

INVESTIGATING THE PRESENCE OF *Pfkelch* GENE
MUTATIONS IN UGANDAN CHILDREN WITH SEVERE
MALARIA

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Artemisinin resistance was first observed in Southeast Asia (SEA) and could pose a threat to malaria treatment all over the world. Recently mutations in the propeller region of *Pfkelch13* gene have been used as a genetic marker for resistance observed in SEA. We investigated the presence of mutations in the *Pfkelch* gene in children in Kampala, Uganda with severe malaria (SM) treated with intravenous quinine, or with asymptomatic *P.falciparum* infection (AP) treated with artemether-lumefantrine. We sequenced the *Pfkelch* gene (2178bp) in 157 children with SM and 49 children with AP infection. In children with SM and AP we identified 106 (60.8%) and 27 (55.1%) parasites with mutations upstream of the *Pfkelch13* propeller region. The two most prevalent mutations were 142NN (26.1% in SM, 33% in AP) and K189T (16.5% in SM, 12.2% in AP). In SM, only a single infection had a mutation in the propeller region (A578S), while in AP, mutations in the propeller region included A578S (n=1) and S522C (n=1). In children with SM, parasites with 142NN insertion compared to 3D7 *Pfkelch13* parasites had lower parasite density (p=0.02) and lower parasite biomass (p=0.03). Children with SM who either had 142NN or K189T mutation cleared parasites after quinine treatment faster than those with the 3D7 *Pfkelch13* genotype (P<0.001 for both mutations compared to 3D7). In this cohort mutations, upstream of the *Pfkelch13* propeller region were common. Future studies will assess the presence of *Pfprt* and *Pfmdr* mutations in this cohort, and how these relate to the *Pfkelch13* mutations and to parasite clearance.

Chandy C. John, MD MS, Chair

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List of Abbreviations

WHO – World Health Organization

SEA – Southeast Asia

DDT – Dichlorodiphenyltrichloroethane

ITN – Insecticide treated bed nets

IRS – Indoor residual spraying

ACT – Artemisinin based combination therapy

RBC – Red blood cell

iRBC – Infected red blood cell

CM – Cerebral malaria

SMA – Severe malarial anemia

CC – Community children

BCS – Blantyre coma score

GCS – Glasgow coma score

CSF – Cerebrospinal fluid

DHA – Dihydroartemisinin

SNPs – Single nucleotide polymorphisms

LP – Lumbar puncture

CBC – Complete blood count

PfPI3K – *P. falciparum* Phosphatidylinositol 3 kinase

PfPI3P – *P. falciparum* Phosphatidylinositol 3 phosphate

PfHrp2 – *P. falciparum* Histidine rich protein 2

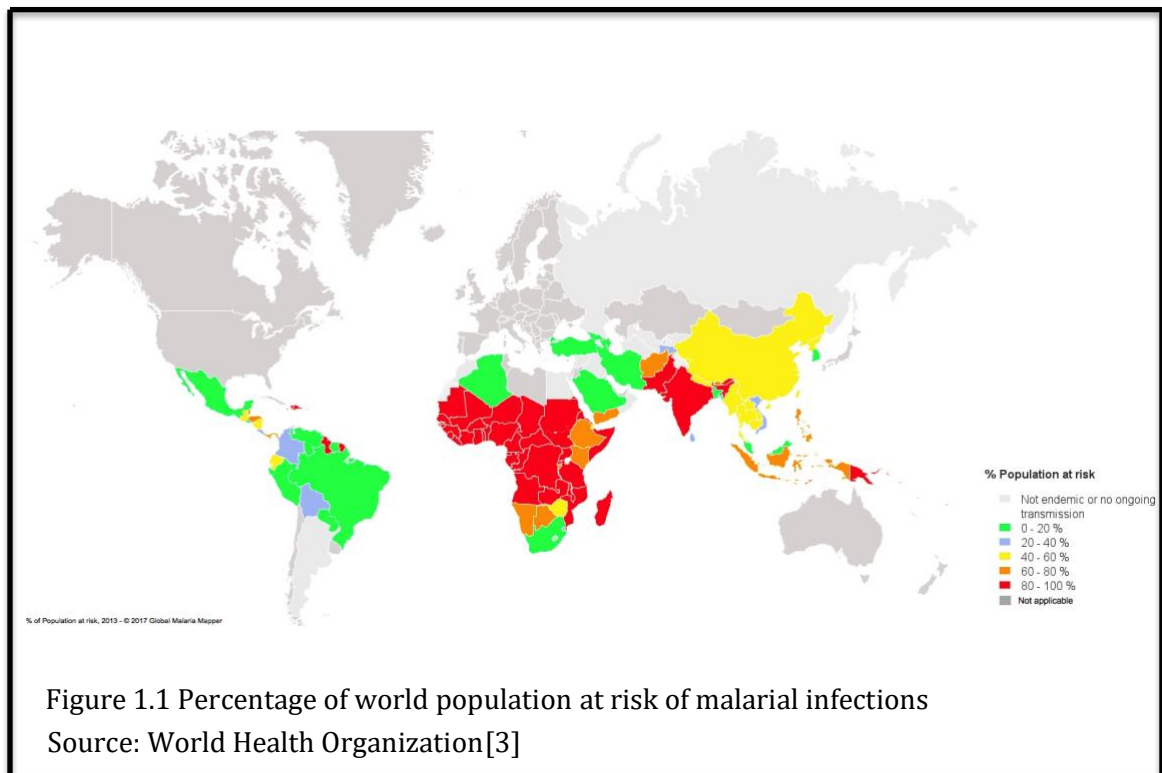
INTRODUCTION

1.1 A general overview

Malaria is one of the major health concerns facing most of the developing world. It is caused by an infection with a protozoan parasite of the genus *Plasmodium*. It was first identified in the blood smear of an Algerian patient in 1880 as the cause of malaria [7]. In 1897 while working in India, Ronald Ross identified plasmodial oocysts in the guts of mosquitoes, hence identifying mosquitoes as the vector of malaria [8]. Since the first half of the 20th century efforts to eliminate malaria have been on going. In 1955 the Global Malaria Eradication Program was launched by the World Health Organization (WHO) in all parts of the world except for Africa. This program involved residual spraying and systemic detection and treatment of cases. This program was later abandoned due to financial and technical issues. However, even with once highly effective drugs such as chloroquine and insecticides such as dichlorodiphenyltrichloroethane (DDT) human malaria still persisted.

In 2015, there were 212 million new cases of malaria reported worldwide and Africa accounted for 90% of these cases [9]. In the same year there was an estimate of 429,000 malaria deaths worldwide and 92% of these occurred in Africa, followed by Southeast Asia (SEA) (6%) and the Eastern Mediterranean Region (2%) [9]. However, malaria case incidence has been dropping rapidly and between 2000 – 2015, there was a 21% reduction globally [9]. During the same time period malaria related mortality rates fell by an estimated 29% globally and 31% in the African Region [9]. The reasons for the decline in malaria cases this century is due to increased interventions like insecticide treated bed nets (ITNs), indoor residual spraying of insecticides (IRS) and the use of newer effective drugs like artemisinin-based combination therapies (ACTs) [9]. Yet malaria remains an important public health issue around the world (Figure 1.1) due to various factors like poverty, lack of adequate treatment policies around the world and also emergence of drug resistant strains.

Malaria related mortality is mainly associated with children under the age of 5 years [9]. An estimated 303,000 deaths of children under the age of 5 due to malaria of which 292,000 were from the African Region in 2015 [9]. Cerebral malaria is the main driver of malaria related mortality in children.



1.2 Parasite life-cycle

Human infections can be caused by five *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. The majority of the severe malaria cases are due to *P. falciparum* infections. The human infection begins when an infected female *Anopheles* mosquito injects *P. falciparum* sporozoites into the skin of an individual. Within minutes, these sporozoites travel to the liver and invade hepatocytes. It is here that the sporozoites initiate intracellular replication, which is known as the pre-erythrocytic development (10-12days). This is followed by the release of merozoites into the bloodstream, where they infect red blood cells (RBCs) and

initiate the asexual parasite multiplication stage. Upon invasion of the RBCs, the merozoites develop either through the sexual or asexual cycle.

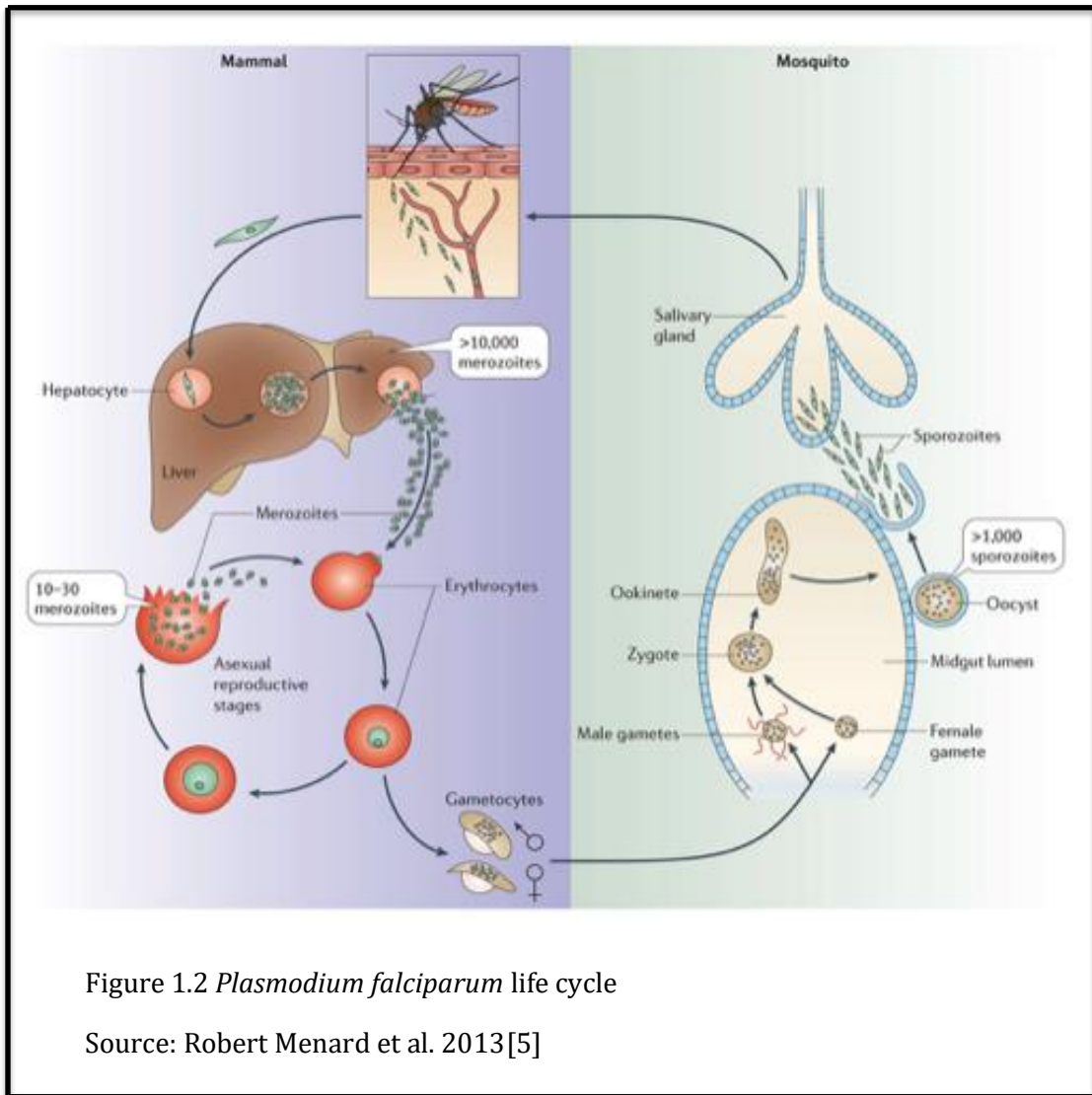


Figure 1.2 *Plasmodium falciparum* life cycle

Source: Robert Menard et al. 2013[5]

Under certain conditions that are not well understood a small number of infected red blood cells (iRBCs) commit to the sexual cycle and develop into gametocytes. These gametocytes can be picked up by another mosquito to restart the transmission cycle. Most of the merozoites commit to the asexual blood stage within the iRBC. This involves various stages from early ring stage to trophozoite and finally the schizont stage within 48 hours. At the end of the 48-hour cycle, the iRBCs burst to release merozoites into the circulation and infecting more RBCs (Figure

1.2). The rupture of the iRBCs is mainly responsible for the clinical symptoms like periodic waves of fever and anemia.

1.3 Malaria pathogenesis

Clinical symptoms of malaria are due to the infection of human red blood cells by parasite merozoites. The clinical symptoms of malaria generally result in uncomplicated or severe malaria. Uncomplicated malaria caused by *P. falciparum* generally begins with an irregular fever, which may occur daily with flu-like symptoms, diarrhea and anorexia. These patients are generally positive for parasites in their blood smear, but will not appear very ill. To the contrary, severe malaria is the main cause of fatal cases predominantly due to *P. falciparum* infections. Severe malarial anemia and cerebral malaria are the severe forms of the disease, however, there are other clinical manifestations that vary in the severity and outcome. These disease manifestations are associated with the parasite species and the treatment facilities available.

P. falciparum infected erythrocytes are involved with a process called sequestration, where IEs adhere to the endothelial cells in the microvasculature of various organs. This results in impaired tissue perfusion, microcirculatory obstruction and activation of inflammatory cells resulting in severe disease.

Rupture of iRBCs to release merozoites which further target erythrocytes and continue with the asexual life-cycle of the parasite. During this rupture, there are toxins and parasite products that further cause the activation of immune cells resulting in symptoms associated with a malarial infection.

1.4 Severe Malaria

Around 1-2% of the malarial infections lead to severe malaria [10] of which the mortality rate of in adults is 18.5% [11] and 9.7% in children [12]. The most common manifestations of severe malaria include cerebral malaria (CM), severe malarial anemia (SMA) and metabolic acidosis [10]. Severe malaria is observed in

adults in areas of low transmission, whereas in areas of stable transmission like many areas in Sub-Saharan Africa, tolerance to infection is observed in adults due to continuous exposure to infectious mosquitoes [13]. These adults have low levels of infection and are often asymptomatic [14]. However, children below the age of five living in high transmission areas are susceptible to developing severe malaria due to the lack of a sufficient immune response which is developed over multiple malaria infections and necessary for an effective response by the acquired immune system [15].

Cerebral Malaria

The World Health Organization (WHO) defines CM as impaired consciousness upon *P. falciparum* infection with the absence of any other cause of coma. CM is graded based on two clinical scores a *Blantyre coma score* (BCS) and a *Glasgow come score* (GCS). BCS and GCS consist of 3 categories: eye movement, verbal response and motor response. BCS uses a scale of 0-2, where 0 being the least responsive while GCS uses a scale of 1-6, where 1 is the least responsive for each of the categories. A BCS of less than 3 is used to determine impaired consciousness in children under the age of 5 and GCS of less than 11 in children older than 5 years of age [16]. In our study the definition of CM is restricted to children who remain unconscious at least one hour after convulsions and after receiving a bolus of glucose; this is to avoid enrolling children with hypoglycemia or febrile convulsions. We also perform Gram stain and cultures of cerebrospinal fluid (CSF) to rule out bacterial meningitis as the cause of coma.

Nearly 9% of malarial admissions in Africa are associated with CM [17]. African children who survived an episode of CM often had long-term cognitive deficits (approximately 21%) [18]. Children with a larger number of seizures before admission and with longer coma duration and cognitive deficits [18]. The exact biological mechanisms underlying the cognitive deficits are still not clearly understood, however it is hypothesized that parasite sequestration in the cerebral microvasculature causes decreased blood flow resulting in increased hemorrhage.

This is one of the main factors in pathogenesis and resulting changes in the surrounding tissue, which may explain the neural dysfunction [19]. High levels of cytokines and chemokines also lead to exaggerated inflammation may also contribute to the severity of the disease [19]. Currently, no adjunctive treatment for CM in humans has decreased mortality or neurologic complications [20] and patients who are identified with CM are treated with intravenous antimalarials to reduce the parasite burden as quickly as possible.

Severe Malarial Anemia

The WHO defines SMA as having detectable levels of *P. falciparum* in peripheral blood and hemoglobin levels $\leq 5\text{g/dL}$ [16], while normal hemoglobin levels in children ranges from 11-13g/dL. In African children severe malaria is generally associated not only with malaria, but also HIV-1 infection, hookworm infections, vitamin deficiencies [21].

In Sub-Saharan Africa, SMA poses a substantial burden causing approximately 20% of *P. falciparum* related hospitalizations [17]. SMA related mortality is lower in an urban setting like Kampala, Uganda where access to good medical facilities is easier. Children who received adequate medical attention with severe anemia showed a lower mortality rate as compared to those who did not [22]. Adjunctive treatments for SMA have been limited, as rapid blood transfusion in combination with antimalarials usually reverses the severe anemia episode and reduces mortality. Associated symptoms like lactic acidosis and hypoxia are treated whenever presented by SMA patients [16].

1.5 Treatment

Efforts to treat malarial infections have always had hurdles, in part due to the development and spread of drug resistant strains of *P. falciparum*. This species of parasites has developed some level of resistance against almost every antimalarial treatment regimen introduced to date (Figure 1.3) [6]. Drugs like chloroquine and sulfadoxine-pyrimethamine, which were initially used as a first line treatment of *P.*

falciparum malaria, have now been rendered ineffective in most malaria-endemic areas due to the emergence of resistant strains against these drugs [23].

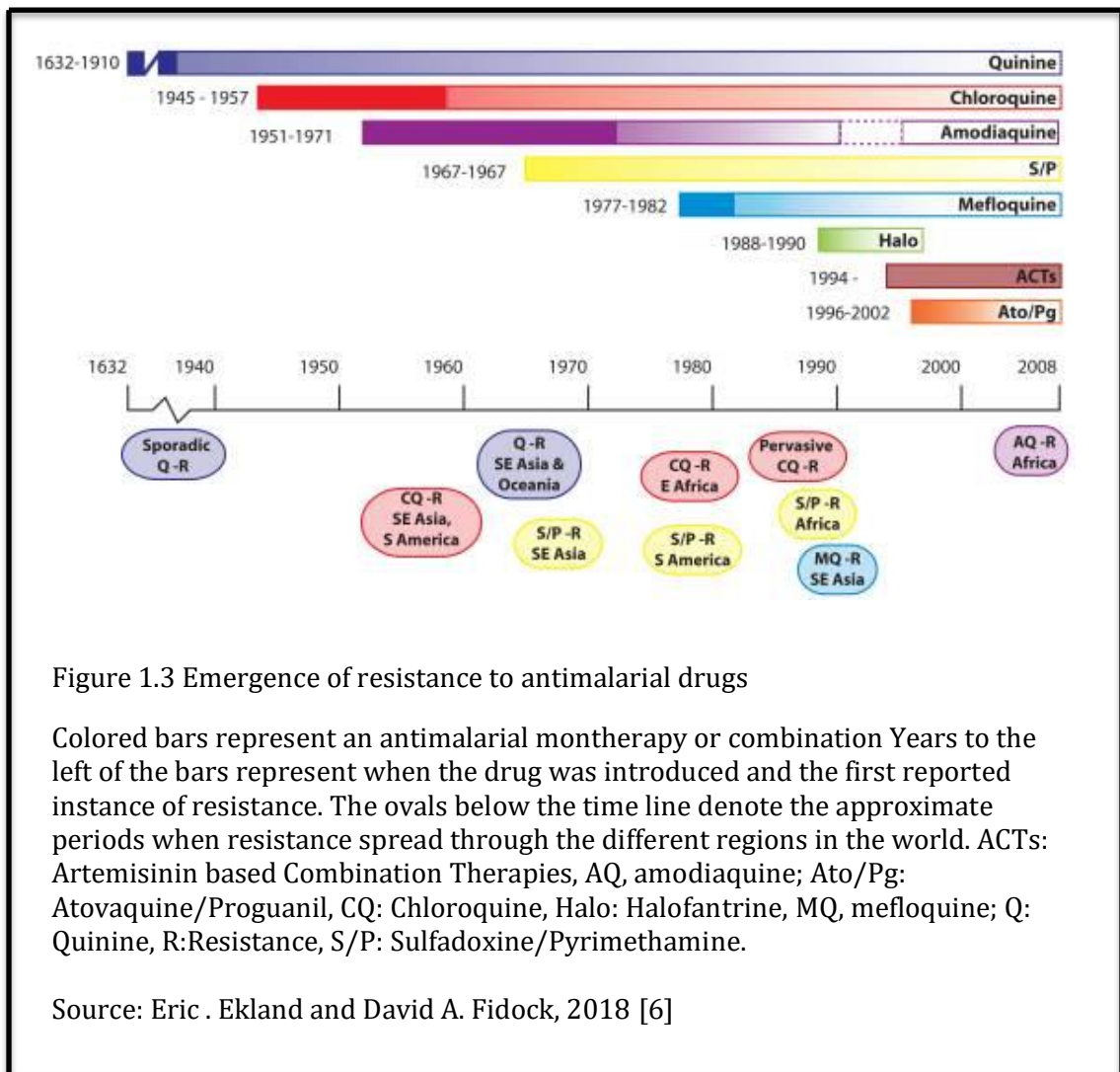
Quinine

Quinine has been the primary drug used in the treatment of malaria since the introduction of chinchona bark to European medicine in the 1630s. In the early 1800s, the chinchona bark was dried and ground to a powder and mixed into a liquid, before the patient consumed it. In 1820, quinine was extracted from the bark and this purified quinine was used to treat malaria. Quinine is rapidly absorbed when consumed orally or through intravenous infusions, within 1-3 hours [24]. The half-life of quinine is approximately 11 hours [25], however, there are variations based on the age of the subjects and type of malaria. Quinine was the main drug used to treat malaria until the 1920s, when more effective synthetic drugs like chloroquine became readily available and were extensively used in the 1940s [26].

However, quinine was the most reliable drug used to treat severe malarial cases until recently; however, it is being replaced by artemisinin combination therapies (ACTs). With quinine treatment reports indicated there was a decrease in coma recovery times in severe malaria cases; however, there was no rise in mortality rates [27]. Hence quinine was still considered the best drug for treatment of severe malaria. Hospital treatment with quinine involves parenteral quinine administration through intramuscular injection or a slow rate-controlled intravenous infusion [28]. Drawbacks to the use of parenteral quinine treatment include pain at the site of intramuscular injection and an increased risk of developing lethal tetanus [29]. Quinine treatment also resulted in side effects such as hypoglycemia in children [30].

Chloroquine

In the beginning of the 20th century, there was a demand for an alternate drug to quinine for the treatment of malaria. This led to development of one of the most important synthetic drug called chloroquine in 1934 [31]. Chloroquine proved to be one of the most effective drugs ever deployed against an infectious disease [32]. It was used for both treatment and prophylaxis. It is a potent schizonticide, effective against the asexual blood stage of all strains of *Plasmodia* [33].



Artemisinin

In 2005, the WHO began recommending the use of ACTs (artemisinin combination therapy) as the first line of treatment for *P. falciparum* infections. This involves the use of an artemisinin derivative (artesunate or artemether), which is highly potent and short-lived along with a long-acting antimalarial such as lumefantrine, mefloquine or amodiquine. Treatment using ACTs have dramatically reduced malaria burden in the world [9] and has reduced mortality and morbidity associated with malaria [34-36]. Currently, cases of severe malaria are also being treated with ACTs due to the reduced rate of mortality compared to quinine treatment [11, 12].

Artemisinin was discovered in 1971, which initiated a new age of antimalarial drug therapy. It was isolated from the Chinese medicinal herb *Artemisia annua*. Later in 1979, the crystal structure was determined to show the presence of a peroxide bridge. The exact mechanism of artemisinin action is not known and is an active area of research, however, it is hypothesized that this peroxide bridge is responsible for the drug action [37]. It is highly insoluble in oil and water and hence is administered orally.

There are semisynthetic derivatives/analogs of artemisinin such as dihydroartemisinin (DHA), artesunate, artemether and arteether, which are all regarded as potent drugs against malaria. The derivatives can be administered to patients via intramuscular injection. They are fast acting and have a short half-life of around 1-5 hours depending on the type of derivative. Regardless of the specific type of artemisinin or derivative administered, all these compounds are metabolized into DHA.

However, in 2009, a study conducted in SEA demonstrated slow parasite clearance times in individuals treated with artemisinin monotherapy and ACTs. The observed delayed parasite clearance times *in vivo* was termed artemisinin resistance [38, 39]. The spread of artemisinin-resistant (delayed clearance) *P. falciparum* to surrounding regions is now threatening the world's malaria control and elimination

efforts [40]. Spread of resistant strains from Cambodia to Greater Mekong Subregion and to Africa has happened before with chloroquine and sulphadoxine-pyrimethamine [41, 42].

1.6 Malaria drug resistance

Antimalarial drugs have been key to malaria control especially due to the lack of an effective vaccine against malarial infections. As mentioned earlier (1.5) there have been various drugs used in the treatment of malaria, however, resistance to these drugs have been a major concern throughout history (Figure 1.3).

Quinine resistance

Resistance to quinine was first reported in Brazil and later in SEA [27, 43]. Quinine resistance is associated with polymorphisms in several transporters.

Polymorphisms associated with *pfmdr1*, *pfcr1* are linked to decreased quinine sensitivity. In addition increase in *pfmdr1* copy number can also lead to quinine resistance [44].

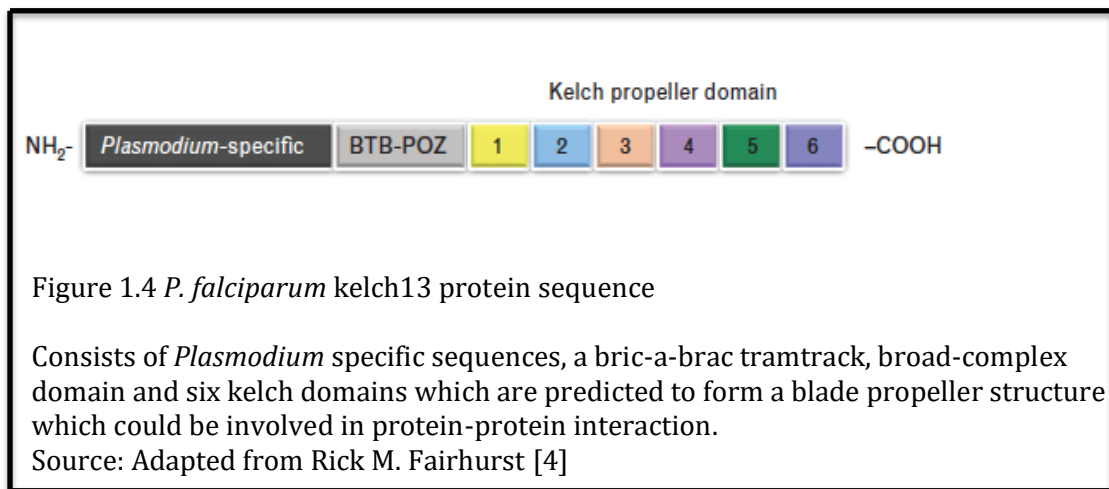
Chloroquine resistance

Due to the high availability and widespread use of chloroquine, there were cases of resistance being reported in the late 1950s at the Cambodia-Thailand border [45] and in the 1980s it had spread to all areas with *P. falciparum* malaria. As a result quinine remained and continued to be the drug used in the treatment of severe malaria.

In Africa, spread of chloroquine resistant strains became a major health problem and drug policies and formulation shifted to the use of combination therapy. This is when two or more drugs are administered simultaneously to a patient. This has a dual effect, as it can improve the efficiency of the treatment due to the different half-lives of these drugs and at the same time reduce the chances of developing resistant strains [46-48].

Artemisinin resistance (Delayed Clearance)

The emergence of artemisinin resistance in SEA resulted in efforts to find the molecular markers associated with this phenomenon. A study conducted in 2014 used an artemisinin-resistant laboratory adapted strain and performed whole genome sequencing to compare the genome of this strain with the clinical strains from Cambodia. They identified non-synonymous single nucleotide polymorphism (SNP) within the kelch propeller region of the *PF3D7_1343700* locus (*Pfkelch-13*)(Figure 1.4) [49].



Since the identification of *PF3D7_1343700* as molecular marker for artemisinin resistance, there have been several studies across regions in the world aimed at finding all the possible SNPs that can be associated with delayed parasite clearance times [50]. Only non-synonymous SNPs in the propeller region of the *Pfkelch-13* gene have been associated with delayed clearance [50] based on the parasite clearance half-life. The most common SNPs in the propeller region of the *Pfkelch-13* isolated from SEA: Cysteine 580 Tyrosine (C580Y), Arginine 539 Threonine (R539T), Tyrosine 493 Histidine (Y493H) and Isoleucine 543 Threonine (I543T) [49].

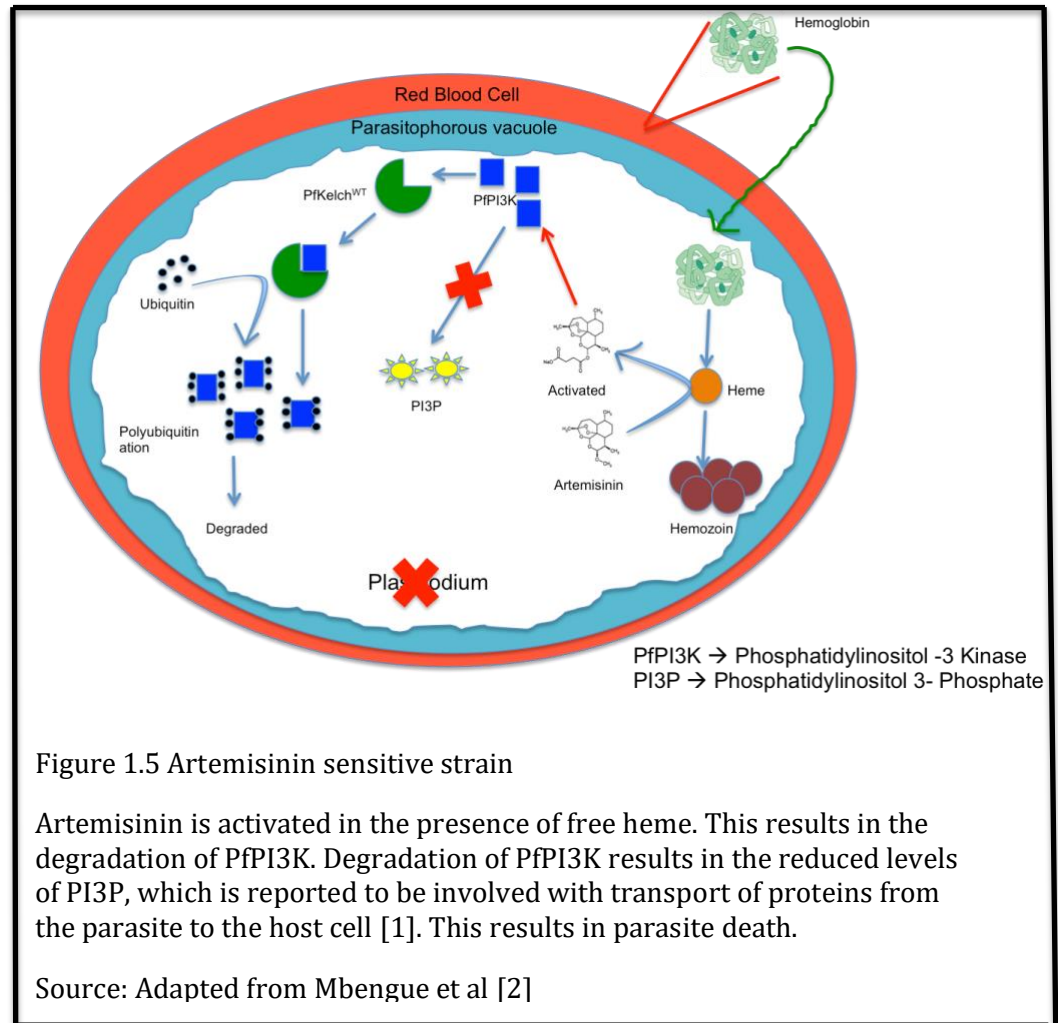
Alternatively, recent studies using culture adapted clinical isolates from SEA have identified parasites with increased ring-stage survival times in the absence of mutations in the propeller domain of the *Pfkelch13* gene [51].

The mutant strains were mainly associated with regions in SEA, and were not observed in Africa, however a study conducted in children with severe malaria in Uganda, Africa found the presence of an alanine residue at position 578 replaced by a serine (A578S) mutation at low frequency was associated with delayed parasite clearance (>72hr) time upon ACT treatment [52]. Since this initial observation, there have been many studies conducted in Africa, to determine whether the presence of mutant parasites is increasing and spreading, as this might result in a major public health crisis in Africa [52-55].

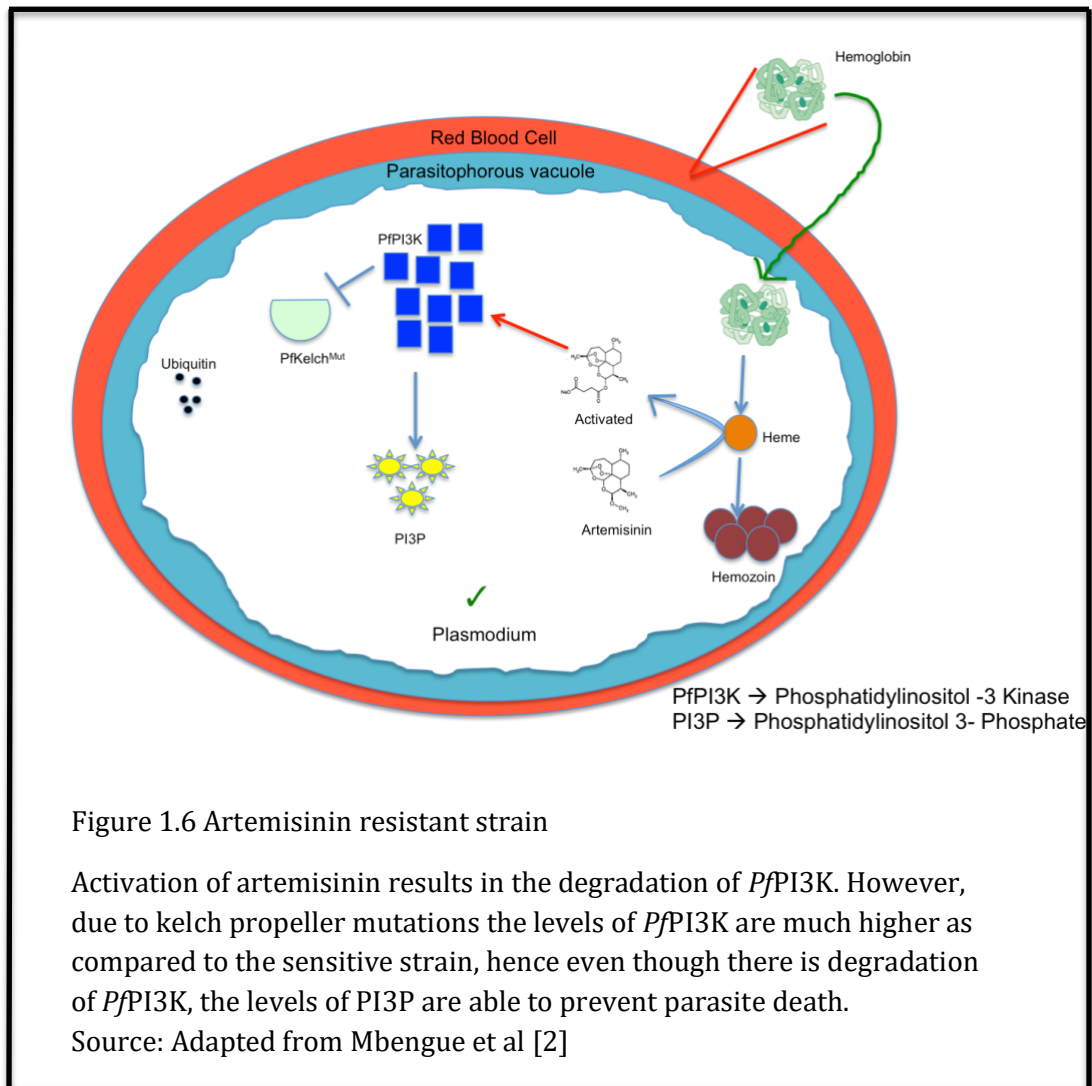
1.7 Mechanism of artemisinin resistance

We do not have a complete understanding about the mechanism involved in artemisinin resistance. There are a few proposed mechanisms:

- 1) A transcriptomics study of isolates from patients in SEA with malaria parasites with delayed parasite clearance times in response to artemisinin treatment had an increase in the 'unfolded protein response' pathway [56]. This could be a mechanism by which the parasite survives artemisinin action, by repairing the damaged proteins. Hence further studies into the cell stress response might give a better understanding of the role played by the stress response during artemisinin exposure.
- 2) Another study provided evidence that artemisinin targets the *P. falciparum* phosphatidylinositol-3-kinase (*PfPI3K*) and that *PfPI3K* binds to *PfKelch13* [2]. In case of artemisinin-sensitive strains, *PfKelch13* binds to *PfPI3K* and this cause *PfPI3K* to undergo polyubiquitination resulting in its proteosomal degradation. These sensitive parasites have low levels of *P. falciparum* phosphatidylinositol-3-phosphate (*PfPI3P*), which is a product of *PfPI3K* activity (Figure 1.5) [2].



Artemisinin resistant strains containing polymorphisms in *PfKelch13* are unable to bind to *PfPI3K*, leading to an increase in the *PfPI3K* levels. Increased concentrations of PI3P are hypothesized to enable parasite survival during artemisinin drug pressure (Figure 1.6) [2].



1.8 Research objectives:

Studies conducted in SEA have reported resistance to most of the drugs used to treat malarial infections. SEA is a region of intermediate malaria transmission as a result of which individuals in this region have lower parasite burden. Artemisinin has been used to treat patients with uncomplicated malaria [57-60]. Resistant strains of the parasite to artemisinin treatment have also mainly been observed in SEA. These studies have identified mutations in the propeller domain of the *Pfkelch13* gene associated with delayed parasite clearance times. In the past, resistance to malarial drug treatment have developed in SEA and gradually spread across to Sub-Saharan Africa.

In our study conducted in Uganda between 2008 and 2013 children with severe malaria were treated with intravenous quinine. However, in 2005 there was a change in the treatment policy in Uganda towards the use of ACTs for uncomplicated malaria [61, 62]. Here we were interested in investigating the presence of parasites with mutations in the *Pfkelch13* gene in our study population. In addition, a study conducted between 2011 and 2013 in Uganda by our group found a mutation (A578S) in the *Pfkelch13* gene was associated with delayed parasite clearance in children with severe malaria who were treated with artemisinin [52]. This was the first time evidence of delayed parasite clearance time was documented in Sub-Saharan Africa and in a population with severe malaria (high parasite burden), rather than uncomplicated malaria.

In the current study we investigate:

1. The presence of mutations in the *Pfkelch13* gene in children with severe malaria in Uganda, with high parasite burden. Our goal was to detect whether the A578S mutation was seen in a population that, unlike the original study population in which we found the A578S mutation, were not being treated with artesunate for severe malaria, but were being treated with ACTs for uncomplicated malaria.

2. Whether *Pfkelch13* mutations, including the A578S mutation, are associated with changes in parasite clearance times in children treated with quinine. The use of ACTs outside this study group could also have an impact on the prevalence of these mutations. However, there is no known reason why these should affect parasite clearance times in children treated with quinine. In this study, we assess whether there is any association, with the idea that if an association with delayed clearance is seen, it may reflect mechanisms by which these mutations also affect quinine treatment effects on parasite clearance, or that these mutations are in linkage disequilibrium with other mutations that lead to delayed parasite clearance with quinine, or could reflect an association of the mutations with other factors such as impaired immunity that might affect parasite clearance. The long-term goal of this preliminary study is to determine whether mutations in the *Pfkelch13* gene are present in other areas of Uganda and whether any mutations seen have effects on parasite clearance with a non-artemisinin containing intervention (quinine).

METHODS AND MATERIAL

2.1 Study description

Study area location

The CMR01 study was conducted at Mulago National Referral and Teaching Hospital in Kampala, Uganda. Uganda is a landlocked country in East Africa. It is bordered by Kenya to the east, Tanzania to the south, Democratic Republic of the Congo to the west, Rwanda to the southwest and South Sudan to the north. In 2015, Uganda had a population of 39,032,000, with a per capita income of \$1 based in 2013 record [63].

According to data from 2015, Uganda is an area of high transmission with more than 1 case of malaria per 1000 individuals in a population. There were 7,137,662 (range 4,500,000 – 13,000,000) reported cases of malaria, with nearly all of them being attributable to *P. falciparum* [63]. During the same period there were 6,100 cases of reported deaths with estimates of 4,300 – 17,000 [63].

2.2 Main study design

Ethics Statement

The Ugandan National Council for Science and Technology (UNCST), The Makerere University School of Medicine Research and Ethics Committee and the University of Minnesota Institutional Review Board approved this study. Written informed consent was obtained from the parents or guardians of the participants enrolled in the local language.

Selection of individuals

The study was conducted between 2008-2013 and children were enrolled into three groups cerebral malaria (CM, n=269), severe malarial anemia (SMA, n=232) and community children (CC, n=213). The enrolled children were between the ages of 18 months and 12 years and if they met the WHO definition for CM or SMA, were recruited from the Acute Care Unit at Mulago Hospital.

Cerebral malaria was defined when a child was diagnosed with: 1) coma (Blantyre Come Score ≤ 2); 2) presence of *P. falciparum* infection on a blood smear; 3) no response to a glucose one hour after administration if hypoglycemic; 4) in a state of coma at least one hour after termination of seizure or after administration of anticonvulsants. Exclusion criteria for CM included: 1) white blood cell count > 5 cells/ μ l in cerebral spinal fluid (CSF); 2) presence of bacterial infections by Gram stain and/or culture positive for CSF. For children suspected with CM a lumbar puncture (LP) was performed to rule out bacterial meningitis, unless the physician on duty deemed it unsafe or the parents or guardians of the child did not agree to have an LP performed.

Severe malarial anemia was defined as: 1) presence of *P. falciparum* on a blood smear; 2) levels of serum hemoglobin was ≤ 5 g/dl. Exclusion criteria in SMA included: 1) the children had any signs of impaired consciousness during the physical exam (GCS <15 for children older than 5 years of age and BCS <5 for children ≤ 5 years of age; 2) had seizures prior to admission. Some children presented both CM and SMA (22% of CM); these children were enrolled into the CM group.

Community children were enrolled into the study from the neighborhood or extended family of a child with CM. These children were also between the age of 18 months to 12 years of age and currently healthy. The community children were

excluded if they had any active illness or had an illness in the past 4 weeks requiring medical care. At the time of enrollment, a blood smear was prepared for the community children, and those with *P. falciparum* on the smear were sent home with antimalarials. These children were indicated as asymptomatic parasitemic (AP).

Other exclusion factors for all children included: 1) any known chronic illness requiring medical care; 2) any developmental delay; 3) prior history of head trauma, coma, cerebral palsy, or hospitalization due to malnutrition.

The children enrolled in this study were followed up for 2 years, and were asked to return to Mulago hospital in the event of any illness. Over this span of time 14 children were admitted with CM, 26 were admitted with SMA, and 3 CC were admitted to the hospital.

Clinical treatment

The children enrolled in this study underwent a medical history and physical examination. Children with severe malaria were treated based on the Ugandan Ministry of Health treatment guidelines at the time, which involved intravenous quinine treatment until the patient is alert and then oral quinine for hospitalized patients; and artemisinin combination therapy, usually with artemether-lumefantrine for outpatients. Currently there is no recommended adjunctive treatment for severe malaria; therefore, these children were provided supportive and symptomatic care, as needed, which mainly included anticonvulsants for CM. Children with SMA received a blood transfusion. The blood units provided to the hospitals in Uganda were screened as negative for HIV-1, syphilis, hepatitis B and hepatitis C [64].

Standard laboratory and clinical testing

A complete blood count (CBC) was performed on all the enrolled children using a COULTER® Ac·T™ 5diff CP (Cap Pierce) hematology analyzer. Immediately upon

enrollment the lactate, blood glucose and hemoglobin levels were also evaluated using hand held devices (lactate monitor, glucometer and hemo control). Only upon consent from the parents or guardians were the results included in the study. HIV-1 testing was also performed upon consent from the parents or guardians. Three immunochromatographic tests (Determine, STAT-PAK and Uni-Gold) tests were performed and the diagnosis was based on the Uganda National HIV testing algorithm. Stool samples from the enrolled children were examined by microscopy for the presence of motile trophozoites, helminthic ova or larva, red blood cells, protozoan cysts.

Thick and thin blood smears were made to look for malaria parasites, this was done according to a standard protocol [65]. Fresh blood smears were prepared and checked for parasites by microscopy every 24 hours after intravenous quinine treatment. The parasite clearance times were calculated based on the absence of parasite in the blood smear after treatment. Parasite density was calculated using microscopy, however, this only provides an estimate of the circulating parasites in the blood. In the case of *P. falciparum* infection, infected red blood cells can sequester in the blood microvasculature of various organs, we also measured the levels of *P. falciparum* histidine rich protein-2 (*PfHrp-2*), which is a marker of overall parasite biomass. This parasite protein is released upon rupture of infected red blood cells at the end of the 48-hour asexual cycle. We quantified the levels of Hrp-2 using the Malaria Ag CELISA (Cellabs, Brookvale, Australia) and this provided an estimate of the sequestered parasite biomass [66]

Specimen collection and storage

Upon enrollment and at 6-month and 12-month follow up visits blood samples were collected in EDTA tubes. Plasma was prepared from whole blood tubes and was made into aliquots of 1 – 1.5 ml and stored at -80 °C. For patients diagnosed with severe malaria, blood was again collected at 24 hours and stored at -80 °C. Dried

blood spots were collected from all the patients upon enrollment, 6-month and 12-month follow ups, which were stored at 4°C.

2.3 Sample processing and DNA extraction

Samples were collected during the main study (section 2.2) from Uganda. The work here utilized dried blood spots from children at enrollment as shown in a schematic diagram (Figure 2.1). We selected 50 children each from CM and SMA groups having high parasite burden (*PfHrp2* levels) and 50 children with low parasite biomass from each CM and SMA groups.

The rationale behind this was:

- *De novo* mutations might occur during the asexual stage of the parasite life cycle. This increases the probability of more than one resistant parasite in an infection upon drug treatment in individuals with high parasite burden. Hence we looked at children with high parasite burden.
- Resistance (delayed parasite clearance) to ACTs was first observed in patients treated for uncomplicated malaria in SEA. These patients had low parasite burden, however, there was selection of resistant strains in this population. Hence we also looked at children with lower parasite burden.

Genomic DNA at enrollment was isolated from dried blood spots (Whatman 903 Protein Saver Card, Cardiff, UK) for children with CM, SMA and CC using the QIAamp 96 DNA Blood Kit (Qiagen, Hilden, Germany).

However, *Pfkelch13* genotyping was performed in only 78 (CM), 79 (SMA) and 49 (CC) samples in each of these groups. We were unable to amplify 18 (CM), 15 (SMA) and 26 (CC) samples as DNA isolated from dried blood spots had very low genomic DNA concentrations and also low quality DNA (Table 2.1, Table 2.2 and Table 2.3). 4 samples from CM and 6 samples from SMA group were ineligible for this study.

2.4 PCR amplification of *Pfkelch13*.

Amplification of the *Pfkelch13* gene was performed using a nested PCR protocol[49].

A diagrammatic representation is shown in (Figure 2.2).

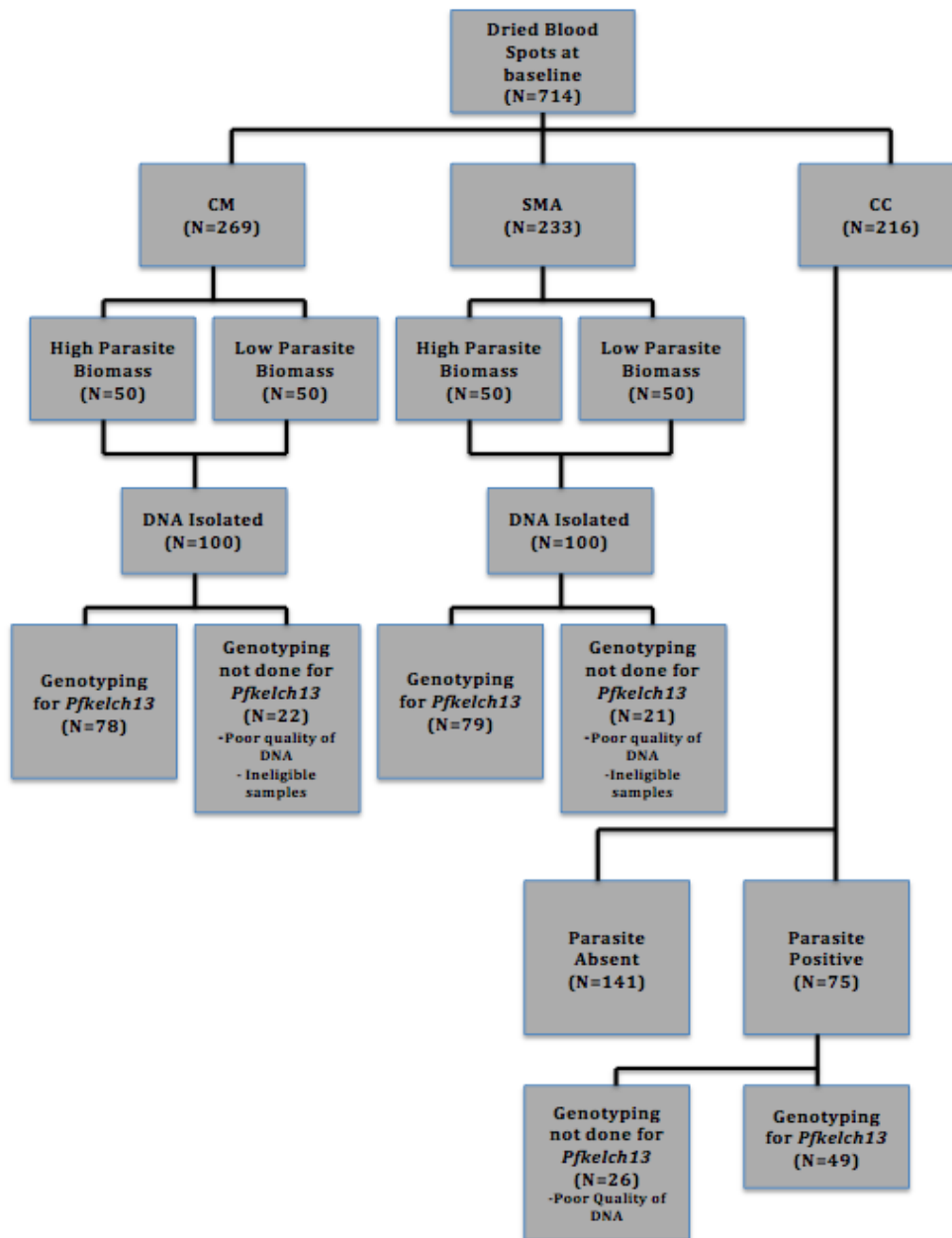


Figure 2.1 Schematic representation of the current study design

18 Samples in CM had low quality DNA. 4 Samples were ineligible

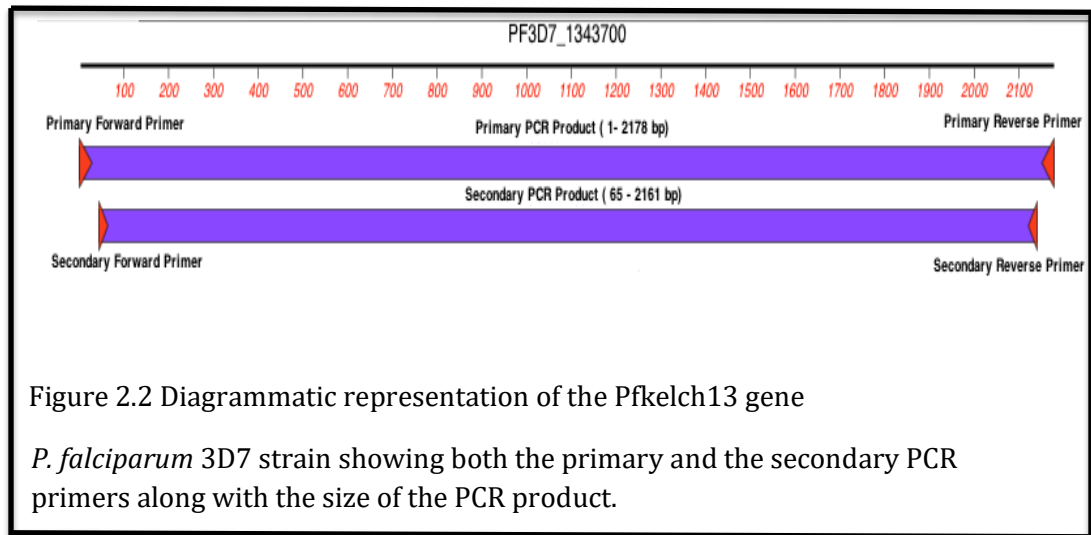
15 Samples in SMA had low quality DNA. 6 Samples were ineligible

Abbreviations: CM, Cerebral Malaria; SMA, Severe Malarial Anemia; CC, Community Control; Hrp2, Histidine Rich Protein 2

Table 2.1 Differences between the children with cerebral malaria (CM) that were tested and not tested			
	Tested (n =78)	Not Tested (n =18)	pa
<i>P. falciparum</i> peripheral blood density (parasites/ μ l), median (IQR)	53680 (16200 – 279240) (n =75)	38880 (26320 – 125900) (n =17)	0.9719
Total Parasite Biomass (<i>PfHrp2</i>)(ng/ml), Median (IQR)	1825.2 (309.6 – 7486.5)	7126.3 (470.4 – 12502.7)	0.2867
No. of Sample with High Parasite Biomass, (%)	39 (78)	10 (20)	NA
No. of Sample with Low Parasite Biomass, (%)	39 (78)	8 (16)	NA
<p>^a Due to non-normality Wilcoxon Rank-Sum Test was performed Abbreviations: <i>PfHrp2</i> (<i>Plasmodium falciparum</i> Histidine Rich Protein -2); IQR (Interquartile Range); n (Number); NA (Not Applicable) 4 children were ineligible from this group</p>			

Table 2.2 Differences between the children with severe malarial anemia (SMA) that were tested and not tested			
	Tested (n =79)	Not Tested (n =15)	P^a
<i>P. falciparum</i> peripheral blood density (parasites/ μ l), median (IQR)	36820 (4360 – 117880)	14120 (7820 – 35180)	0.2116
Total Parasite Biomass (<i>PfHrp2</i>)(ng/ml), Median (IQR)	2469.6 (140.0 – 3725.7) (n =78)	3051.5 (264 – 5929) (n =14)	0.5755
No. of Sample with High Parasite Biomass, (%)	42 (84)	8 (16)	NA
No. of Sample with Low Parasite Biomass, (%)	37 (74)	7 (14)	NA
^a Due to non-normality Wilcoxon Rank-Sum Test was performed Abbreviations: <i>PfHrp2</i> (<i>Plasmodium falciparum</i> Histidine Rich Protein -2); IQR (Interquartile Range); n (Number); NA (Not Applicable) 6 children were ineligible from this group			

Table 2.3 Differences between the children with asymptomatic infection (CC) that were tested and not tested			
	Tested (n =49)	Not Tested (n =26)	P^a
<i>P. falciparum</i> peripheral blood density (parasites/ μ l), median (IQR)	0 (0 – 1240) (n =48)	0 (0 – 330) (n =24)	0.1333
Total Parasite Biomass (<i>PfHrp2</i>)(ng/ml), Median (IQR)	4.8 (4.8 – 73.2) (n =44)	4.8 (4.8 – 4.8) (n =24)	0.2695
^a Due to non-normality Wilcoxon Rank-Sum Test was performed Abbreviations: <i>PfHrp2</i> (<i>Plasmodium falciparum</i> Histidine Rich Protein -2); IQR (Interquartile Range); n (Number)			



A list of primers used for the primary amplification and secondary reaction is shown in table 2.4 and table 2.6. A total volume of 25µl reaction mixture was set for each primary PCR reactions in the PCR tubes. The reaction mixture was composed of 5X Phusion HF Buffer (5µl) supplied by (Thermo Scientific, USA), 5µM of forward and reverse primers (1µl each) supplied by (Eurofins Genomics, USA), 25mM dNTP (1.0µl) supplied by (Clontech, USA), 3mM MgCl₂ (1.0µl) supplied by (Thermo Scientific, USA) and 2U/µl Phusion polymerase (0.5µl) supplied by (Thermo Scientific, USA). 5µl of genomic DNA was added into each reaction mixture and 10.5µl of distilled water was used to make the volume up to 25µl. The cycling conditions used for the primary PCR amplification is shown in table 2.5.

Table 2.4 List of primers used for the primary PCR amplification of Pfk13 gene

Oligonucleotide	Sequence 5' - 3'	Tm (°C)
KelchForwardN-1 (Forward Primer)	ATGGAAGGAGAAAAAGTAAAAACAAAAG	58.8
KelchReverseN-1 (Reverse Primer)	TATATTTGCTATTAACGGAGTGACC	60.0

Table 2.5 Primary PCR amplification cycling conditions for the *Pfkelch13* gene

Gene	Denaturing Temperature	Annealing Temperature	Extension Temperature
<i>Pfkelch13</i>	94°C for 30 seconds	60°C for 90 seconds	72°C for 90 seconds

The secondary PCR reaction for the *Pfkelch13* gene was performed using a total volume of 25µl of reaction mixture in each PCR tube. The mix contained 1µl of primary PCR product, 10X PCR buffer (Mg⁺) (3.2µl) supplied by Clontech, USA, 2mM dNTP mixture (2.5µl), 5µM of forward and reverse primers (1.25µl each) supplied by (Eurofins Genomics, USA) as shown in table 2.6 and 5U/µl Takara Ex Taq® Hot Start Version supplied by (Clontech, USA). The cycling conditions used in the secondary PCR reactions are shown in table 2.7.

Table 2.6 List of primers used in the secondary PCR reaction of the *Pfkelch13* gene

Oligonucleotide	Sequence 5' - 3'	Tm (°C)
K13 - 1 (Forward Primer)	GGGAATCTGGTGGTAACAGC	62.4
K13 - 4 (Reverse Primer)	CGGAGTGACCAAATCTGGGA	62.4

Table 2.7 Secondary PCR amplification cycling conditions for the *Pfkelch13* gene

Gene	Denaturing Temperature	Annealing Temperature	Extension Temperature
<i>Pfkelch13</i>	94°C for 30 seconds	60°C for 120 seconds	72°C for 120 seconds

Agarose Gel Electrophoresis

The PCR products obtained from the secondary PCR reactions were analysed on a 1% agarose gel (1gm agarose + 100ml 1X TBE buffer). A 1kb ladder supplied by (Invitrogen, USA) was used to confirm the PCR product obtained using AlphaImager HP supplied by (ProteinSimple, USA).

2.5 DNA sequencing

The amplified DNA product obtained after the secondary PCR was then PCR purified using QIAquick PCR Purification Kit (Qiagen, Germany). The sequencing reactions were carried out using Sanger Sequencing (SimpliSeq™ DNA Sequencing, Quintarabio, USA). Each sample was sequenced using 6 different primers supplied by (Eurofins Genomics, USA). The list of primers used for sequencing is provided in table 2.8. The sequencing reactions were prepared in PCR tubes with 10µl of sample and 5µl of each primer/reaction.

Table 2.8 List of primers used for sequencing of the Pfkclch13 gene

Oligonucleotide	Sequence 5' - 3'	Tm (°C)
K13 - 1	CGGAGTGACCAAATCTGGGA	62.4
Kelch13_2 for seq	GATATGAGTGTATTAGATTCGAAC	57.7
Kelch13_3 for seq	GATACTTATGAAAAGAAAATTATTG	53.1
Kelch13_4 for seq	GAAAAAGAAAAATATTATCAAGAA	50.9
Kelch13_5 for seq	CAACAATGCTGGCGTATGTGTACACC	66.2
Kelch13_6 for seq	GATAATAAAATTTATGTCATTGGTGG	56.7

The sequencing data obtained after sequencing was analyzed using the software MacVector.

2.6 Statistical tests

Laboratory and clinical finding for children in the different disease groups were compared using the chi-square test in case of categorical variables. When comparing two independent groups due to non-normality of the data Wilcoxon-Rank-Sum Test was performed and for more than two groups the Kruskal-Wallis Test was performed. We also transformed parasite density and parasite load levels to common logs (log to the base 10) for better graphical representation of the data. Kaplan – Meier Survival analysis was also performed for parasite clearance times. All these statistical tests were performed using StataSE and the graphs were prepared using SAS (SAS Institute, USA).

RESULTS

3.1 The study and baseline characteristics

Between 2008 and 2013 a total of 269 children with CM, 232 children with SMA and 213 community controls (CC) were enrolled. Of these we used 100 samples (50 high *PfHrp2* and 50 low *PfHrp2*) from CM, 100 samples (50 high *PfHrp2* and 50 low *PfHrp2*) from SMA and 75 children from the asymptomatic CC groups to isolate the genomic DNA from dried blood spots. We were unable to amplify the *Pfkelch13* gene from 18 samples from the CM, 15 from the SMA and 26 from the asymptomatic CC groups due to low concentration of genomic DNA and low quality DNA and were excluded from the analysis (Table 2.1, 2.2 and 2.3). There were 4 ineligible samples in the CM group, and 6 ineligible samples in the SMA group due to the lack of follow-up data, which were excluded from the analysis. In total we were able to amplify and genotype the *Pfkelch13* gene in 78 samples from CM, 79 from SMA and 49 from asymptomatic (CC) groups (Figure 2.1).

Children with SMA were younger than both children with CM and CC ($P=0.0014$; Table 3.1). The peripheral blood density of parasites was the highest in children with CM as compared to SMA ($P=0.0356$). Community children had negligible levels of parasitemia in peripheral blood as detected by microscopy (Table 3.1). Previously it has been documented that children with severe malaria generally have higher levels of parasite biomass due to potential sequestration of the parasite within the vasculature, which may not be adequately represented using the measurements of the parasite levels in the peripheral blood [67]. So, we also measured the concentration of *PfHRP2*, which gives an estimate of the overall parasite biomass. The parasite biomass (*PfHRP2* concentration) differed among all the groups, with the highest concentration occurring in children with SMA (CM vs SMA vs CC) ($P<0.0001$; Table 3.1). Children with CM and SMA did not show any difference in the parasite clearance times (Table 3.1). Deaths caused by malaria were only observed in children with CM.

Table 3.1 Characteristics of children with cerebral malaria (CM), severe malarial anemia (SMA) and asymptomatic community children (CC)

	Cerebral Malaria (CM, n=78)	Severe Malarial Anemia (SMA, n=79)	Community Children (CC, n=49)	P^a
Age (months), median (IQR)	41.8 (30.0 – 58.0)	33.3 (23.4 – 53.9)	48.0 (34.3 – 61.2)	0.0018 ^b
Sex, male n (%)	51(65)	53(67)	25 (50)	0.154
<i>P. falciparum</i> peripheral blood density (parasites/μl), median (IQR)	53680 (16200 – 279240) (n =75)	36820 (4360 – 117880)	0 (0 – 1240) (n =48)	<0.0001 ^c
Total Parasite Biomass (<i>PfHrp2</i>)(ng/ml), Median (IQR)	1825.2 (309.6 – 7486.5)	2469.6 (140.0 – 3725.7) (n =78)	4.8 (4.8- 73.2) (n =44)	<0.0001 ^c
Parasite clearance time (hours), median (IQR)	48 (24 – 72) (n =58)	48 (24 – 72) (n =66)	NA	0.8590
Prior Artemisinin Exposure, n (%)	15 (20) (n =76)	9 (13) (n =68)	NA	0.296
Death, n (%)	15 (19)	0 (0)	NA	<0.0001
Mutants, n (%)	46 (59)	44 (56)	27 (55)	0.884

^a Due to non-normality Kruskal – Wallis Test was performed when comparing three groups and Wilcoxon Rank-Sum Test when comparing two groups. Chi-squared test was used for categorical variables.

^b All the pairs of groups differ significantly, except between CM and SMA (however close to significance).

^c All the pairs of groups differ significantly.

Abbreviations: *PfHrp2* (*Plasmodium falciparum* Histidine Rich Protein -2); IQR (Interquartile Range); n (Number); NA (Not Applicable)

3.2 Detection of *PfKelch13* gene mutations

Whole gene sequencing was done on a total of 205 enrollment samples. 157 samples (78.5%) were from children with severe malaria and forty-eight samples (64%) were from children with an asymptomatic parasite infection (CC).

Ninety children (57%) with severe malaria had non-synonymous mutations in the *kelck13* gene; all the polymorphisms are shown in Table 3.2. One infection showed the presence of two *kelch13* mutations upstream of the propeller region of the *Pfkelch13* gene.

The A578S mutation in the propeller region of the *Pfkelch13* gene was identified in a single infection. A578 has been previously observed in Africa and Asia, and in some clinical cases has been associated with delayed clearance [50, 52, 68, 69]. We did not observe any of the mutations observed in SEA which have been associated with *in-vitro* delayed parasite clearance times.

Table 3.2 Nonsynonymous mutations observed in the *Pfkelch13* gene in 157 children with severe malaria (CM and SMA)

Polymorphisms	Reference Sequence (3D7)	Mutant Sequence ^a	No. of isolates/Total isolates (%)
G112E	GGA	<u>G</u>AA	1 (0.6)
N132S	AAT	<u>A</u>GT	2 (1.3)
142N ^b		<u>A</u>AT	4 (2.5)
142NN ^b		AAT <u>A</u>AT	41 (26)
N142D	AAT	<u>G</u>AT	2 (1.3)
K189N	AAA	<u>A</u>AT	3 (1.9)
K189T	AAA	<u>A</u>CA	26 (16.5)
R255K	AGA	<u>A</u>AA	7 (4.5)
L258M	TTG	<u>A</u>TG	3 (1.9)
K189T & Q271H	AAT & CAG	<u>A</u>CA & <u>C</u>AT	1 (0.6)
A578S	GCT	<u>T</u>CT	1 (0.6)

^a Mutated bases are in bold and underlined.

^b Insertion.

G:Glycine, E:Glutamic acid, N:Asparagine, S:Serine, D:Aspartic acid, K:Lysine, T:Threonine, R:Arginine, L:Leucine, M:Metheonine, Q:Glutamine, H:Histidine, A:Alanine

There were also synonymous mutations in the *Pfkelch13* gene (4%) in children with severe malaria (CM and SMA). Three of these infections were associated with a synonymous and a nonsynonymous mutation. A list of these synonymous mutations is provided in (Table 3.3).

Table 3.3 Synonymous and nonsynonymous mutations observed in the <i>Pfkelch13</i> gene in 157 children with severe malaria (CM and SMA)			
Polymorphisms	Reference Sequence (3D7)	Mutant Sequence ^a	No. of isolates/Total isolates (%)
K189T & L251L	AAA & CTA	<i>ACA</i> & CTG	1 (0.6)
K189T & V219V	AAA & GTA	<i>ACA</i> & GTT	1 (0.6)
K189N & L119L	AAA & TTG	<u>AAT</u> & CTG	1 (0.6)
G220G	GGT	GGC	1 (0.6)
T474T	ACA	ACC	1 (0.6)
G496G	GGT	GGG	1 (0.6)

^a Synonymous mutated bases are italicized and nonsynonymous mutated bases are in bold and underlined.
K:Lysine, T:Threonine, V:Valine, G:Glycine

Nonsynonymous mutations were observed in 54% of community control children who were asymptomatic infected with malaria. Most of the nonsynonymous mutations (50%) were observed upstream of the propeller regions of the *Pfkelch13* gene. There were two CC children with parasite mutations in the propeller region of the *Pfkelch13* gene. One infection had the A578S, which was also observed in an infection associated with the severe malaria group, and the other infection had a serine residue replaced at position 522 by a cysteine (S522C). The S522C mutation has also been previously observed in Africa [70, 71]. All the nonsynonymous mutations are provided in Table 3.4. There were no synonymous mutations associated with asymptotically infected CC children.

Table 3.4 Nonsynonymous mutations observed in the <i>Pfkelch13</i> gene in 49 asymptomatic community control (CC) children			
Polymorphisms	Reference Sequence (3D7)	Mutant Sequence ^a	No. of isolates/Total isolates (%)
G112E	GGA	<u>G</u> A	1 (2)
142N ^b		AAT	1 (2)
142NN ^b		AAT AAT	16 (33)
K189T	AAA	A <u>C</u> A	6 (12.5)
N197D	AAT	<u>G</u> AT	1 (2)
S522C	AGT	<u>T</u> GT	1 (2)
A578S	GCT	<u>T</u> CT	1 (2)

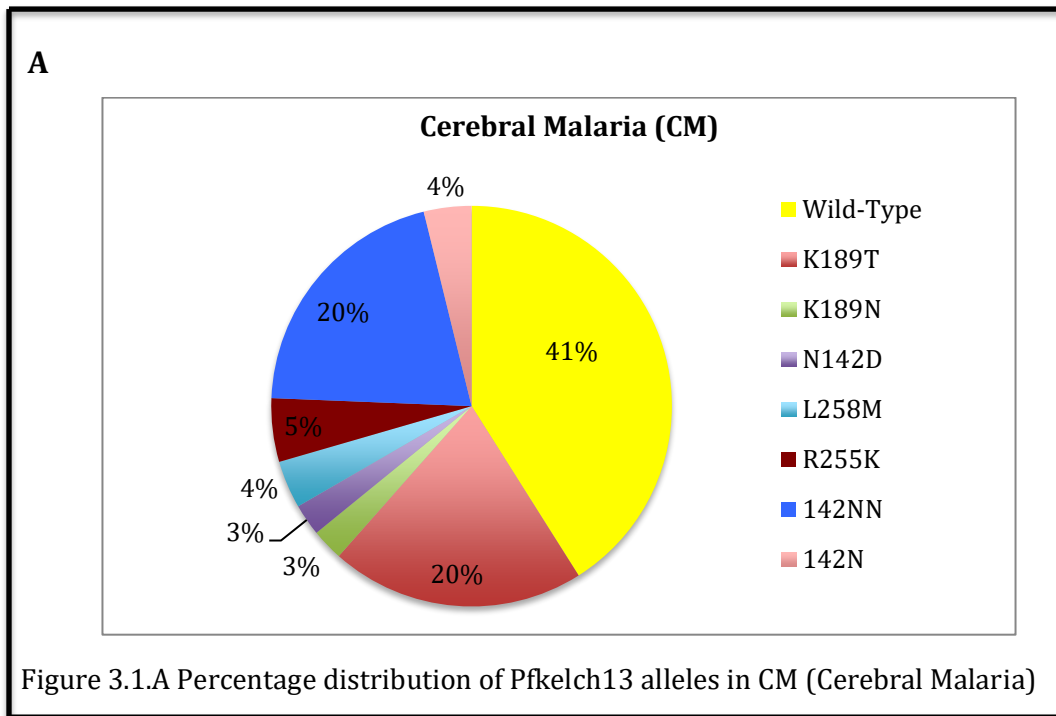
^a Mutated bases are in bold and underlined
^b Insertions
G:Glycine, E:Glutamic acid, N:Asparagine, K:Lysine, T:Threonine, S:Serine, C:Cysteine, A:Alanine

3.3 Frequency distribution of *Pfkelch13* alleles in CM, SMA and CC children

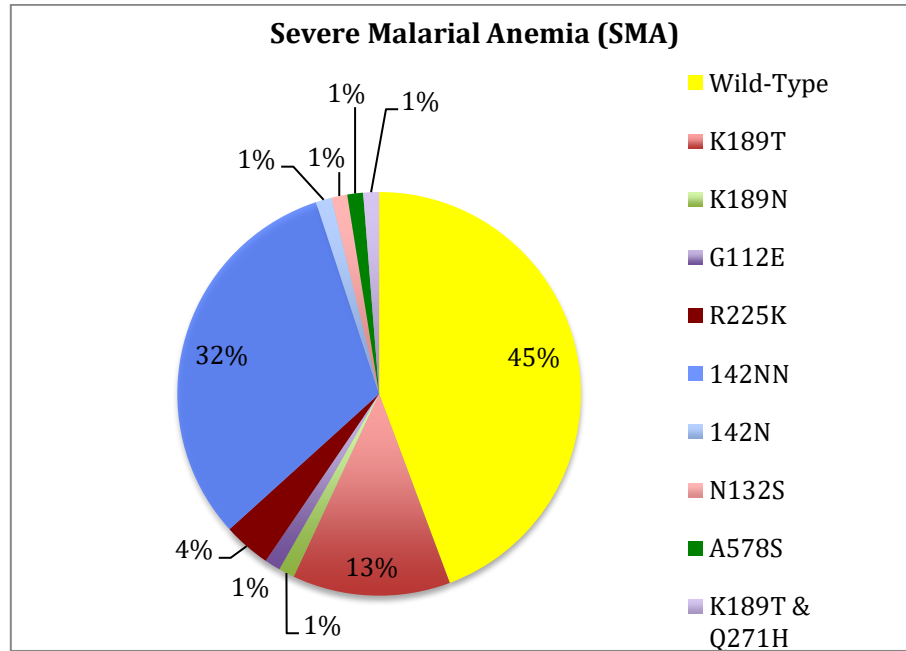
We observed malarial infections in CM, SMA and asymptomatic CC children with mutations in the *Pfkelch13* gene. The two most prevalent forms of mutations were lysine residue replaced at position 189 by a threonine (K189T) (n=32) and at position 142 double insertion of arginine residue (142NN) (n=57) insertion. In children with CM, 20% of the infections had the K189T (n=16) mutation and the 142NN (n=16) insertion each (Figure 3.1A). Children with SMA had 13% of the infections with K189T (n=10) mutations and 32% with the 142NN (n=25) insertion (Figure 3.1B, Table 3.5). Similarly, in CC children the prevalence of K189T mutation was 13% (n=6) and 33% for the 142NN (n=16) insertion (Figure 3.1C). The A578S mutation in the propeller region of the *Pfkelch13* gene was identified in one child belonging to SMA and asymptomatic CC groups each. The S522C mutation was identified solely in one kid with an asymptomatic infection in the CC group.

Table 3.5 Distribution of K189T, 142NN and wild-type strains in children with CM, SMA and asymptomatic community control (CC)				
	K189T	142NN	Wild-Type	p^a
Cerebral Malaria (CM), n (%)	16 (20)	16 (20)	32 (41)	
Severe Malarial Anemia (SMA), n (%)	10 (13)	25 (32)	35 (44)	0.368
Asymptomatic Controls (CC), n (%)	6 (12)	16 (33)	22 (45)	

^a Chi-squared test was used for categorical variables



B



C

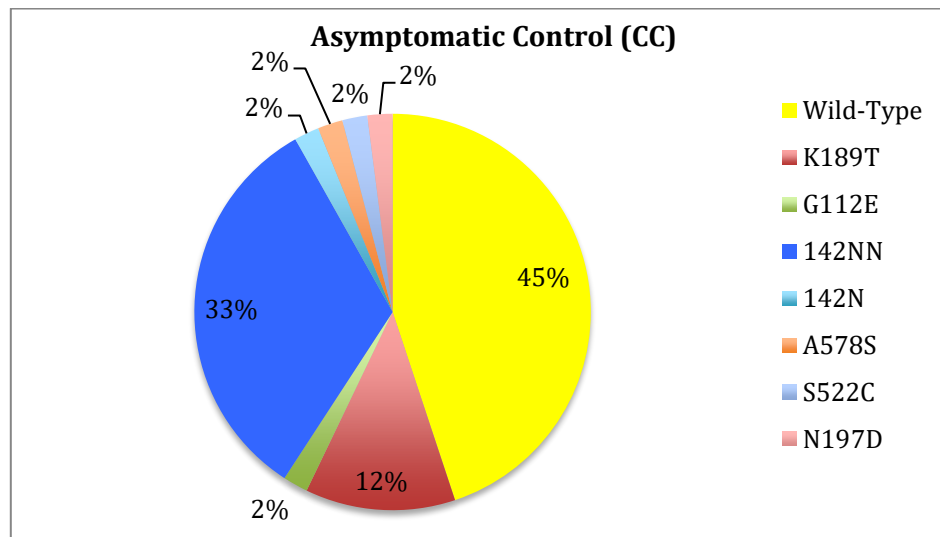


Figure 3.1.B Percentage distribution of Pfkclch13 alleles in SMA (Severe Malarial Anemia)

Figure 3.1.C Percentage distribution of Pfkclch13 alleles in CC (Community Children)

3.4 Differences in the characteristics of children infected with different *Pfkelch13* mutants in severe malaria (CM and SMA).

The distributions of the most prevalent *Pfkelch13* genotype (K189T, 142NN, other mutants and 3D7) among children with CM & SMA are shown in Table 3.5. In children with severe malaria parasites with the 142NN insertion compared to 3D7 (wild-type) *Pfkelch13* genotype had reduced parasite clearance times (median, interquartile range, in hours: 24, [24 – 48] vs 48, [48 – 72], $P = 0.0001$) upon quinine treatment (Table 3.6). Similarly, parasites with the K189T mutations when compared to the 3D7 *Pfkelch13* genotype had reduced clearance time (median, interquartile range, in hours: 24, [24 – 48] vs 48, [48 – 72], $P = 0.0258$). However, there was no significant difference observed between the K189T and 142NN insertion groups (median, interquartile range, in hours: 24, [24 -48], $P=0.4764$). Further, there was no significant difference in parasite clearance times upon quinine treatment between the wild-type and other mutant groups (median, interquartile range, in hours: 48, [48 – 72], $P = 0.7256$).

In children with severe malaria, parasites with the 142NN insertion also had lower parasite biomass when compared to 3D7 *Pfkelch13* genotype (*Pf*HRP2, ng/ml, median [IQR]; 370.8 [125.5 – 3463.2] vs 3134.4 [273.6 – 6842.4], $P = 0.0307$; Figure 3.2A). Similarly, there was a difference in the parasite biomass between the participants with parasites having the 142NN insertion when compared to the K189T mutation groups (*Pf*HRP2, ng/ml, median [IQR]; 370.8 [125.5 – 3463.2] vs 3134.4 [600 – 6684.2], $P = 0.0182$). Severe malaria infections with 142NN insertion had lower peripheral parasite density when compared to 3D7 *Pfkelch13* genotype (median [IQR], 23110 parasites/ μ l [2000 – 65820] vs 55350 parasites/ μ l [12300 – 224600], $P = 0.0207$; Figure 3.2B). Equally, there was a difference in the parasite density between the infections having the 142NN insertion and K189T mutation groups (median [IQR], 23110 parasites/ μ l [2000 – 65820] vs 46920 parasites/ μ l [20060 – 107820], $P = 0.0445$).

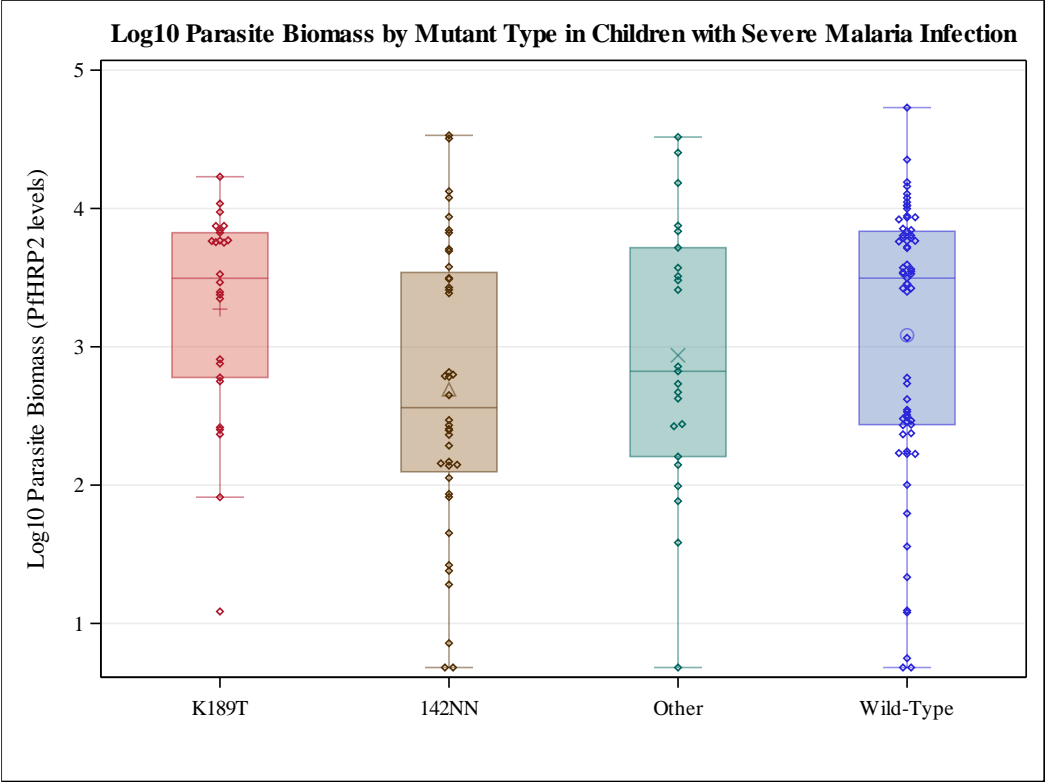
Table 3.6 Characteristics of children with K189T, 142NN, Other Mutants, Wild-Type (3D7) in children with severe malarial infection					
	K189T (n =26)	142NN (n =41)	Other Mutants (n =23)	Wild-Type (n =67)	P^a
Age (months), Median (IQR)	35.9 (23.0 -48.7)	41.1 (28.1 – 57.6)	36.5 (30.5 – 57.8)	39.3 (25.0 – 49.7)	0.7755
Sex (male), n (%)	19(73)	33 (80)	14 (61)	38 (57)	0.064
<i>P. falciparum</i> peripheral blood density (parasites/μl), median (IQR)	46920 (20060 – 107820) (n =25)	23110 (2000 – 65820) (n =40)	52800 (3560 – 328180)	55350 (12300 – 224600) (n =66)	0.0989 ^b
Total Parasite Load (PfHrp2)(ng/ml), Median (IQR)	3134.4 (600 – 6684.2)	370.8 (125.5 – 3463.2) (n =40)	664.9 (160.8 – 5206.9)	3134.4 (273.6 – 6842.4)	0.0703 ^b
Parasite clearance time (hours), median (IQR)	24 (24 – 48) (n =19)	24 (24 – 48) (n =37)	48 (48 – 72) (n =17)	48 (48 – 72) (n =51)	0.0003 ^c
Prior Artemisinin exposure, n (%)	1 (5) (n =22)	7 (18) (n =39)	4(18) (n =22)	12(20) (n =61)	0.422
Deaths, n (%)	4(15)	1(2)	4(17)	6(9)	0.166

^a Kruskal – Wallis Test was performed. Chi-squared test was used for categorical variables.
^b For differences between groups having a P<0.10, we performed exploratory post-hoc analysis.
^c Log-rank Test for equality of survivor functions was performed between the groups. Children who died were censored.
Abbreviations: PfHrp2 (*Plasmodium falciparum* Histidine Rich Protein -2); IQR (Interquartile Range); n (Number).

3.5 Frequency distribution of parasite clearance times in children with severe malaria (CM and SMA)

57.89% of the children with an infection (CM and SMA) harboring the K189T mutation in the *Pfkelch13* gene cleared the parasites within 24 hours (Figure 3.3A and Figure 3.3B). Similarly children infected with the parasite carrying the 142NN insertion (64.86%) in the *Pfkelch13* gene also cleared the parasite in 24 hours (Figure 3.3A and Figure 3.3B). In contrast only 29.41% of the infections with the wild-type (3D7 strain) for the *Pfkelch13* gene cleared in 24 hours (Figure 3.3A and Figure 3.3B), while 47.06% of the infection took 72-96 hours to clear. All the other mutations identified on the *Pfkelch13* gene were grouped together and only 29.41% of them cleared the parasite in 24 hours. The clearance times for the other mutants are shown in Appendix 1 (Figure 4.1).

A



B

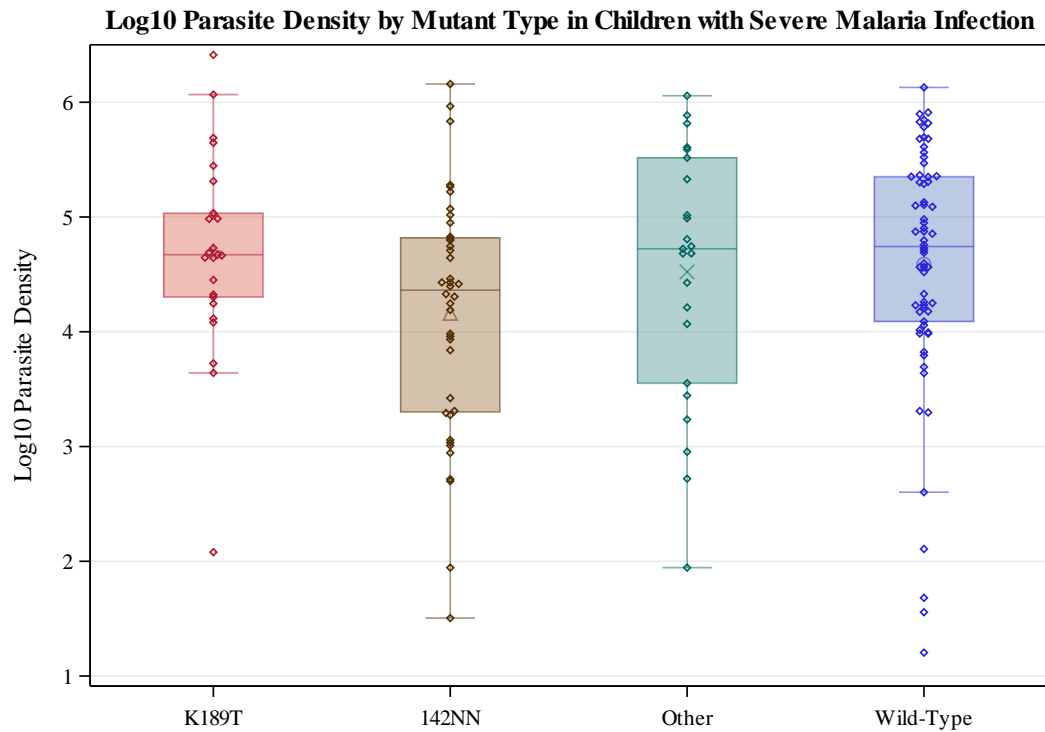


Figure 3.2.A Difference in the parasite biomass in children with severe malaria

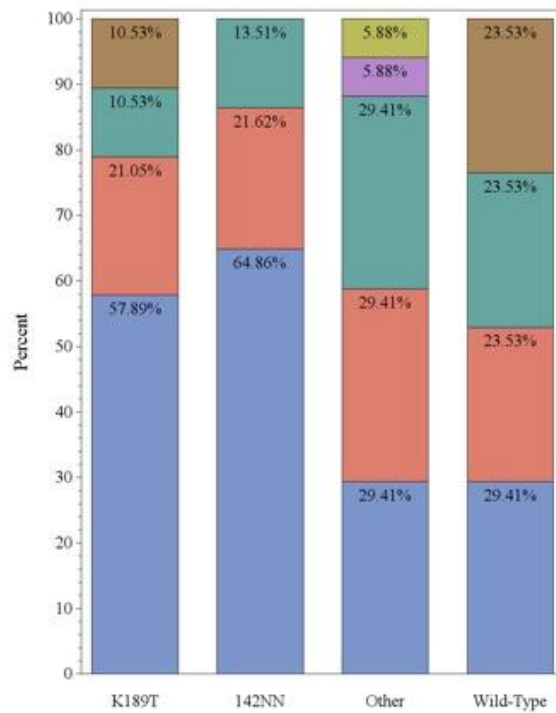
Differences in the parasite biomass (PfHRP2) (transformed log to the base 10 in children with severe malaria between 142NN and wild-type ($p=0.0307$); and between K189T and 142NN

Figure 3.2.B Difference in the parasite density in children with severe malaria

Difference in the parasite density (transformed log to the base10) in children with severe malaria between 142NN and wild-type($p=0.0207$); and between K189T and 142NN

Other: K189N, N142D, L258M, R255K, 142N insertion, N132S, A578S, K189T & Q271H

Frequency of Parasite Clearance Time by Mutant Type in Children with Severe Malaria Infection



Parasite Clearance Time (Hours) 24 48 72 96 120 144

Figure 3.3.A Differences in the frequency of parasite clearance times between infections with K189T, 142NN, Other mutations and wild-type
 Other: K189N, N142D, L258M, R255K, 142N insertion, N132S, A578S, K189T & Q271H

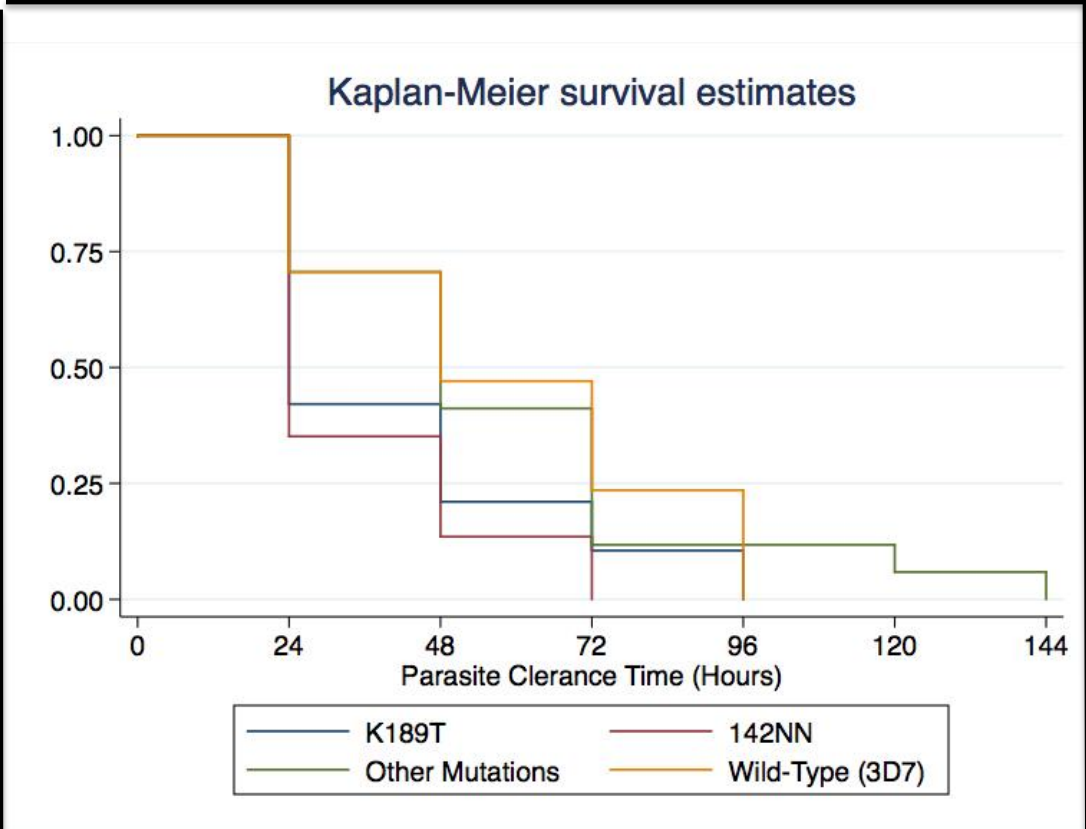


Figure 3.3.B Kaplan-Meier survival analysis of different types of mutations

Parasite clearance times for infections with different types of mutations: K189T, 142NN, other mutations and wild-type. More than 50% of the infections with the K189T and 142NN mutations cleared within 24 hours. Some infections with other mutations cleared parasites in >96 hours.

The log - rank test for equality of survivor functions between the groups was significant with a p value of 0.0003. The children who died were censored during the analysis.

DISCUSSION

Most studies have looked at mutations in the *Pfkelch13* associated with delayed parasite clearance upon artemisinin treatment. In the CMR01 study conducted between 2008 and 2013 in Uganda, children with severe malaria were treated with quinine upon enrollment. In this preliminary study conducted to investigate the prevalence of *Pfkelch13* mutations in the CMR01 study. Treatment of malaria using artemisinin and its derivatives has led to the emergence of *P. falciparum* mutants with delayed clearance times in SEA. Parasite strains showing delayed clearance times correlated with *in vitro* assays after artemisinin treatment [72]. Study conducted by Hawkes *et al*, found that the presence of the mutation A578S in the *Pfkelch13* gene was associate with delayed parasite clearance (>72hours) in children with severe malaria treated with artesunate: the three children with delayed parasite clearance times all had the A578S mutation [52]. This A578S mutation has previously been observed in *P. falciparum* malaria in Ethiopia and Bangladesh, but has not been associated with prolonged parasite clearance time with ACT treatment [50, 69]. In our study we found only a single infection had the presence of A578S mutation in the severe malaria group and had a parasite clearance time of 72 hours upon quinine treatment. A recent research study assessing mutant parasites with the A578S mutation showed that these mutants did not confer *in vitro* resistance to artesunate [71]. Our study could not test for resistance to artesunate since the children were treated with quinine. The mutations A578S was present at low frequency (2/206 infections). Future studies will need to determine if the A578S mutation is present more frequently in children who are treated with artesunate for severe malaria, and if there is any evidence in these cohorts to support the association between A578S and delayed parasite clearance time.

Our sequencing results of the *Pfkelch13* gene did not provide any evidence of mutations associated with delayed parasite clearance in SEA [49, 50, 57, 60, 73] suggesting that the mutations previously associated with delayed parasite clearance in Asia have not become prevalent in our study area. This could be due to the recent introduction of ACTs as a drug to treat *P. falciparum* related malaria in Africa. Furthermore, among our asymptomatic community controls we found two children with infections carrying the A578S and S522C mutation each. The S522C mutation has been previously reported in Uganda [74] however; *in vitro* studies have not been conducted using these mutants.

Studies conducted in SEA where ACTs have been used to treat malarial infections since the 1990s and were the first to report cases of delayed parasite clearance [38]. Delayed parasite clearance to artemisinin treatment was associated with mutation in the propeller domain of the *Pfkelch13* gene [49]. Furthermore, studies observed that mutations in the propeller domain of the *Pfkelch13* gene were associated with delayed parasite clearance, while mutations upstream of the propeller domain may not have a role in delayed parasite clearance times with artemisinin treatment [50].

The two most prevalent mutations were the K189T and the 142NN insertion in our cohort. These mutations have been identified previously in Bangladesh and Senegal [74, 75] where malarial infections were being treated with ACTs. These mutations were, however, not associated with delayed parasite clearance upon treatment with ACTs. In our study we found the children infected with the mutant parasite having the 142NN insertion had lower parasite biomass and lower parasite density as compared to infections with wild-type parasite. Upon administration of quinine the infections with K189T mutants and 142NN insertion cleared faster (24 hours) as compared to infections with wild-type parasite (48 hours). This faster clearance suggests that these mutant parasites might be more sensitive to quinine compare to wild-type strains. The lower parasite density and parasite biomass in 142NN mutant infections could indicate a possible fitness cost associated with these parasites. To

the contrary this could also suggest higher virulence associated with these strains, despite low parasite burden and parasite density were able to cause severe malaria. These were very interesting and unexpected results. The role-played by these mutations in the *Pfkelch13* gene and its association with quinine treatment have not been studied before.

Genes like *Pfmdr1*, *Pfnhe1* and *Pfcrt* that have been associated with efficacy of quinine treatment. *In vitro* studies conducted on strains of parasites with mutations in these genes that have shown reduced sensitivity to quinine treatment [44, 76-78]. Increased *Pfmdr1* copy number has been associated with decrease in quinine sensitivity [76]. Previous reports from Uganda have found a modest association between mutations in the *Pfmdr1* gene and quinine sensitivity [78]. The number of repeats in *Pfnhe* also determined the sensitivity to *in vitro* quinine treatment [77]. Other *in vitro* studies have also suggested mutations in the *Pfcrt* gene could also result in decreased quinine sensitivity [79]. Mutations in *Pfkelch13* could be in linkage disequilibrium with mutations in other genes that could affect the parasite clearance times upon quinine treatment.

Naturally acquired host immunity is another key factor to consider in parasite clearance times. Multiple infections eventually lead to a much better immune response to malaria infection, but do not lead to sterile immunity. Immunity to malaria parasite in individuals in regions of high transmission is more pronounced as compared to individuals from medium or low transmission areas. These factors could also contribute to faster parasite clearance times in our cohort.

With the recent adoption of ACTs as the first line of treatment in most countries in Africa, surveillance of *Pfkelch13* mutations is required to stymie the spread of mutant strains associated with delayed parasite clearance times with artemisinin. There have been few reports of mutations in the propeller domain of the *Pfkelch13* gene in Mali, Ethiopia and Uganda [52, 53, 55], however, in very low frequency.

Further investigation in the role played by other mutations in the *Pfkelch13* gene using *in vitro* assays might provide evidence regarding parasite clearance times and use of ACTs in malarial treatment. Similarly, investigating other mutations in other genes might help in understanding the various proposed mechanism in delayed clearance to artemisinin treatment.

Appendix A

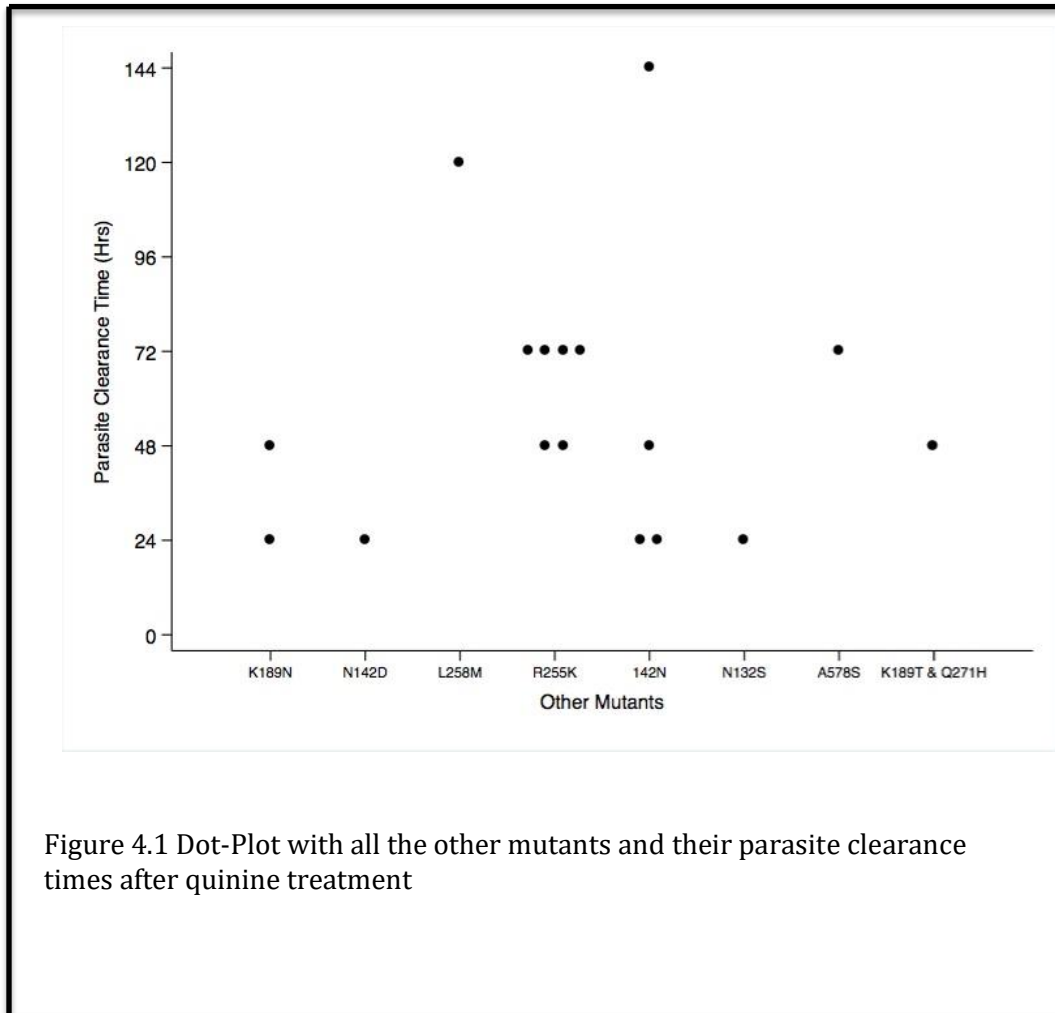


Figure 4.1 Dot-Plot with all the other mutants and their parasite clearance times after quinine treatment

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CURRICULUM VITAE

Adnan Gopinadhan

Education:

Master's Degree in Molecular Genetics

University of Leicester (UK)

Research Project: Evaluation of Meningococcal Carriage Isolates for Genetic Variation in a Family of Phase Variable Surface Proteins.

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Bachelor's Degree in Technology (Biotechnology)

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Research Project: Determination of oxytetracycline from edible Prawns using validated HPLC method.

- Supervised by Dr. Sasikumar Menon (Assistant Director-Therapeutic Drug Monitoring Lab. Mumbai, Ind.)

Presentation & Conferences:

- Presented a poster at Midwest Neglected Infectious Diseases Meeting, University of Notre Dame, USA (27th – 28th August 2017)
- Presented a poster at Indiana Biology of Intracellular Pathogen Retreat at IUSM, USA (23rd June 2017)
- Presented a paper on “Evaluation of Meningococcal Carriage Isolates for Genetic Variation in a Family of Phase Variable Surface Proteins” at the University of Leicester, UK (3rd Aug 2009).
- Presented research papers on *Neisseria meningitides* at Lab Meetings during my Masters Course at the University of Leicester, UK (19th May 2009).
- Participated in the International symposium on Model Organisms and Stem Cells in Development, Regeneration and Disease, organized by NCBS, TIFR, Bangalore, India (23rd - 25th Feb 2008).

- Presented a research paper on “Detection of oxytetracycline in Aquaculture Prawns using a validated HPLC method” at DY Patil University, India (23rd Aug 2007).
- Attended a two day seminar on sector wise analysis of Indian Biotech Industry, organized by S.I.E.S. College of Management Studies, (20th – 21st Sep 2006).
- Participated in a four days training program in Applied Genetics and Genetic Counseling organized by Center for Research in Mental Retardation (CREMERE) (31st Aug – 3rd Sep 2005).
- Participated in a two days 2nd Global Medical Forum Symposium Advances in Genetic aspects, Diagnosis and Treatment of GI and Breast Cancer, organized by Jas Lok Hospital and Hinduja Hospital in collaboration with Stanford University (1st – 2nd Oct 2005).
- Attended one-day seminar on Animal cell culture, organized by Vivekanand Education Society’s Department of Microbiology, (27th Nov 2004).

Awards:

- Won a poster award at Midwest Neglected Infectious Diseases Meeting held at the University of Notre Dame in August 2017.