated as shown in **figure 4.5 (B)**. The superimposition of the wild-type (green) and mutant (purple) homology models resulted in structural changes in the overall topology of the protein, as shown in **figure 4.5 (C)**. The c4yy8B template chosen resulted in a model with a confidence score of 100% based on homology assessment and model prediction quality.

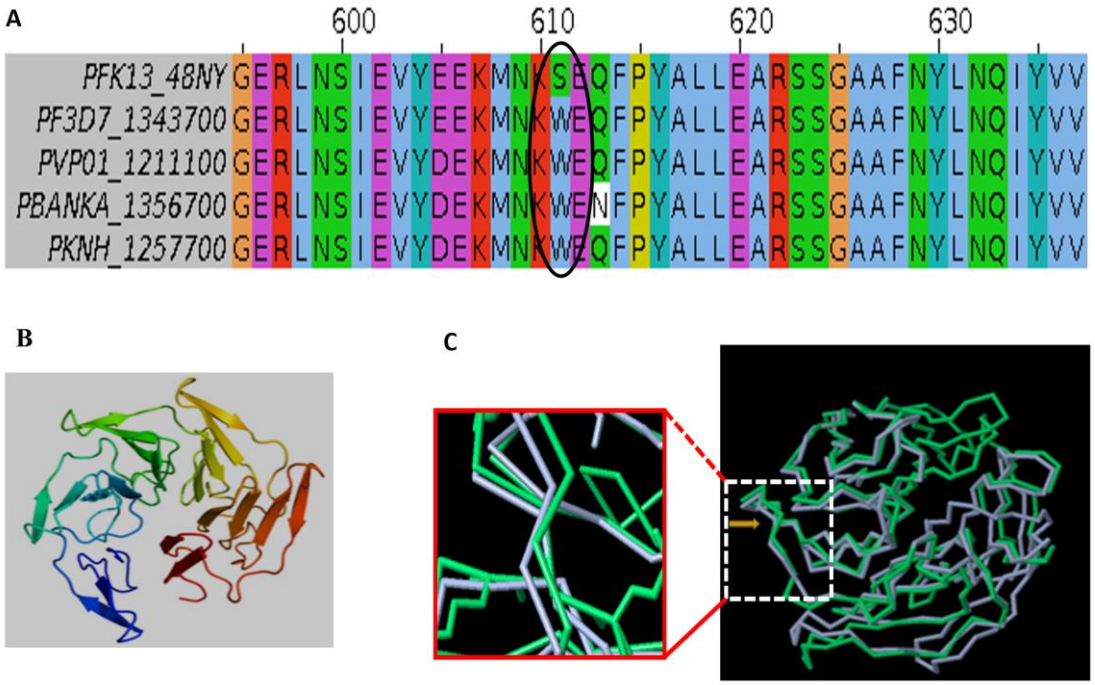


Figure 4.5: Multiple sequence alignment and 3D protein homology modeling

A. Multiple sequence alignment (MSA) of the isolate (PFK13_48NY) with W611S SNP (circled in red) alongside four curated reference strains selected from PlasmoDB. The selected strains included *Plasmodium: falciparum* (PF3D7_1343700_Pf), *vivax* (PVP01_1211100_P.v),*berghei* (PBANKA_1356700) and *knowlesi* (PKNH_1257700). The MSA was carried out using MEGA-X software. From MSA, the 611S SNP lies in a highly conserved region. **B.** Predicted 3D model of the *P. falciparum* K13 propeller protein. **C.** Shows the homology models' superimposition between the wild type (green color) and mutated proteins (Purple). The modeling and superimposition were carried out using phyre 2 online protein structure prediction tool. The arrow shows the location of the W611S mutation.

4.3 Increased prevalence of *Pf*ap2mu-160N/T allele

The PCR amplification of the *Pf*ap2mu gene yielded the expected band size of 560bp, as shown in Figure 4.6

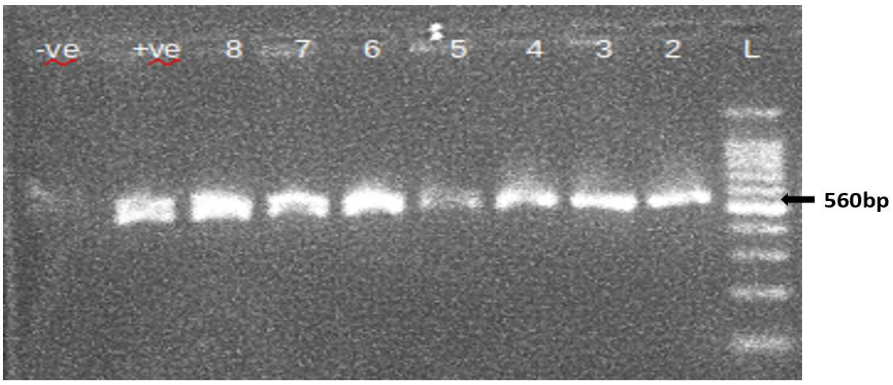


Figure 4.6: PCR products of Pfap2mu gene resolved on 2% agarose gel. Negative Lane1 – Negative control (nuclease-free water), Lane2 – positive control (3D7 strain of P. falciparum), Lane 3-9 (selected sample isolates) and Lane 10 – 100bp DNA ladder.

The multiple sequence alignment of nucleotides and amino acid sequences indicated the non-synonymous mutation (S160N), as shown in **Figures 4.7 and 4.8.**

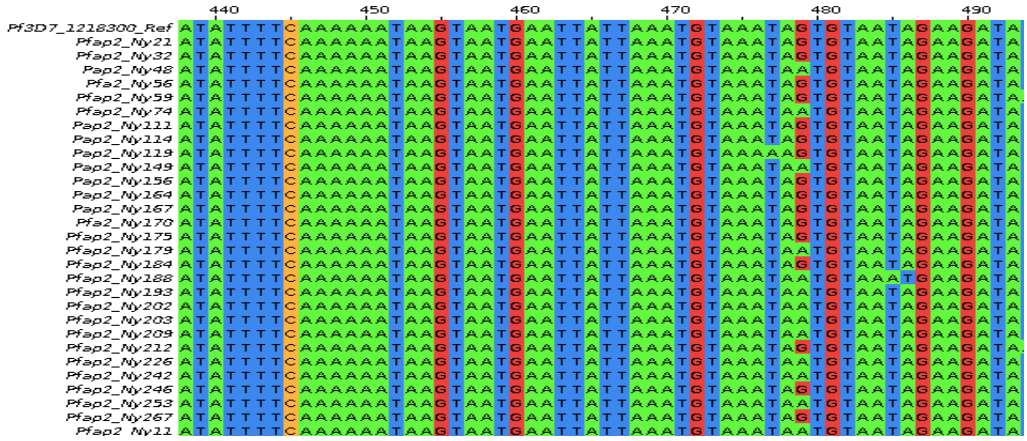


Figure 4.7: Multiple sequence alignment of Pfap2mu nucleotide sequences showing SNPs.

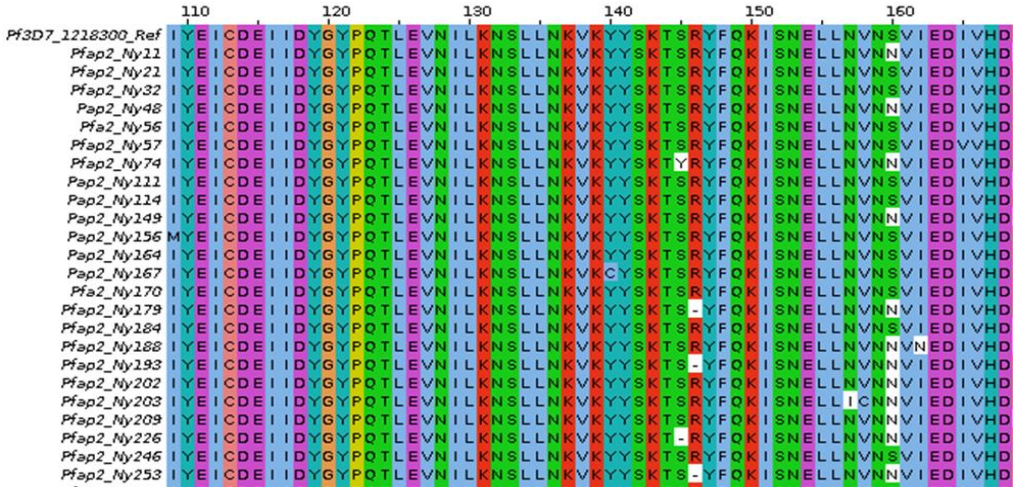


Figure 4.*8*: Multiple sequence alignment of *Pfap2mu* protein sequences showing nsSNP (S160N).

Out of the 47 samples analyzed, fourteen samples harbored the S160N/T, translating to (29%). The SNP is a crucial change closely associated with ACT delayed clearance, as shown in **Figure 4.8,** which was high compared to the previous study from Kenya.

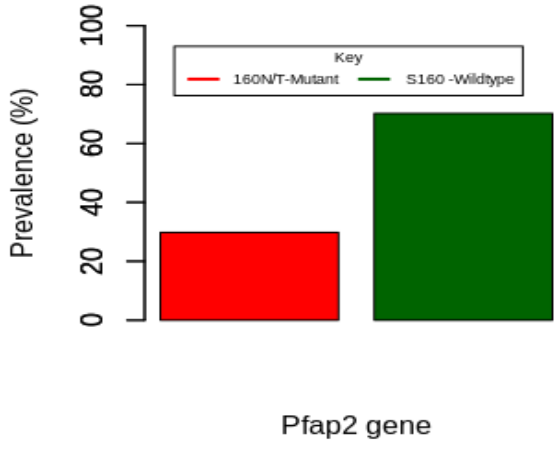


Figure 4.*9*: The prevalence of S160N/T SNP in the *P. falciparum* associated adaptor protein 2-mu subunit (*Pf*ap2-mu) gene from samples collected in Nyando 2015.

4.4 Enhanced prevalence of wild-type alleles of *Pf*crt-K76

The PCR amplification of the *Pf*crt gene yielded the expected band size of 145bp, as shown in Figure 4.10

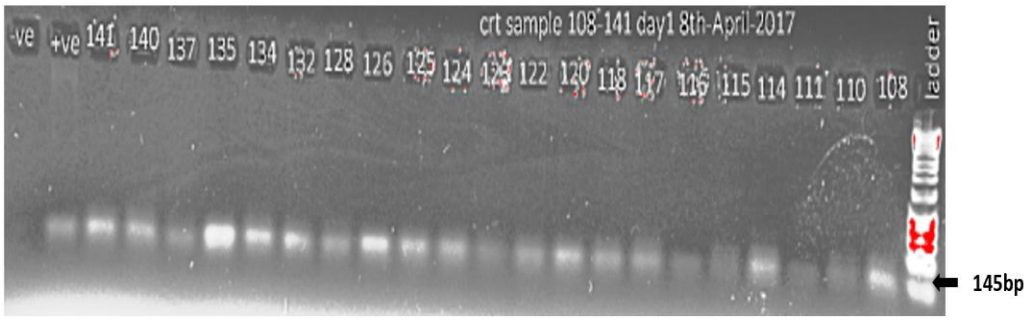


Figure 4.10: PCR products of Pfcrt-K76 gene resolved on 2% agarose gel. Lane1 – Negative control (nuclease-free water), Lane2 – positive control (3D7 strain of P. falciparum), Lane 3-13 (selected sample isolates) and Lane 14 – 100bp DNA ladder.

Figure 4.10: PCR products of Pfcrt-K76 gene resolved on 2% agarose gel. Lane1 – Negative control (nuclease-free water), Lane2 – positive control (3D7 strain of *P. falciparum*), Lane 3-13 (selected sample isolates) and Lane 14 – 100bp DNA ladder.

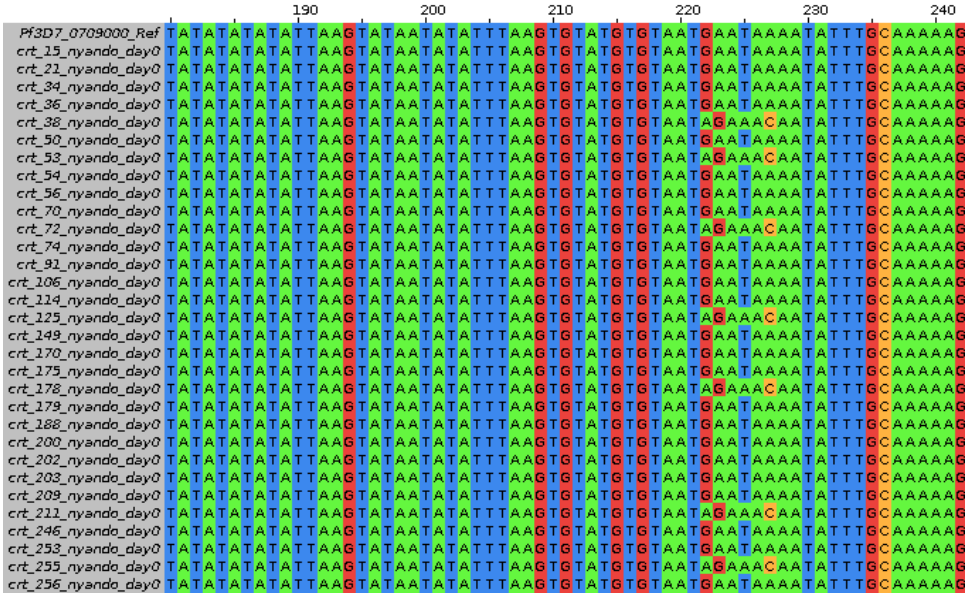


Figure 4.11: Multiple sequence alignment of Pfcrt nucleotide sequences showing SNPs.

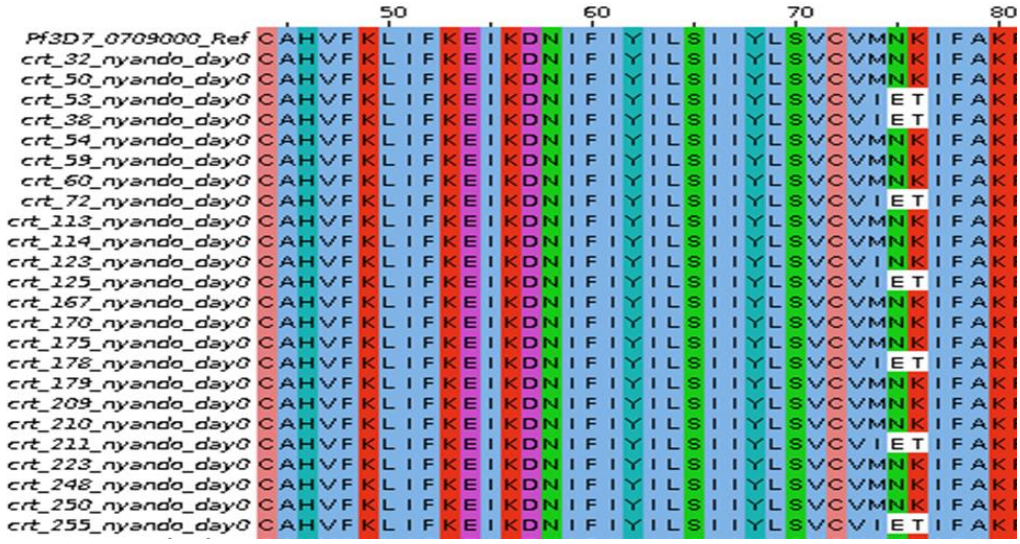


Figure 4.12: Multiple sequence alignment of Pfcrt amino acid sequences indicating the CVMNK/CVIET haplotype.

The wildtype CVMNK (codons 72-76) haplotype of the *Pf*crt gene was confirmed as present in 87 of the 94 samples analyzed (92.56%). The remaining 7 of the 94 samples (7.44%) were CVIET alone, as indicated in **Figure 4.13.** The frequency of the *Pf*crt-76T primary mutation associated with CQ resistance in the $C_{72}V_{73}I_{74}E_{75}T_{76}$ haplotype significantly decreased ($P < 0.001$) from 41% (Kiarie *et al.*,2015) to 7.44% in the current study.

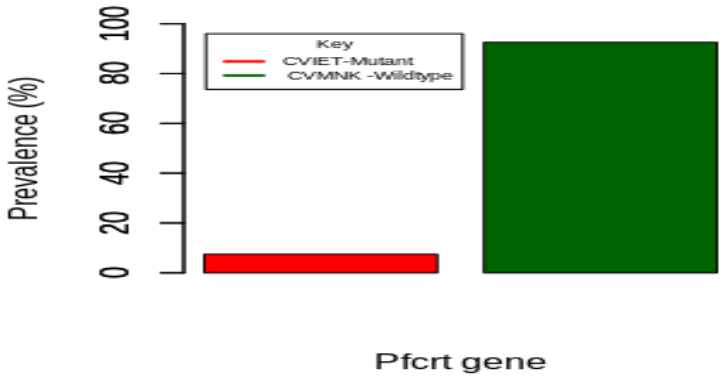


Figure 4.13: The prevalence of SNPs in the P. falciparum chloroquine resistance transporter (Pfcrt) gene from codons 72-76 (CVMNK/CVIET haplotype).

4.5 Increased prevalence of *Pfmdr1* N86 and D1246

The PCR amplification of *Pf*mdr1 yielded the expected band size of 560bp, as shown in Figure 4.14

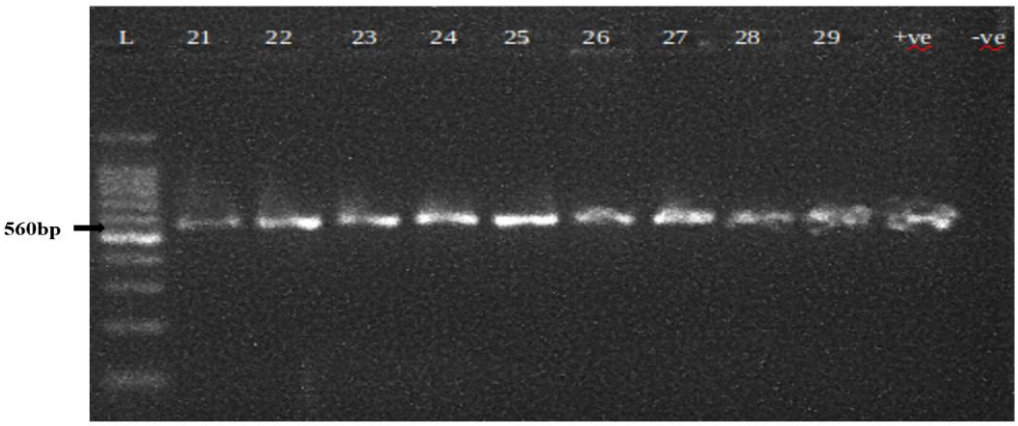


Figure 4.14: PCR products for samples 21-29 (Lane 2-10), 100bp DNA ladder (Lane 1), 3D7 P. falciparum Positive control (Lane 12) and Negative control – nuclease free water (Lane 12)

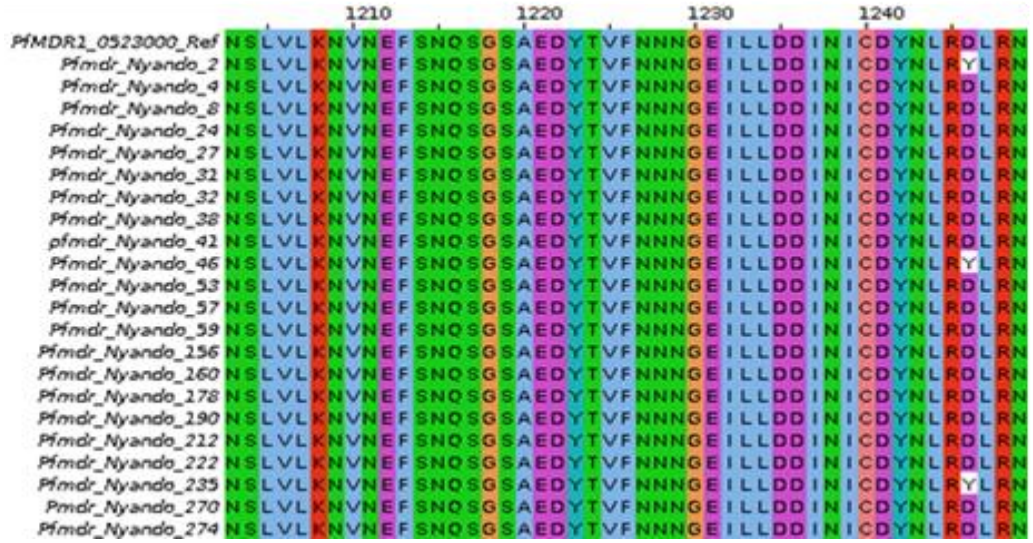


Figure 4.15: Multiple sequence alignment of Pfmdr1 nucleotides showing SNPs

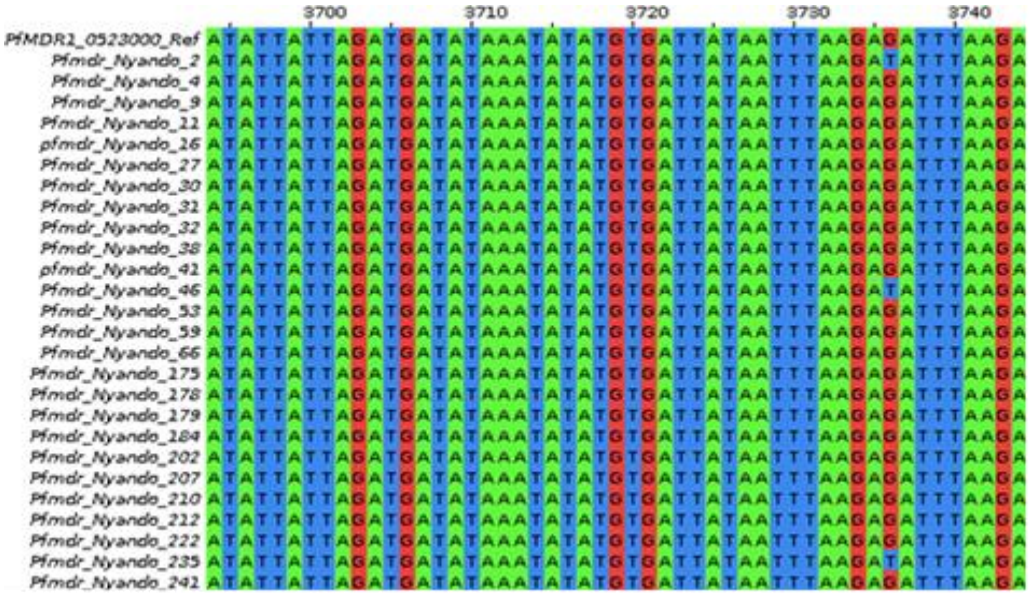


Figure 4.16: Multiple sequence alignment of Pfmdr1 nucleotides showing SNPs.

This study did not detect a mutation at codon N86Y, 1034C, and 1042N in the *Pf*mdr1 gene. Likewise, 91 of the 94 samples analyzed harbored the wildtype D1246 allele (96.81%), and only three samples carried the 1246Y mutant allele (3.19%) (**Figure 4.17**). There was no significant increase in the wild-type allele.

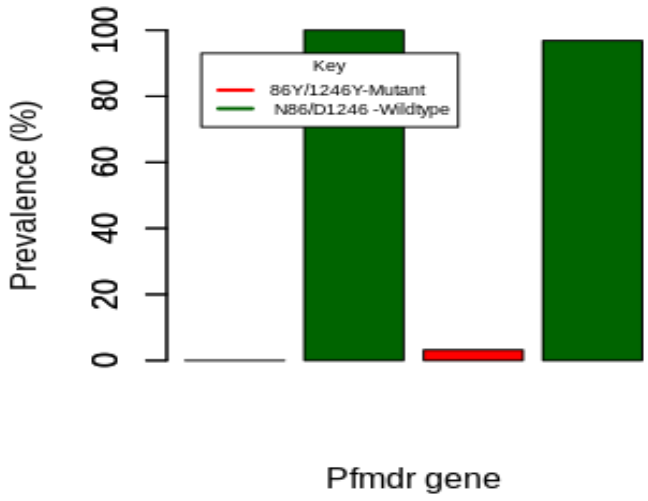


Figure 4.17: The prevalence of N86Y and D1246 SNPs in the P. falciparum multidrug resistance 1 (Pfmdr1) gene from samples collected in Nyando in 2015

CHAPTER FIVE

DISCUSSION

5.1 Detection of a potential functional novel mutation in the kelch propeller region

The study reports new non-synonymous (W611S) and synonymous (C469T) mutations in the K13 propeller domain. These mutations were in isolates of *P. falciparum* from patients in high malaria-endemic regions where the first-line of treatment for malaria over the last decade has been artemether-lumefantrine (Baird, 2005; WHO, 2019). Artemisinin and, by extension, ACT resistance manifests a delayed parasite clearance after administering the ACTs' recommended dosage (Noedl *et al.,* 2008; Suresh & Haldar, 2018). Across the malaria-endemic region of Africa, ACTs generally remain efficacious (Amambua-Ngwa *et al.,* 2017; Hemming-Schroeder *et al.,* 2018); however, the emergence of parasite tolerant to the long-acting moiety of the ACTs (Cooper et al., 2015; Amambua-ngwa *et al.,* 2018) indicate a slow filtering of ACTs resistant parasites in the field. In Kenya, the initial report on the failure of ACTs to clear parasites was reported in 2013 (Beshir *et al.,* 2013). Analysis of the residue parasites targeting the hot spot genes associated with multidrug resistance parasites found guiding patterns of mutation in *Pf*crt, *Pf*mdr1, *Pf*ubp1, and *Pf*ap2-mu genes (Henriques *et al.,* 2014a). To date, it is now evident that multidrug-resistant parasite lineage has spread across South East Asia (Hamilton *et al.,* 2019).

In line with previous studies in African settings, this study adds to the existing evidence that there is a reservoir for k13 polymorphisms worldwide by identifying a novel mutation in the k13 propeller region. For instance, a study by Isosumi *et al.,* (2015) identified four novel non-synonymous mutations (M442V, N554S, A569S, A578S) in the k13 propeller region. Another study by wamae *et al.,* (2019) identified several SNPs at low frequency in the propeller region that included C469T, V487T,

A578S, V589T, and K189T (over >10%), located at the N-terminal of the k13 gene. The K189T mutation has also been reported at a lower frequency (1.6%) in studies in Bangladesh and China-Myanmar border (Mohon *et al.,* 2014). Additionally, the K189T has been reported at a higher frequency and T149S (6.3%) in Dakar, Senegal (Torrentino-Madamet *et al.,* 2014). Another study by Ocan *et al.,* 2016 in Uganda among symptomatic patients identified two non-synonymous mutations (S522R, G533C) and one synonymous mutation, Q509Q. A recent study carried in Rwanda showed a clonal expansion of the R561H mutation (Uwimana *et al.,* 2020). Additionally, a study by Kamau *et al.* (2015) reported a total of 22 unique SNPs, of which seven were nonsynonymous. Altogether, these mutations' frequency appears to be low within African settings and are not associated with artemisinin resistance since their impact is poorly understood.

This study identifies two new mutations, W611S, and C469T, which occur in the K13 propeller domain and may be associated with slow clearance of *P. falciparum* parasite following AL treatment. However, the precise impact of these mutations on parasite drug responses will require further analysis of under dihydroartemisinin treatment *in vitro*. The switch from a non-polar tryptophan into polar serine at position 611 enhances the protein's interaction with other proteins, which may ultimately alter the protein's biological function.

Previous studies have identified background mutation in genes closely associated with multidrug-resistant phenotypes (Miotto *et al.,* 2015). Within the endemic malaria region, such as the Western part of Kenya, three key drivers influence the emergence and spread of the drug-resistant parasite (Maraka *et al.,* 2020). First, the presence of asymptomatic infections resulting from semi-immunity sustains both the parasite transmission and incubation of different mutant parasite populations. Second, the high transmission of *P. falciparum* is associated with the slow fixation of functional mutations due to the high sexual recombination of the parasite

populations. Third, the high prevalence of CQ mutants may provide harbinger mutations that induce or antagonize the fixation of functional K13 mutations. Here, the argument is that the parasites may be undergoing selection sweep under the three biological drivers for the emergence and spread of the functional mutant parasite.

In the Cambodia region of South East Asia, the non-synonymous mutation; C580Y, R539T, R543I, and Y493H in the K13 gene are associated with artemisinin resistance in the k13 gene reported in Cambodia (Spring *et al.,* 2015). To date, these mutations have not been detected in the African parasite population, suggesting that the artemisinin-resistant parasite may arise through a functional mutation different from those of the South East Asia region (Maraka *et al.,* 2020). Indeed, the drug-resistant parasite population's selective sweeps between the two regions also differ, and resistance to ACTs within the African setting might arise independently.

5.2 An upsurge of S160N SNP of the *Pf*ap2-mu gene

In this study, the prevalence of SNP at codon S160N/T in the *Pf*ap2-mu in the parasite population isolated from individuals in the Western region of Kenya is trending upward (29%) compared to the previously published prevalence of 18% in the same region (Henriques *et al.,* 2014a). The need to study this gene was driven by a previous report that had noted reduced ACT responsiveness in the Kenya population and had linked the reduced parasite clearance to 160N/T SNP in the *Pf*ap2-mu gene (Beshir *et al.,* 2017). The ap2-mu gene, encoding the adaptor protein two complex's mu chain, was initially identified using artemisinin-resistant *P. chabaudi* (Henriques *et al.,* 2013). Subsequent studies confirmed the mutation in ap2-mu association with artemisinin resistance in the human malaria parasite *P. falciparum in vitro* (Henriques, Van Schalkwyk, *et al.,* 2015). The adaptor protein 2 complex facilitates the formation of clathrin-coated vesicles required to transfer parasite materials between different cell membrane compartments (Owen, Collins, &

Evans, 2004). Mutations in the ap2-mu sub-unit reduce the binding affinity of the membrane cargo, consequently decreasing the efficiency of endocytic trafficking of membrane proteins.

Thus, our data suggest a slow but steady replacement of artemisinin susceptible parasite population with less susceptible parasite lineage. Interestingly, and in line with the global trend of reciprocal selection sweep of CQ sensitive parasite following continuous use of the ACTs, the isolates with S160N mutation also carried *Pf*crt CVMNK haplotype (Blasco *et al.*, 2017). The rise in the S160N/T mutation and concomitant selection of *Pf*crt CVMNK wild-type haplotype might be explained in the context of continued use of ACTs in the region. Importantly, our data may also indicate that CQ is a future option specifically to antagonize ACTs resistance selection. A study by Blasco *et al.* (2017) explains that the reciprocal resistance between CQ and the ACT suggests that a triple combination of AL and CQ may significantly protect against the rapid emergence and spread of drug-resistant *P. falciparum* parasites in the field. However, the 160N/T SNP appears to be restricted in Africa since it has not been in SEA, where artemisinin resistance is frequent (MalariaGEN, 2016). Recently, another potential SNP in the *ap2-mu* gene, I592T (not determined in this study), has been shown by Henrici *et al.*, (2019) to enhance the parasite's ring-stage survival following DHA pulsing.

5.3 Near reversion of mutant to wildtype alleles at Key SNPs in the *Pf*crt and *Pf*mdr genes associated with CQ sensitivity

Over the last two decades since the withdrawal of CQ, the reemergence of *Pfcrt* K76 and *Pfmdr1* N86 wildtype parasite lineage in African has been slow (Ocan *et al.*, 2019). Our study reveals a plummeting prevalence of CQ resistance; this is based on the 7% prevalence of *Pf*crt CVIET haplotype associated with CQ resistance compared to the 93% of wildtype CVMNK haplotype. Also, studies in Kenya over

the last ten years have shown a steady but sluggish decline of the mutant parasite carrying *Pfcrt* 76T mutation to 41% from as high as 63% and 95% (Mwai *et al.,* 2009; Ocan *et al.,* 2019). In line with recent studies (Kiarie *et al.,*2015; Lucchi *et al.,*2015; Kublin *et al.,*2016), we have shown an increase in the selection of *Pf*crt K76 SNP, which is associated with chloroquine-sensitive parasites after removal of chloroquine drug pressure from the market, 19 years later. Our focus on *Pfmdr1* reveals that the study area's parasites predominantly carried the wild-type allele of the *Pf*mdr1-86N (100%) and *Pf*mdr2-1246D (~97%). The *Pf*mdr1-N86Y and *Pf*mdr1 D1246Y mutation modify and argue CQ resistance (Dokomajilar *et al.,* 2006).

This study agrees with studies carried out in both the Western and coastal regions of Kenya that have shown a steady decrease in Chloroquine-resistant alleles, *crt* 76T, *mdr1* 86Y, and 1246Y. For instance, in the coastal area (Kilifi), the *crt* 76T and *mdr1* 86Y alleles have decreased from 88% to 63% and 75% to 54% from 1998 to 2008, respectively (Mang'era *et al.,* 2012). A recent study by Wamae *et al.,* (2019) reported a 99% reversion of chloroquine-sensitive parasite in Kilifi (coastal region). Likewise, the same observation has been made in western Kenya in which the *crt* 76T, *mdr1* 86Y, and 1246Y mutant alleles decreased from 86% to 2%, 92% to 1%, and 67% to 6% amid the years 2003 and 2014 respectively (Achieng *et al.,* 2015).

Additionally, another study carried out by Eyase *et al.,* (2013) in western Kenya showed a steady decline in the prevalence of the parasites with *Pf*mdr-1 86Y mutation from 45% in 2008 to 15%. The reversion to CQ- sensitive parasites carrying the wildtype alleles: *Pf*crt K76, *Pf*mdr1-N86, and D1246 in the two regions can be argued in two ways. First, lumefantrine has been shown through various studies to select wild-type alleles in the *Pf*crt-76K, *Pf*mdr1 N86, and D1246 alleles (Malmberg *et al.,*2013). Here, the argument is that the dominance of wild-type parasites N86, N1042, D1246 of the *Pf*mdr1 gene in this study is linked to increased

pressure from the lumefantrine portion of the AL that selects reciprocal mutation to that of CQ pressure. Secondly, this study adds to the increasing evidence that the policy change from CQ to AL in 2006 has eventually reduced the CQ drug pressure in the population and reduced the CQ-resistant parasites' fitness cost. The selection of these wild-type alleles is attributed to AL's continued use in malaria treatment in western Kenya.

Overall, the selection of new mutations in the K13 propeller domain and an increased frequency in the ap2-mu mutation, genes closely associated with artemisinin resistance, highlight the rising need to closely monitor a functional mutation's fixation that would render the ACTs ineffective. Importantly, there is an urgent need to evaluate the impacts of these mutations on drug response. These follow-up studies will expose critical genetic markers that may be used for genetic surveillance of the drug-resistant parasite population, contributing to and accelerating malaria elimination efforts.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This study found novel synonymous mutations in the K13-propeller region, coupled with increased *Pf*ap2-160N/T mutation. In this view, continued surveillance is required to monitor the possibility of unprecedented ACT resistance in the future. Findings suggest that the SNPs in the *Pf*crt and *Pf*mdr1 genes change in favor of Chloroquine sensitivity in western Kenya, which on the other hand, might be working against AL.

6.2 RECOMMENDATIONS

The impact of W611S mutation on drug response and its effect on the functional role of the K13 protein needs further interrogation. This study shows that the parasites in the western part of Kenya are changing in favor of CQ sensitivity. Therefore, support the possibility of reintroducing chloroquine or other compounds in the same class to areas where they have been withdrawn and replaced by AL.

REFERENCES

Abdulla, S., Oberholzer, R., Juma, O., Kubhoja, S., Machera, F., Membi, C., ... & Tanner, M. (2008). Safety and immunogenicity of RTS, S/AS02D malaria vaccine in infants. *New England Journal of Medicine*, *359*(24), 2533-2544.

Achieng, A. O., Muiruri, P., Ingasia, L. A., Opot, B. H., Juma, D. W., Yeda, R., ... & Kamau, E. (2015). Temporal trends in prevalence of Plasmodium falciparum molecular markers selected for by artemether–lumefantrine treatment in pre-ACT and post-ACT parasites in western Kenya. *International Journal for Parasitology: Drugs and Drug Resistance*, *5*(3), 92-99.

Amambua-Ngwa, A., Jeffries, D., Amato, R., Worwui, A., Karim, M., Ceesay, S., ... & D'Alessandro, U. (2018). Consistent signatures of selection from genomic analysis of pairs of temporal and spatial Plasmodium falciparum populations from The Gambia. *Scientific reports*, *8*(1), 1-10.

Amambua-Ngwa, A., Okebe, J., Mbye, H., Ceesay, S., El-Fatouri, F., Joof, F., ... & D'Alessandro, U. (2017). Sustained ex vivo susceptibility of Plasmodium falciparum to artemisinin derivatives but increasing tolerance to artemisinin combination therapy partner quinolines in The Gambia. *Antimicrobial agents and chemotherapy*, *61*(12).

Amato, R., Lim, P., Miotto, O., Amaratunga, C., Dek, D., Pearson, R. D., ... & Fairhurst, R. M. (2017). Genetic markers associated with dihydroartemisinin–piperaquine failure in Plasmodium falciparum malaria in Cambodia: a genotype–phenotype association study. *The Lancet Infectious Diseases*, *17*(2), 164-173.

Amek, N., Bayoh, N., Hamel, M., Lindblade, K. A., Gimnig, J. E., Odhiambo, F., …

& Vounatsou, P. (2012). Spatial and temporal dynamics of malaria transmission in rural Western Kenya. *BioMed Central*, *5*(86), 1–13. https://doi.org/10.1186/1756-3305-5-86

Aminake, M. N., & Pradel, G. (2013). Antimalarial drugs resistance in *Plasmodium falciparum* and the current strategies to overcome them. *Microbial pathogens and strategies for combating them. Formatex*, *2013*(1), 1-774.

Anderson, T. J., & Roper, C. (2005). The origins and spread of antimalarial drug resistance: lessons for policymakers. *Acta tropica*, *94*(3), 269-280.

Apinjoh, T.O., Ouattara, A., Titanji, V. P. K., Djimde, A., & Amambua-Ngwa, A. (2019). Genetic diversity and drug resistance surveillance of *Plasmodium falciparum* for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? *Malaria Journal*, *18*(2017). https://doi.org/10.1186/s12936-019-2844-5

Aponte, J. J., Aide, P., Renom, M., Mandomando, I., Bassat, Q., Sacarlal, J., … & Alonso, L. P. (2007). Safety of the RTS, S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double-blind, randomized controlled phase I/IIb trial. *The Lancet*, *370*(9598), 1543-1551.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C.M., Bout, D.M., Menard, S., Rogers, W.O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Bras, J.L., Berry, A., Barale, JC., Fairhurst, R.M., Benoit-Vical, F., Mercereau-Puijalon, O&Didier, M (2014). A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature*, *505*(7481), 50–55.

Baird, J. K. (2005). Effectiveness of antimalarial drugs. *New England Journal of Medicine*, *352*(15), 1565-1577.

Bates, I., Fenton, C., Gruber, J., Lalloo, D., Lara, A. M., Squire, S. B., Theobald, S., Thomson, R., & Tolhurst, R. (2004). Vulnerability to malaria, tuberculosis, and HIV/AIDS infection and disease. Part 1: determinants operating at individual and household level. *The Lancet infectious diseases*, *4*(5), 267-277.

Bayoh, M. N., Akhwale, W., Ombok, M., Sang, D., Engoki, S. C., Koros, D., Walker, E. D., Williams, H. A., Burker, H., Armstrong, G. L., Cetron, M. S., Weinberg, M., Breinman, R., & Hamel, M. J. (2011). Malaria in Kakuma refugee camp, Turkana, Kenya: facilitation of *Anopheles arabiensis* vector populations by installed water distribution and catchment systems. *Malaria Journal*, *10*(1), 149. https://doi.org/10.1186/1475-2875-10-149

Maes, P., Harries, A. D., Van den Bergh, R., Noor, A., Snow, R. W., Tayler-Smith, K., ... & Allan, R. (2014). Can timely vector control interventions triggered by atypical environmental conditions prevent malaria epidemics? A case-study from Wajir County, Kenya. *PloS one*, *9*(4), e92386.

Beshir, K. B., Sutherland, C. J., Sawa, P., Drakeley, C. J., Okell, L., Mweresa, C. K., … & Bousema, T. (2013). Residual *Plasmodium falciparum* parasitemia in Kenyan children after artemisinin-combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. *Journal of Infectious Diseases*, *208*(12), 2017–2024. https://doi.org/10.1093/infdis/jit431

Biamonte, M. A., Wanner, J., & Le, K. G. (2013). Bioorganic & Medicinal Chemistry Letters Recent advances in malaria drug discovery. *Bioorganic & Medicinal Chemistry Letters*, *23*(10), 2829–2843.

Blasco, B., Leroy, D., & Fidock, D. A. (2017). Antimalarial drug resistance: Linking *Plasmodium falciparum* parasite biology to the clinic. *Nature medicine*, *23*(8), 917–928. https://doi.org/10.1038/nm.4381

Bloland, P. B. (2001). *Drug resistance in malaria*. Chamblee, GA, United States of America. Retrieved from WHO/CDS/CSR/DRS/2001.4

Brasil, P., Zalis, M. G., de Pina-Costa, A., & Siqueira, A. M. (2017). Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: A molecular epidemiological investigation. *Lancet Glob Health, 5*(10), 1038-1046. http://dx.doi.org/10.1016/S2214-109X(17)30333-9

Chandramohan, D., Jaffar, S., & Greenwood, B. (2002). Use of clinical algorithms for diagnosing malaria 1, *7*(1), 45–52.

Cooper, R. A., Conrad, M. D., Watson, Q. D., Huezo, S. J., Ninsiima, H., Tumwebaze, ..., & Rosenthal, P. J. (2015). Lack of artemisinin resistance in *Plasmodium falciparum* in Uganda based on parasitological and molecular assays. *Antimicrobial agents and chemotherapy*, *59*(8), 5061-5064.

Cowman, A. F., Karcz, S., Galatis, D., & Culvenor, J. G. (1991). A P-glycoprotein homolog of *Plasmodium falciparum* is localized on the digestive vacuole. *The Journal of Cell Biology*, *113*(5), 1033–42.

Depinay, J. O., Mbogo, C. M., Killeen, G., Knols, B., Beier, J., Carlson, J., Dushoff, J., Billingsley, P., Mwambi, H., Githure, J., Toure, A. M., & Mckenzie, F. E. (1805). Dynamics for the analysis of malaria transmission. *Malaria Journal*, *3*(29), 1–21. https://doi.org/10.1186/1475-2875-3-29

Djimdé, A., Doumbo, O. K., Cortese, J. F., Kayentao, K., Doumbo, S., Diourté, Y., … & Wellems, T. E., (2001). A molecular marker for chloroquine-resistant

falciparum malaria. *New England journal of medicine*, *344*(4), 257-263.

Dokomajilar, C., Nsobya, S. L., Greenhouse, B., Rosenthal, P. J., & Dorsey, G. (2006). Selection of *Plasmodium falciparum* pfmdr1 Alleles following Therapy with Artemether-Lumefantrine in an Area of Uganda where Malaria Is Highly Endemic, *50*(5), 1893–1895. https://doi.org/10.1128/AAC.50.5.1893

Eyase, F. L., Akala, H. M., Ingasia, L., Cheruiyot, A., Omondi, A., Okudo, C., Juma, D., Yeda, R., Andagalu, B., Wanja, E., Kamau, E., Schnabel, D., Bulimo, W., Waters, N. C., & Walsh D. S. (2013). The Role of Pfmdr1 and Pfcrt in Changing Chloroquine, Amodiaquine, Mefloquine, and Lumefantrine Susceptibility in Western-Kenya *P. falciparum* Samples during 2008 – 2011, *8*(5). doi.org/10.1371/journal.pone.0064299

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, M. B., Sidhu, A. S., Naude, B., Deitsch, K. W., SU, X., Wootton, J. C., Roepe, P. D., & Wellens, T. E.(2000). Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell*, *6*(4), 861–871. https://doi.org/10.1016/S1097-2765(05)00077-8

Fischer, A., Lejczak, C., Lambert, C., Servais, J., Makombe, N., Rusine, J., … & Arendt, V. (2004). Simple DNA Extraction Method for Dried Blood Spots and Comparison of Two PCR Assays for Diagnosis of Vertical Human Immunodeficiency Virus Type 1 Transmission in Rwanda. *Journal of Clinical Microbiology*, *42*(1), 16–20. https://doi.org/10.1128/JCM.42.1.16

Foote, S. J., Thompson, J. K., Cowman, A. F., & Kemp, D. J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P.*

falciparum. *Cell*, *57*(6), 921–30.

Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., Scherf, A., & Lopez-Rubio, J. J. (2014). Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nat Biotechnol*, *32*(8), 819–821. https://doi.org/nbt.2925 [pii]\r10.1038/nbt.2925

Girard, M. P., Reed, Z. H., Friede, M., & Kieny, M. P. (2007). A review of human vaccine research and development: malaria. *Vaccine*, *25*(9), 1567-1580.

Goldring, J. C. M. & J. P. D. (2013). Malaria Rapid Rapid Diagnostic Malaria Diagnostic Test Performance Performance Test. *J. Med. Microbiol*, *62*, *n*(2012), 1491–1505.

Greenwood, B. M., Bojang, K., Whitty, C. J., & Targett, G. A. (2013). The burden of malaria. *Group (CHERG)*, *14*, 16.

Haldar, K., Bhattacharjee, S., & Safeukui, I. (2018). Drug resistance in *Plasmodium*. *Nature Reviews Microbiology*, *16*(3), 156-170. https://doi.org/10.1038/nrmicro.2017.161

Hamilton, W. L., Amato, R., van der Pluijm, R. W., Jacob, C. G., Quang, H. H., Thuy Nhien, N. T.,. …& Miotto, O. (2019). Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. *The Lancet Infectious Diseases*, *3099*(19), 10–15. https://doi.org/10.1016/S1473-3099(19)30392-5

Hartmeyer, G. N., Stensvold, C. R., Fabricius, T., Marmolin, E. S., Hoegh, S. V., Nielsen, H. V., … & Vestergaard, L. S. (2019). *Plasmodium cynomolgi* as cause of malaria in tourist to Southeast Asia, 2018. *Emergence of Infectious Disease*, *25*(10), 1936-1939. https://doi.org/10.3201/eid2510.190448.

Hemming-Schroeder, E., Umukoro, E., Lo, E., Fung, B., Tomás-Domingo, P., Zhou, G., Zhong, … & Yan, G.(2018). Impacts of antimalarial drugs on *Plasmodium falciparum* drug resistance markers, Western Kenya, 2003–2015. *The American journal of tropical medicine and hygiene*, *98*(3), 692-699.

Henrici, R.C., Edwards, R.L., Zoltner, M., van Schalkwyk, D.A., Hart, M.N., Mohring, F., & Baker, D.A., (2019). Modification of an atypical clathrin-independent AP-2 adaptin complex of *Plasmodium falciparum* reduces susceptibility to artemisinin. *bioRxiv*, p.621078

Henriques, G., Hallett, R. L., Beshir, K. B., Gadalla, N. B., Johnson, R. E., Burrow, R., … & Sutherland, J. (2014). The directional selection at the pfmdr1, pfcrt, pfubp1, and pfap2mu loci of *Plasmodium falciparum* in Kenyan children treated with ACT. *The Journal of infectious diseases*, *210*(12), 2001-2008.

Henriques, G., Martinelli, A., Rodrigues, L., Modrzynska, K., Fawcett, R., Houston, D. R., … & Cravo, P. (2013). Artemisinin resistance in rodent malaria - Mutation in the AP2 adaptor μ-chain suggests the involvement of endocytosis and membrane protein trafficking. *Malaria Journal*, *12*(1).

Henriques, G., van Schalkwyk, D. A., Burrow, R., Warhurst, D. C., Thompson, E., … & Sutherland, C. J. (2015). The mu subunit of *Plasmodium falciparum* clathrin-associated adaptor protein 2 modulates in vitro parasite response to artemisinin and quinine. *Antimicrobial agents and chemotherapy*, *59*(5), 2540-2547.

Holmgren, G., Gil, J. P., Ferreira, P. M., Veiga, M. I., Obonyo, C. O., & Björkman, A. (2006). Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with selection of pfcrt 76T and pfmdr1 86Y. *Infection, Genetics,*

and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, *6*(4), 309–14.

Imbahale, S. S., Mukabana, W. R., Orindi, B., Githeko, A. K., & Takken, W. (2012). Variation in Malaria Transmission Dynamics in Three Different Sites in Western Kenya. *Journal of Tropical Medicine, 2012,* 664-671.

Imwong, M., Pukrittakayamee, S., Looareesuwan, S., Pasvol, G., Poirriez, J., White, N. J., & Snounou, G. (2001). Association of Genetic Mutations in *Plasmodium vivax* dhfr with resistance to Sulfadoxine-Pyrimethamine: Geographical and Clinical Correlates, *45*(11), 3122–3127.

Imwong, M., Suwannasin, K., Kunasol, C., Sutawong, K., Mayxay, M., Rekol, H., ... & Dondorp, A. M. (2017). The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong subregion: a molecular epidemiology observational study. *The Lancet Infectious Diseases*, *17*(5), 491-497.

Isozumi, R., Uemura, H., Kimata, I., Ichinose, Y., Logedi, J., Omar, A. H., & Kaneko, A. (2015). Novel mutations in the K13 propeller gene of artemisinin-resistant *Plasmodium falciparum. Emerging infectious diseases*, *21*(3), 490.

Jain, P., Chakma, B., Patra, S., & Goswami, P. (2014). Potential biomarkers and their applications for rapid and reliable detection of malaria. *BioMed research international*, *2014*.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, … & Djimde, A. A. (2014). K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. *The Journal of infectious diseases*, *211*(8), 1352-1355.

Kelley, L., Mezulis, S., Yates, C., Wass, M. N., Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modelling, prediction and analysis. *Nature Protocols, 10*(6), 845-858. https://doi.org/10.1038/nprot.2015.053

Kelly-hope, L. A., Hemingway, J., & Mckenzie, F. E. (2009). Environmental factors associated with the malaria vectors Anopheles gambiae and Anopheles funestus in Kenya. *Malaria Journal*, *8*(268), 1–8. https://doi.org/10.1186/1475-2875-8-268

Kiarie, W. C., Wangai, L., Agola, E., Kimani, F. T., & Hungu, C. (2015). Chloroquine sensitivity: diminished prevalence of chloroquine-resistant gene marker pfcrt - 76 13 years after cessation of chloroquine use in Msambweni, Kenya. *Malaria Journal*, *14*(328), 1–7. https://doi.org/10.1186/s12936-015-0850-9

Killeen, G. F., Smith, T. A., Ferguson, H. M., Mshinda, H., Abdulla, S., Lengeler, C., & Kachur, S. P. (2007). Preventing childhood malaria in Africa by protecting adults from mosquitoes with insecticide-treated nets. *PLoS Medicine*, *4*(7), e229.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology Evolution, 35*(6), 1547-1549. https://doi.org/10.1093/molbev/msy096

Lu, F., Culleton, R., Zhang, M., Ramaprasad, A., Von Seidlein, L., Zhou, H., & Cao, Y. (2017). The emergence of indigenous artemisinin-resistant *Plasmodium falciparum* in Africa. *New England Journal of Medicine*, *376*(10), 991-993.

Lucchi, N. W., Komino, F., Okoth, S. A., Goldman, I., Onyona, P., Wiegand, R. E.,

… & Kariuki, S. (2015). *In vitro* and molecular surveillance for antimalarial drug resistance in *Plasmodium falciparum* parasites in Western Kenya reveals sustained artemisinin sensitivity and increased chloroquine sensitivity. *Antimicrobial agents and chemotherapy*, *59*(12), 7540-7547.

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., … & Lopez, R. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*, 47(1), 636-641. https://doi.org/10.1093/nar/gkz268

MalariaGEN Plasmodium falciparum Community Project, (2016). Genomic epidemiology of artemisinin-resistant malaria. *elife*, *5*, p.e08714.

Malmberg, M., Ngasala, B., Ferreira, P. E., Larsson, E., Jovel, I., Hjalmarsson, … & Mårtensson, A. (2013). Temporal trends of molecular markers associated with artemether-lumefantrine tolerance/resistance in Bagamoyo district, Tanzania. *Malaria Journal*, *12*(1), 103.

Mang'era, C.M., Mbai, F.N., Omedo, I.A., Mireji, P.O. & Omar, S.A., (2012). Changes in genotypes of *Plasmodium falciparum* human malaria parasite following withdrawal of chloroquine in Tiwi, Kenya. *Acta tropica*, *123*(3), pp.202-207.

Maraka, M., Akala, H. M., Amolo, A. S., Juma, D., Omariba, D., Cheruiyot, A., … & Andagalu, B. (2020). A seven-year surveillance of epidemiology of malaria reveals travel and gender are the key drivers of dispersion of drug-resistant genotypes in Kenya. *PeerJ*, *8*, 1-27. https://doi.org/10.7717/peerj.8082

Marshall, J. M., & Taylor, C. E. (2009). Malaria Control with Transgenic

Mosquitoes, *6*(2). https://doi.org/10.1371/journal.pmed.1000020

Martin, R. E., & Kirk, K. (2004). The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. *Molecular Biology and Evolution*, *21*(10), 1938–49.

Miotto, O., Amato, R., Ashley, E. A., Macinnis, B., Almagro-Garcia, J., Amaratunga, C., … & Kwiatkowski, D. P. (2015). Genetic architecture of artemisinin-resistant *Plasmodium falciparum. Nature Genetics*, *47*(3), 226–234. doi.org/10.1038/ng.3189

Mohon, A.N., Alam, M.S., Bayih, A.G., Folefoc, A., Shahinas, D., Haque, R. and Pillai, D.R., (2014). Mutations in *Plasmodium falciparum* K13 propeller gene from Bangladesh (2009–2013). *Malaria Journal*, *13*(1), 1-6.

Muwanguzi, J., Henriques, G., Sawa, P., Bousema, T., Sutherland, C. J., & Beshir, K. B. (2016). Lack of K13 mutations in *Plasmodium falciparum* persisting after artemisinin combination therapy treatment of Kenyan children. *Malaria Journal*, *15*(1), 36.

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S. M., Ward, S., Kokwaro, G., Sasi, P., Marsh, K., Borrmann, S., Mackninnon, M., & Nzila, A. (2009). Chloroquine resistance before and after its withdrawal in Kenya. *Malaria Journal*, *8*(1), 106.

MalariaGEN Plasmodium falciparum Community Project. (2016). Genomic epidemiology of artemisinin resistant malaria. *elife*, *5*, e08714.

Newsholme, P., Stenson, L., Sulvucci, M., Sumayao, R., & Krause, M. (2011). Amino acid metabolism. In *Comprehensive Biotechnology* (pp. 3-14). Elsevier.

Kublin, J. G., Cortese, J. F., Njunju, E. M., G. Mukadam, R. A., Wirima, J. J., Kazembe, P. N., Djimde, A. A., Kouriba, B., Taylor, T. E., & Plowe, C. V. (2003). Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *The Journal of infectious diseases*, *187*(12), 1870-1875.

Noedl, H., Se, Y., Schaecher, K., Smith, B. L., Socheat, D., & Fukuda, M. M. (2008). Evidence of Artemisinin-resistant malaria in Western Cambodia. *New England Journal of Medicine*, *359*(24), 2619–2620.

Nosten, F., & White, N. J. (2007). Artemisinin-based combination treatment of falciparum malaria. *American Journal of Tropical Medicine and Hygiene*, *77*(SUPPL. 6), 181–192. https://doi.org/77/6_Suppl/181 [pii]

Nsanzabana, C., Djalle, D., Guérin, P. J., Menard, D., & Gonzalz, I. J. (2018). Tools for surveillance of anti-malarial drug resistance: an assessment of the current landscape. *Malaria Journal*, *17*(75), 1-16. https://doi.org/10.1186/s12936-018-2185-9

Nyunt, M. H., Kyaw, M. P., Win, K. K., Myint, K. M., & Nyunt, K. M. (2013). Field evaluation of HRP2 and pan pLDH-based immunochromatographic assay in therapeutic monitoring of uncomplicated *falciparum* malaria in Myanmar. *Malaria Journal*, *12*(1), 123.

Ocan, M., Akena, D., Nsobya, S., Kamya, M. R., Senono, R., Kinengyere, A. A., & Obuku, E. A. (2019). Persistence of chloroquine resistance alleles in malaria-endemic countries: a systematic review of burden and risk factors. *Malaria Journal*, *18*(1), 76.

Ocan, M., Bwanga, F., Okeng, A., Katabazi, F., Kigozi, E., Kyobe, S., Ogwal-

Okeng, J., & Obua, C. (2016). Prevalence of K13-propeller gene polymorphisms among *Plasmodium falciparum* parasites isolated from adult symptomatic patients in northern Uganda. *BMC infectious diseases*, *16*(1), 428.

Okara, R. M., Sinka, M. E., Minakawa, N., Mbogo, C. M., Hay, S. I., & Snow, R. W. (2010). Distribution of the main malaria vectors in Kenya. *Malaria Journal*, *9*(69), 1–11. https://doi.org/10.1186/1475-2875-9-69

Owen, D. J., Collins, B. M., & Evans, P. R. (2004). Adaptors for Clathrin Oats: Structure and function. doi.org/10.1146/annurev.cellbio.20.010403.104543

Palacpac, N., & Horii, T. (2020). Malaria vaccines: facing unknowns. *F1000Research*, *9(*296). 1-9. https://doi.org/10.12688/f1000research.22143.1

PlasmoDB. (2020). PlasmoDB: *Plasmodium* informatics resources. Retrieved from https://plasmodb.org/plasmo/app/

Phyo, A. P., Ashley, E. A., Anderson, T. J. C., Bozdech, Z., Carrara, V. I., Sriprawat, K., … & Nosten, F. (2016). Declining Efficacy of Artemisinin Combination Therapy Against *P. falciparum* Malaria on the Thai-Myanmar border (2003-2013): The Role of Parasite Genetic Factors. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *63*(6), 784–791.

Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K., & Cowman, A. F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, *403*(6772), 906–9.

Rohrbach, P., Sanchez, C. P., Hayton, K., Friedrich, O., Patel, J., Sidhu, A. B. S., ... & Lanzer, M. (2006). Genetic linkage of pfmdr1 with food vacuolar solute

import in *Plasmodium falciparum*. *The EMBO journal*, *25*(13), 3000-3011.

Sanchez, C. P., McLean, J. E., Rohrbach, P., Fidock, D. A., Stein, W. D., & Lanzer, M. (2005). Evidence for a pfcrt-associated chloroquine efflux system in the human malaria parasite *Plasmodium falciparum. Biochemistry*, *44*(29), 9862-9870.

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., … & Hay, S. I. (2010). The dominant Anopheles vectors of human malaria in Africa, Europe, and the Middle East: occurrence data, distribution maps, and bionomic précis. *Parasites & Vectors*, *3*(117), 1–34. https://doi.org/10.1186/1756-3305-3-117

Sisowath, C., Strömberg, J., Mårtensson, A., Msellem, M., Obondo, C., Björkman, A., & Gil, J. P. (2005). In vivo selection of *Plasmodium falciparum* pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). *The Journal of Infectious Diseases*, *191*(6), 1014–7.

Spring, M. D., Lin, J. T., Manning, J. E., Vanachayangkul, P., Somethy, S., Bun, R., Se, … & Saunders, D. L. (2015). Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an. *The Lancet Infectious Diseases*, *13*(15), 1–9. https://doi.org/10.1016/S1473-3099(15)70049-6

Straimer, J., Gnädig, N. F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A. P., … & Fidock, D. A. (2015). K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science*, *347*(6220), 428-431.

Suresh, N., & Haldar, K. (2018). Mechanisms of artemisinin resistance in

Plasmodium falciparum malaria. *Current opinion in pharmacology*, *42*, 46-54.

Sutherland, C. J., Lansdell, P., Sanders, M., Muwanguzi, J., van Schalkwyk, D. A., Kaur, H., … & Chiodini, P. L (2017). Pfk13-independent treatment failure in four imported cases of *Plasmodium falciparum* malaria treated with artemether-lumefantrine in the United Kingdom. *Antimicrobial agents and chemotherapy*, *61*(3), e02382-16.

Uwimana, A., Legrand, E., Stokes, B.H., Ndikumana, J.L.M., Warsame, M., Umulisa, N., & Campagne, P., (2020). Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. *Nature medicine*, *26*(10), 1602-1608.

Takala-Harrison, S., Jacob, C. G., Arze, C., Cummings, M. P., Silva, J. C., Dondorp, A. M., … & Plowe, C. V. (2015). Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. *Journal of Infectious Diseases*, *211*(5), 670–679.

Tangpukdee, N., Duangdee, C., Wilairatana, P., & Krudsood, S. (2009). Malaria diagnosis: a brief review. *The Korean Journal of parasitology*, *47*(2), 93.

Taylor, S. M., Parobek, C. M., DeConti, D. K., Kayentao, K., Coulibaly, S. O., Greenwood, … & Juliano, J. J. (2014). Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in sub-Saharan Africa: a molecular epidemiologic study. *The Journal of infectious diseases*, *211*(5), 680-688.

Technelysium Pty Ltd. (2018). *ChromasPro version 2.1.8*. Retrieved from https://technelysium.com.au/wp/chromaspro/

Torrentino-Madamet, M., Fall, B., Benoit, N., Camara, C., Amalvict, R., Fall, M., Dionne, P., Fall, K.B., Nakoulima, A., Diatta, B. and Diemé, Y., (2014). Limited polymorphisms in k13 gene in *Plasmodium falciparum* isolates from Dakar, Senegal in 2012–2013. *Malaria journal*, *13*(1), pp.1-5.

Valderramos, S. G., & Fidock, D. A. (2006). Transporters involved in resistance to antimalarial drugs. *Trends in pharmacological sciences*, *27*(11), 594-601.

Venkatesan, M., Gadalla, N. B., Stepniewska, K., Dahal, P., Nsanzabana, C., Moriera, C., ... & ASAQ Molecular Marker Study Group. (2014). Polymorphisms in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for P. falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine. *The American journal of tropical medicine and hygiene*, *91*(4), 833-843.

Wamae, K., Okanda, D., Ndwiga, L., Osoti, V., Kimenyi, K.M., Abdi, A.I., Bejon, P., Sutherland, C. and Ochola-Oyier, L.I., (2019). No evidence of *Plasmodium falciparum* k13 artemisinin resistance-conferring mutations over a 24-year analysis in Coastal Kenya but a near-complete reversion to chloroquine-sensitive parasites. *Antimicrobial Agents and Chemotherapy*, *63*(12).

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*(9), 1189-1191.

Wellems, T. E., Walker-Jonah, A., & Panton, L. J. (1991). Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proceedings of the National Academy of Sciences of the United States of*

America, *88*(8), 3382–6. https://doi.org/10.1073/PNAS.88.8.3382

White, N. J. (2014). Malaria: a molecular marker of artemisinin resistance. *The Lancet*, *383*(9927), 1439-1440.

White, N. J. (2004). Antimalarial drug resistance. *The Journal of Clinical Investigation*, *113*(8), 1084–1092.

Wongsrichanalai, C., & Meshnick, S. R. (2008). Declining Artesunate-Mefloquine Efficacy against Falciparum Malaria on Cambodia - Thailand Border. *Emerging Infectious Diseases*, *14*(5), 716–719.

World Health Organization. (2015). *World Malaria Report 2015*. Geneva.

WHO. (2009). *Methods for surveillance of antimalarial drug efficacy*. Geneva, World Health Organization. Retrieved from http://apps.who.int/iris/bitstr

WHO. (2019). *World malaria report 2019*. Retrieved from https://apps.who.int/iris/rest/bitstreams/1262394/retrieve

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino acids*, *37*(1), 1-17.

Yayon, A., Cabantchik, Z. I., & Ginsburg, H. (1984). Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine. *The EMBO Journal*, *3*(11), 2695–700.

Zimmerman, P. A., & Howes, R. E. (2015). Malaria diagnosis for malaria elimination. *Current opinion in infectious diseases*, *28*(5), 446-454.

APPENDICES

Appendix I:Publication

Infection, Genetics and Evolution 78 (2020) 104121

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Prevalence of mutations in *Plasmodium falciparum* genes associated with resistance to different antimalarial drugs in Nyando, Kisumu County in Kenya

Kelvin B. Musyoka[a,c,*], John N. Kiiru[b], Eva Aluvaala[c], Protus Omondi[d], William K. Chege[c], Terry Judah[b], Daniel Kiboi[a], Joseph K. Nganga[a], Francis T. Kimani[c]

[a] Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[b] Centre for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya
[c] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya
[d] Department of Biochemistry, Microbiology, and Biotechnology, Kenyatta University, Nairobi, Kenya

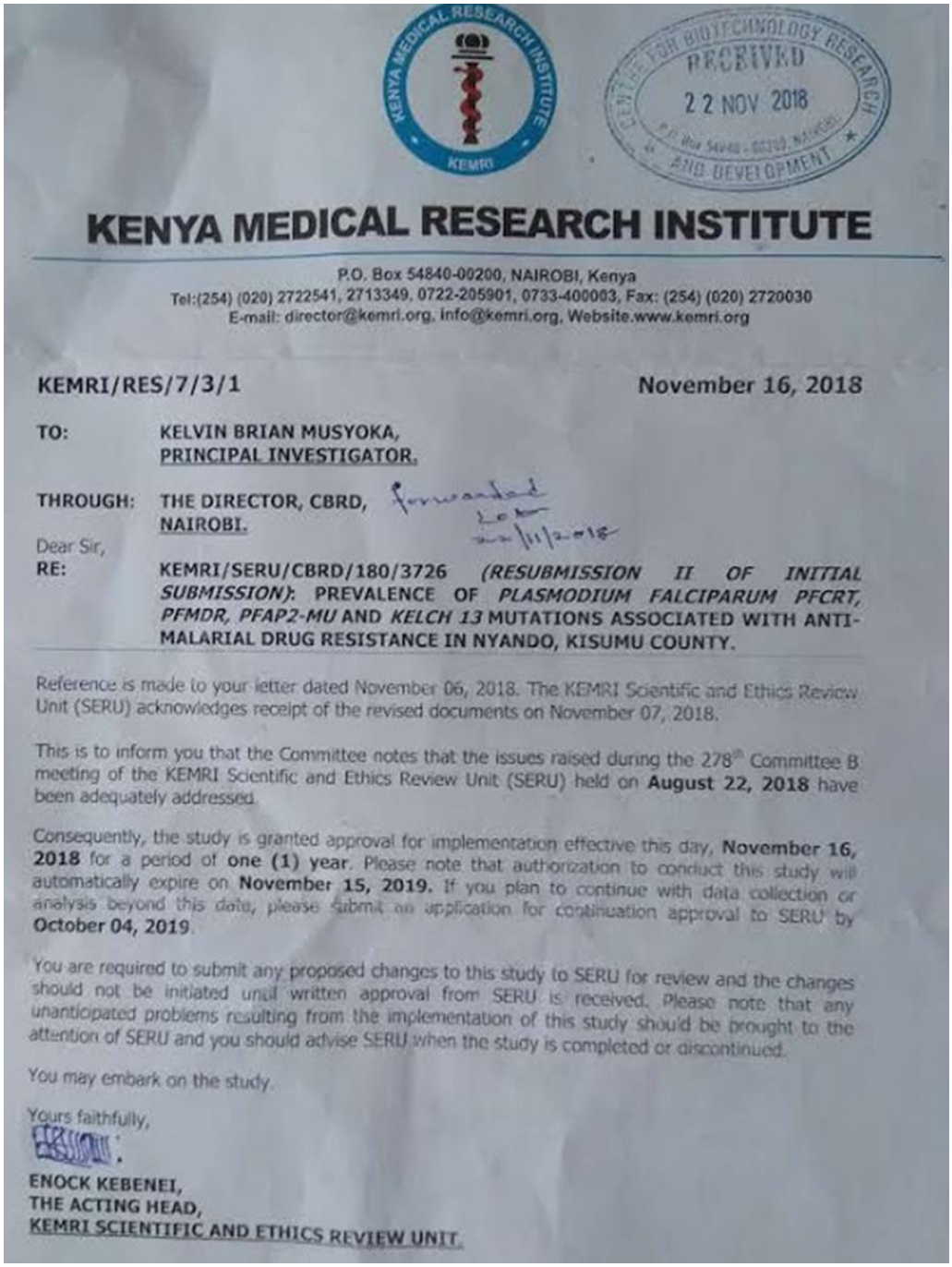
ARTICLE INFO

Keywords:
Plasmodium falciparum
ACT
Delayed clearance
SNPs
Pfap2mu
*Pf*K13

ABSTRACT

Resistance to the mainstay antimalarial drugs is a major concern in the control of malaria. Delayed *Plasmodium falciparum* parasite clearance has been associated with Single Nucleotide Polymorphisms (SNPs) in the kelch propeller region (K13). However, SNPs in the *Pf*-adaptor protein complex 2 mu subunit (*Pf*ap2-mu), *Pfcrt and Pf*mdr1 are possible markers associated with multi-drug resistance. Here, we explored the prevalence of SNPs in the K13, *Pf*ap2-mu, *Pf*crt, and *Pf*mdr1 in 94 dried blood spot field isolates collected from children aged below 12 years infected with *P. falciparum* during a cross-sectional study. The samples were collected in 2015 during the peak malaria transmission season in the Nyando region of Western Kenya before treatment with Artemether-Lumefantrine, the first-line artemisinin-based combination therapy (ACT) in Kenya. However, 47 of the 94 samples had recurrent parasitemia and were interrogated for the presence of the SNPs in K13 and *Pf*ap2-mu. We used PCR amplification and sequencing to evaluate specific regions of K13 (codons 432–702), *Pf*ap2-mu (codons 1–350), *Pf*mdr1 (codons 86, 1034–1246), and *Pf*crt (codons 72–76) gene(s). The majority of parasites harbored the wild type K13 sequence. However, we found a unique non-synonymous W611S change. *In silico* studies on the impact of the W611S predicted structural changes in the overall topology of the K13 protein. Of the 47 samples analyzed for SNPs in the *Pf*ap2-mu gene, 14 (29%) had S160 N/T mutation. The CVIET haplotype associated with CQ resistance in the *Pf*crt yielded a 7.44% (7/94), while CVMNK haplotype was at 92.56%. Mutations in the *Pf*mdr1 region were detected only in three samples (3/94; 3.19%) at codon D1246Y. Our data suggest that parasites in the western part of Kenya harbor the wildtype strains. However, the detection of the unique SNP in K13 and *Pf*ap2-mu linked with ACT delayed parasite clearance may suggest slow filtering of ACT-resistant parasites.

Appendix 2: Ethical clearance



KENYA MEDICAL RESEARCH INSTITUTE

RECEIVED
22 NOV 2018
CENTRE FOR BIOTECHNOLOGY RESEARCH AND DEVELOPMENT

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 November 16, 2018

TO: KELVIN BRIAN MUSYOKA,
PRINCIPAL INVESTIGATOR.

THROUGH: THE DIRECTOR, CBRD, Forwarded
NAIROBI. 22/11/2018

Dear Sir,

RE: KEMRI/SERU/CBRD/180/3726 *(RESUBMISSION II OF INITIAL SUBMISSION)*: PREVALENCE OF *PLASMODIUM FALCIPARUM PFCRT, PFMDR, PFAP2-MU* AND *KELCH 13* MUTATIONS ASSOCIATED WITH ANTI-MALARIAL DRUG RESISTANCE IN NYANDO, KISUMU COUNTY.

Reference is made to your letter dated November 06, 2018. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised documents on November 07, 2018.

This is to inform you that the Committee notes that the issues raised during the 278th Committee B meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **August 22, 2018** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **November 16, 2018** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **November 15, 2019.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **October 04, 2019**.

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

You may embark on the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

Appendix 3: A table showing the frequency of mutation at specific codons in the *Plasmodium falciparum*, chloroquine resistance transporter (CRT), Multi-drug resistance (MDR), kelch 13 (k13), and an adaptor protein 2-mu genes.

Gene (polymorphic codon)	**SNP/Haplotype**			
***Pfcrt* (72,73,74,75,76)**	**SNPs**	**Frequency**	**Haplotypes**	**Frequency**
	K76*	87(92.56%)	CVMNK*	87(92.56%)
	76T	7(7.44%)	CVIET	7(7.44%)
Total= 94				
Kelch 13 propeller region	W611*	46 (97.87%)	None	0
	611S	1(2.13%)	None	0
Total = 47				
***Pf*ap2-mu**	S160*	33 (70.21%)	None	0
	160N/T	14 (29.79%)	None	0
Total = 47				
***Pfmdr1* (codon 86,1246)**	**SNPs**	**Frequency**	**Haplotypes**	**Frequency**
	N86*	94(100%)	None	0
	86Y	0(0%)	None	0
	N1246*	91 (96.81)	None	0
	1246Y	3(3.19%)	None	0
Total =94				

Key: 1. * - Wild-type