Chapter 1: Introduction

1.1 Malaria Overview

1.1.1 Epidemiology

Malaria remains a global public health burden with the highest mortality rate amongst vector-borne diseases, affecting mostly children under five years of age making it the leading cause of child deaths, with an estimated 229 million cases and an estimated 400,000 deaths recorded in 2019 alone (World Health Organisation, 2020; Patriani *et al,* 2019). The disease is transmitted by the female *Anopheles* mosquito and caused by several species of *Plasmodium* protozoan parasites in humans including *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale and Plasmodium knowlesi* (Rocamora and Winzeler, 2020; Su *et al,* 2020). *P. falciparum* remains the most virulent amongst the species of the genus *Plasmodium* accounting for the majority of severe cases and malaria-related deaths in endemic areas (Haldar *et al,* 2018; Kapesa *et al,* 2018).

Transmission was reported to occur in 87 endemic countries in 2019 in which ~94% of the cases are accounted for in the African region and ~3% in South East Asia, with half of the world's population at risk (World Health Organisation, 2020). Areas affected by malaria (Figure 1.1) are mostly low income, tropical and sub-tropical areas, with Africa been the most affected due to a combination of factors including insufficient resources, political and socio-economic instability, which are bottlenecks in implementing efficient malaria control projects (Centres for Disease Control and Prevention, 2020; Fletcher *et al,* 2020).

There has been a significant reduction of malaria burden as a result of global efforts in implementing control intervention to address malaria morbidity and mortality

(Debebe *et al,* 2020). The Global Malaria Eradication Programme, initiated by the World Health Organisation (WHO) in 1955, was a vector control-based campaign using insecticide dichlorodiphenyltrichloroethane (DDT) and chemoprevention with chloroquine. This resulted in elimination of the disease in several parts of Europe, the Americas and Asia, but with no major success in sub Saharan-Africa (World Health Organisation, 2016). Other malaria elimination campaigns have been initiated towards the path to malaria elimination over the past two decades including the Roll Back Malaria initiative, the malaria-focused target of The Millennium Development Goals by the United Nations (UN) and Medicines for Malaria Ventures (MMV) with the aim of dramatically reducing malaria morbidity and mortality (Roll Back Malaria, 2018; Rowe 2017; Wells *et al* 2015). The global increase in intervention has led to a significant decline in malaria mortality by 25% from 2010 to 2016, saving millions of lives (Centre for Disease Control, 2020)

However, the burden of malaria remains high in many endemic areas despite the substantial decline in global spatial distribution and burden since 2000 with over 90% of people within sub-Saharan Africa residing in endemic areas (Weiss *et al,* 2019).

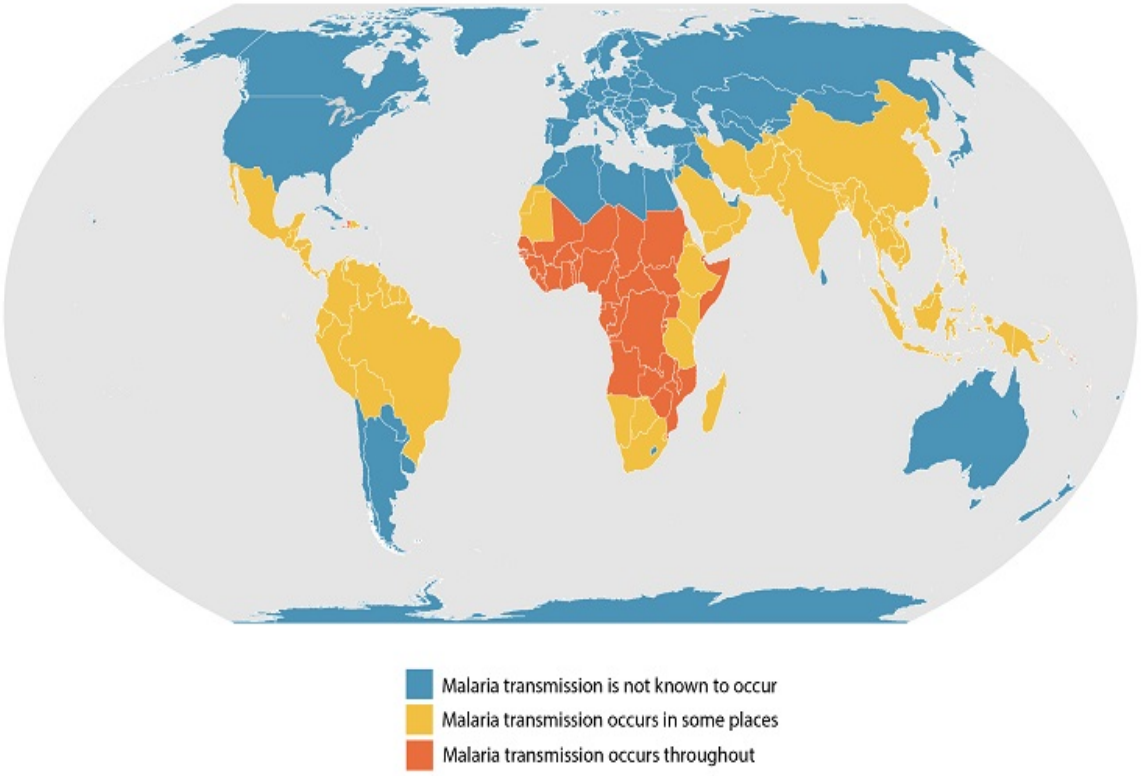

Figure 1.1 Malaria global distribution map showing an approximation of regions of the world with malaria transmission. Figure reproduced from Centres for Disease Control (2020).

1.1.2 Transmission and Vector

Malaria is a vector-borne parasitic disease transmitted by female mosquitoes of several species of the subfamily *Anopheles* during the bloodmeal stage of the reproductive cycle of the mosquito (Liu *et al* 2020; Singh *et al,* 2020). The prevalence of the different *Anopheles* species varies by geographical location with the major vectors being *Anopheles gambiae sensu lato* and *Anopheles funestus.* These are the major species in sub-Saharan Africa where the majority of malaria cases occur with their anthropophilic habits making them a major public health problem (Dahalan *et al,* 2019: Emami *et al,* 2017; Sinka *et al,* 2010).

The difference in behaviour, ecology, physiology and in morphological characters of the mosquito vectors are used for their identification. A combination of these differences is used as criteria to help define species and are the major source of distinction for species identification (Sallum *et al,* 2020). Transmission of parasites to the host by the mosquito occurs during feeding, in which sporozoites are transmitted to the host and sexual forms (gametocytes) are ingested by the mosquito with the blood meal (Yamamoto *et al,* 2016).

1.1.3 *Plasmodium*

Plasmodium spp. which are protozoan parasites belonging to the phylum Apicomplexa are the fundamental agents of malaria with five species of the *Plasmodium* genus causing infection in humans (de Koning-Ward *et al,* 2016). The species that are able to infect humans are *P. vivax, P. malariae, P. knowlesi, P. falciparum* and *P. ovale* which comprises of two genetically variant sympatric species, *Plasmodium ovale curtisi* and *Plasmodium ovale walikeri* (Miller *et al,* 2015). Amongst these species that infect humans, *P. falciparum* has been seen to be the most prevalent and virulent accounting for the majority of severe cases and malaria related mortality (Murphy *et al,* 2020).

1.1.3.1 *Plasmodium ovale*

Plasmodium ovale was first reported as a human-infecting species in 1922 and is geographically distributed across sub-Saharan Africa, the Western Pacific, Timor, and Indonesia with a higher prevalence reported in Nigeria and Papua New Guinea (Kotepui *et al,* 2020). *P. ovale* infections are rarely seen outside of the African region with relatively lower frequency in comparison with other species, thus severity of *P.*

ovale cases are rarely reported (Okafor and Finnigan, 2020). The occurrence of dormant forms of *P. ovale* (hypnozoites) in human infections remains unestablished (Markus, 2015). The two sympatric ovale species, *P. ovale curtisi* and *P. ovale wallikeri* are distinguished by genetic typing but morphologically identical by microscopy (Xia *et al.* 2020).

1.1.3.2 Plasmodium malariae

Plasmodium malariae was first reported as a malaria causative agent in 1880, and occurs in similar geographical locations as *P. ovale* mostly in sub-Saharan Africa, South America, Indonesia, Southeast Asia, and the western Pacific (Collins and Jeffery, 2007). *P. malariae* mono-infections are mostly benign and rarely related to severe cases or mortality, although it mostly occurs as mixed species infections with *P. falciparum* with a higher prevalence of *P. falciparum* than *P. malariae* in these mixed infections. *P. malariae* infections has been associated with long term low-grade chronic infection and nephropathy and anaemia (Yman *et al,* 2019; Lo *et al* 2017).

1.1.3.3 Plasmodium vivax

Plasmodium vivax is the most geographically widespread species and the second primary malaria causative agent in humans that occurs at a higher prevalence in tropical, sub-tropical and temperate zones, mostly in Asia and Latin America although also occurring at significant levels in some parts of Africa (Elgoraish *et al* 2019; Sitali *et al,* 2019). *P. vivax* is not benign despite the high frequency of asymptomatic infections as well as relatively low prevalence estimation and low parasite densities in the peripheral blood (Battle *et al,* 2019; Howes *et al,* 2016). One of the main biological characteristics of *P. vivax* is the formation of undetectable

dormant liver stages known as hypnozoites. These forms cause repeated infection relapses that can occur within weeks to several years of primary inoculation thus posing a big challenge for control and elimination of this species (Twohig *et al,* 2019). The low prevalence of *P. vivax* in Sub-Saharan Africa has been linked to the absence of the Duffy antigen receptor, in the majority of that population, which is a crucial red blood cell receptor for the invasion of *P. vivax* into erythrocytes. However, there have been increasing reports of *P. vivax* infections occurring in Duffy-negative individuals and populations indicating the use of alternate receptors for erythrocyte invasion (Popovici *et al,* 2020; Mendes *et al,* 2011). *P. vivax* has been observed to be less virulent in Duffy-negative Africans in comparison to the Africans harbouring the Duffy antigen receptor (Golassa *et al,* 2020).

1.1.3.4 Plasmodium knowlesi

Plasmodium knowlesi malaria is now classified as a zoonotic disease but was initially strictly associated with simian infection of the long-tailed macaque (*Macaca fascicularis*), pig-tailed macaque (*Macaca nemestrina*), and the banded-leaf monkey (*Presbytis melalophos*), which are the parasite's natural reservoir hosts (Amir *et al,* 2018; Singh and Daneshvar, 2013). The first successful experimental infection of 3 human volunteers with *P. knowlesi* malaria by Knowlesi and Das Gupta in 1930, with the first case of a natural infection of *knowlesi* malaria in humans reported in 1965 in an American tourist that travelled to Peninsular Malaysia (Wesolowski *et al,* 2015; Singh and Daneshvar, 2013). Since the landmark report of a large number of *P. knowlesi* cases in Malaysia, in 2004, there were increasing records of the Simian *P. knowlesi* human infections across South East Asia (Zaw and Lin, 2019; Imai *et al,* 2014). The increasing number of *knowlesi* malaria cases are associated with the

application of molecular methods for diagnosis, which addressed the challenge of misdiagnosis of *P. knowlesi* as *P. malariae*, as well as deforestation resulting in increased exposure of humans to vector mosquitoes that coexist with the macaque (Jeyaprakasam *et al* 2020; Davidson *et al,* 2019; Imai *et al,* 2014). As a result of the occurrence of the natural hosts in these regions, *P. knowlesi* is suspected to be geographically limited to Southeast Asian countries (Scott, 2020). Similar to *P. vivax, P. knowlesi* also invades the erythrocytes with the use of the Duffy antigen receptors. This invasion pathway is likely a contributory factor for the absence of *P. knowlesi* in the highly Duffy-null African population, in combination with the absence of the reservoir Macaque monkeys in Africa (Muh *et al,* 2018; Onyedibe *et al,* 2016). However, the ability of *P. knowlesi* to exploit alternate mechanisms for erythrocyte invasion is unclear (Antinori *et al,* 2012).

1.1.3.5 Plasmodium falciparum

Plasmodium falciparum is less geographically widespread than *P. vivax* but remains the most virulent amongst the five *plasmodium* species that causes malaria in humans, accounting for the majority of malaria morbidity and mortality (Nureye and Assefa, 2020). The significantly higher incidence of malaria in the WHO African region compared to the other parts of the world is as a result of the predominance of *P. falciparum* in sub-Saharan Africa (Battle *et al,* 2019). Severe complications which can be fatal including coma, transient or permanent neurological effects as a result of cerebral malaria is a major complication of *P. falciparum.* Issues in organs such as lungs, kidneys caused by parasite adherence to tissue cells of different stages is also a major complication of *P. falciparum* (Patel *et al,* 2020; Brazier *et al,* 2017; Rénia *et al,* 2012). The occurrence of mixed infections of multiple distinct strains of *P.*

falciparum species as well as co-infection with other plasmodium species, mostly *P. malariae* and *P. vivax* in hyperendemic regions linked to severity and treatment failure has been reported (Hossain *et al,* 2020; Kotepui *et al,* 2020; Zhu *et al,* 2019).

1.1.4 Life Cycle of *Plasmodium falciparum*

The *P. falciparum* life cycle shown in figure 1.2 involves two hosts, both the intermediate human host and the mosquito vector. The life cycle passes through three major stages (pre-erythrocytic stage, erythrocytic stage and the vector stage), and clinical symptoms are associated with continuous rounds of asexual replication in the blood (Toro-Moreno *et al,* 2020; Venugopal *et al,* 2020). The infection starts with the injection of variable amounts of motile sporozoites from the salivary glands of the mosquito into the bloodstream when an infected female *Anopheles* mosquito is taking up a blood meal from the host, marking the initiation of the pre-erythrocytic stage of the cycle (Vaughan *et al,* 2012).

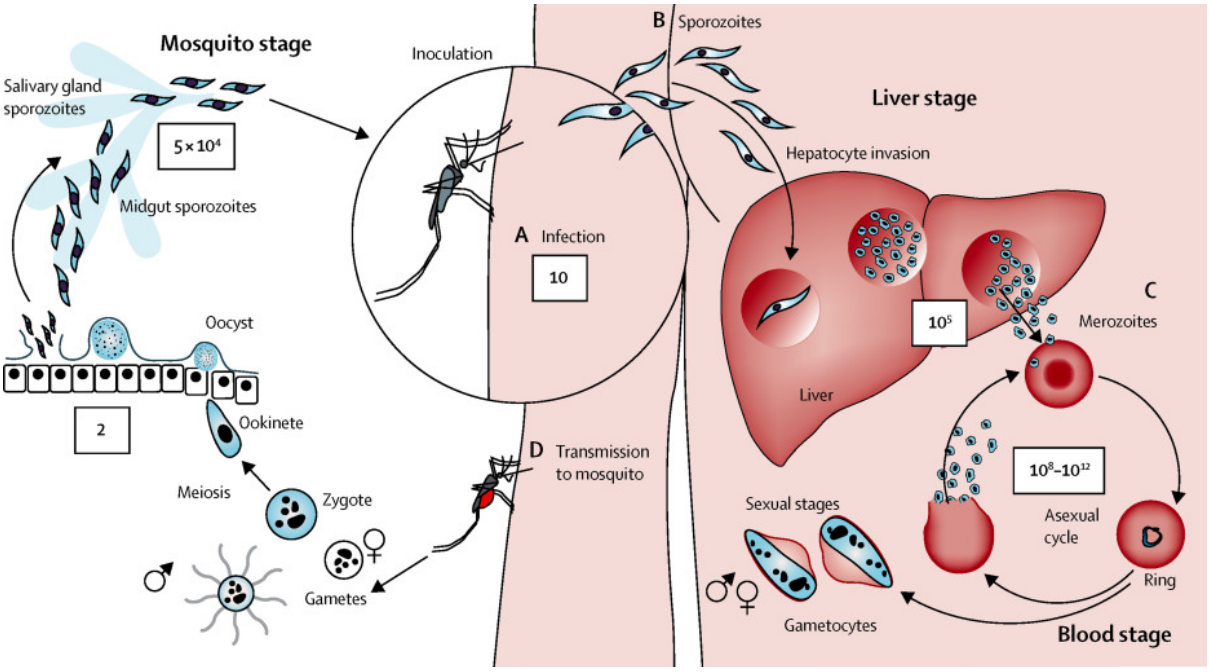

Figure 1.2 Life cycle of *Plasmodium falciparum*. The three main developmental stages of the life cycle: **(A) Pre-erythrocytic stage**: Injection of sporozoites that (**B**) migrate to the liver to generate liver-stage merozoites. **(C) Erythrocytic stage:** Merozoites released into the bloodstream invade RBCs and undergo several rounds of asexual multiplication progressing

through ring, trophozoite and schizont stages. Less than 10% of parasites develop into sexual forms (gametocytes) of the parasite during the erythrocytic stages. (**D**) **Mosquito stage:** Mature gametocytes are taken up by the mosquito, progressing through several developmental stages after fertilisation. Adapted from White *et al* (2014).

1.1.4.1 Pre-Erythrocytic Stage

The sporozoites deposited into the skin by the mosquito migrate to the liver, through the blood circulation, where they invade and develop in the liver cells known as hepatocytes (Osii *et al,* 2020; Putrianti *et al,* 2020). Whilst inside the liver cells, the parasite goes through intra-hepatocytic replication, maturing into hepatic schizonts to produce invasive exoerythrocytic merozoites through a process known as schizogony. (Winer *et al,* 2020; Vaughan and Kappe, 2017; Prudêncio *et al,* 2006).

1.1.4.2 Erythrocytic Stage

During schizogony, merozoites produced after the initial reproductive stage in the liver are released into the bloodstream. These specialised free invasive forms of the parasite of around 1μm in size rapidly invade red blood cells (RBCs) in a multi-process step that involves different phases following initial adhesion to cell membrane through to forming the parasitophorous vacuole membrane (PVM) when the parasite finally enters the host RBC (Blake *et al,* 2020; Paone *et al,* 2020). Interactions between specific parasite ligands and host receptors defines the specific molecular invasion pathway that mediates the invasion of erythrocytes, a key step in parasite growth (Cai *et al,* 2020). The merozoites that successfully invade RBCs develop into ring forms that last for 24 hours before transitioning into trophozoites when RBC contents are taken up, developing a digestive food vacuole visible by microscopy. The trophozoites eventually mature after 12 hours into schizonts after the occurrence of several nuclear divisions. These matured segmented schizonts with multiple nuclei rupture and

release between 16 to 32 daughter merozoites which then invade new RBCs marking the beginning of another 48-hour cycle (Burns *et al,* 2019; White *et al,* 2014). During the different stages of development, the *Plasmodium* parasite takes up haemoglobin, as a source of amino acids, which is digested in the digestive food vacuole of the parasite. This leads to the release of iron-containing heme molecules which are toxic to the parasite and are thus detoxified and made inactive by crystallisation into the malaria pigment, hemozoin under the acidic conditions of digestive vacuole (Kapishnikov *et al,* 2019; Pishchany and Skaar, 2012; Dluzewski *et al,* 2008). The host RBC is drastically remodelled as the intracellular parasites grow and replicate resulting in deformation of the cell which is when clinical symptoms are manifested as a result of repeated rupture of RBCs. Diagnosis occur and parasite clearance by antimalarials is targeted at this stage (Amoah *et al,* 2020; Burns *et al,* 2019; Cowman *et al,* 2012). A relatively low proportion (0-20%) of the blood stage parasites differentiate into sexual forms of gametocyte-committed (gc)-ring-stage parasites. These gc-ring-stage parasites go through five morphologically distinct stages (I–V) (Figure 1.3) to become a mature, transmissible stage V gametocyte (Figure 1.3) over a duration of ~8–12 days (Prajapati *et al,* 2020; Venugopal *et al,* 2020). Progression through the developmental stages of gametocytogenesis that leads to the mature infectious sexual form (gametocyte) is driven by a combination of genetic, epigenetic and environmental factors. The presence of *gametocyte development protein 1 (gdv1)* and the *apetela-2 transcription factor (ap2-g)* have been reported to be required in initiating this sexual phase from asexual stages (de Jong *et al,* 2020; Usui *et al,* 2019).

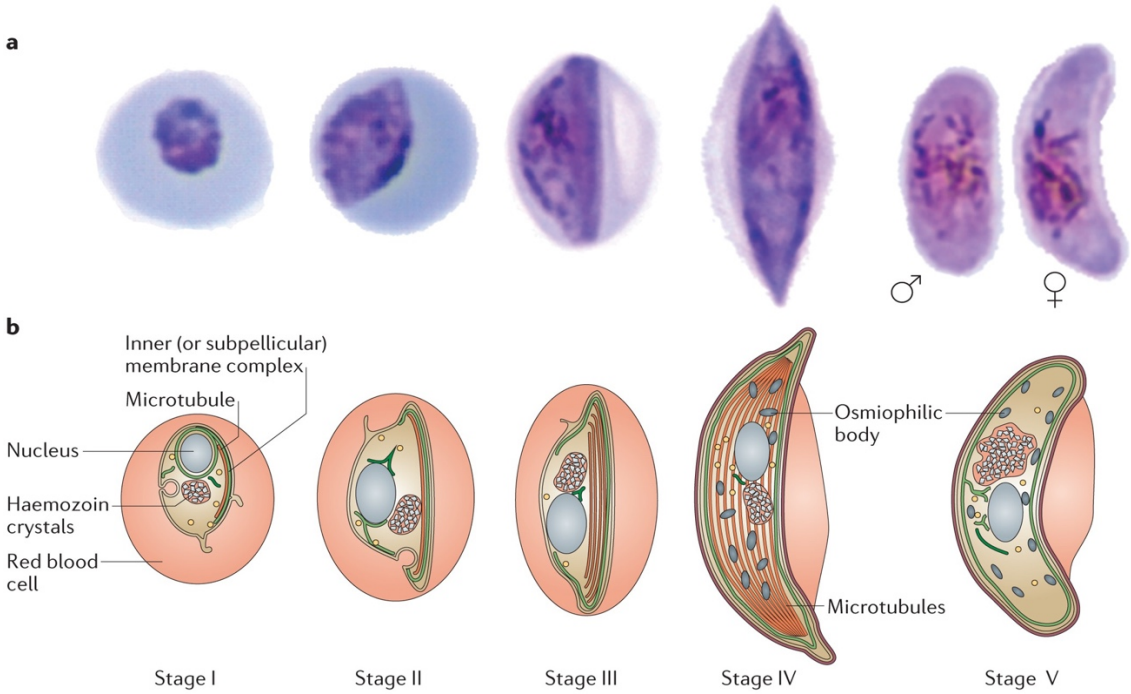

Figure 1.3 Development of mature *Plasmodium falciparum* sexual forms (gametocytes). Gametocytes develop through five distinct morphological stages during the blood stages of the life cycle. Gametocytes differentiate into male and female forms, that are transmissible to mosquitoes at the last stage. Adapted from Josling and Llinás (2015).

1.1.4.3 Mosquito Stage

Sexual differentiation in the blood to form male and female gametocytes during gametocytogenesis is required for transmission of parasites from the human host to the mosquito, and thus is a vital stage in continuity of the transmission cycle. Gametocytes ingested by mosquitoes during a blood meal from the human host mature into gametes, a process that is triggered by environmental factors in the mosquito midgut including exposure to xanthurenic acid, a change in pH, and temperature drop (Josling and Llinás, 2015). Activation and fertilization of male and female gametes occurs forming a zygote that develops into motile ookinete after 16 to 18 hours through meiosis. The mosquito midgut epithelium is invaded by the ookinete that matures into an oocyst on the midgut basal side. This oocyst then produces thousands of sporozoites through asexual replication, which are released and migrate

into the salivary gland of the mosquito enabling their transmission to another human host during a blood meal (Volohonsky *et al,* 2020; Ngotho *et al,* 2019; Josling *et al,* 2018).

1.1.5 Pathogenesis

The clinical manifestations of *P. falciparum* have been associated with the asexual erythrocytic stages of the life cycle which can result in either asymptomatic, uncomplicated or severe malaria (Colvin and Joice Cordy, 2020; Zekar and Sharman, 2020). The common unspecific clinical symptoms displayed as a result of host responses include headache, myalgia, dizziness, fever and chills, which may sometimes progress to severe malaria (Su *et al,* 2020). These manifestations are driven by multiple factors including parasite strain and densities in an infected individual, host genetic susceptibility and resistance (immunogenicity) (Loiseau *et al* 2019; Laishram *et al,* 2012; Mendoca *et al* 2012). Development of symptoms in *P. falciparum* infections occur after 3 to 4 cycles when a threshold of 6 to 20-fold exponential increment of parasitaemia per cycle is reached. These symptoms are manifested after an incubation period of 11-15 days, which varies in other species (White *et al,* 2017). Periodic fever spikes at 48-hour intervals correspond to schizont bursting at the end of the erythrocytic cycle of *P. falciparum*. Cycle length, which is different in other species, influences timing of these fever spikes that are one of the classical clinical manifestations (Smith *et al,* 2020; Bartoloni *et al,* 2012).

Variation of clinical manifestations have also been observed between children and adults although cerebral involvement, kidney dysfunction, and acidosis has been reported to be independent mortality predictors in both categories (Plewes *et al,* 2018). Severe malarial anaemia, cerebral malaria (CM), and respiratory distress syndrome

are the major clinical manifestations or symptoms that have been implicated in severe malaria (Mousa *et al,* 2020). The unique sequestration characteristic of *P. falciparum* infected RBCs to walls of different sized blood vessels is mainly associated with its high virulence compared to the other species (Siciliano and Alano, 2015). The sequestration is a mechanism that protects the parasitised RBCs from filtration by the spleen resulting in host endothelial cell injury and microvascular obstruction thus the development of severe malaria (Lee *et al,* 2019). This leads to severe forms of pathogenesis including cerebral malaria (coma), respiratory distress, severe anaemia, acute renal failure, jaundice, acidosis, hypoxia and hypoglycaemia, which can be fatal. These outcomes are triggered based on the organ involved such as brain, lung, kidney (Ashley *et al,* 2018). It has been reported that aggravated anaemia due to stringent splenic clearance of ring-infected RBCs and uninfected altered RBCs, is associated with reduced risks of severe complications such as cerebral malaria from high parasitaemia. This is as a result of retention of normal and ring-infected RBCs in the spleen, preventing the circulation of a subpopulation of rings to mature and sequester thus leading to reduced parasitaemia (Buffet *et al,* 2011).

Diverse clinical presentations have been observed in *P. falciparum* infections, even with the same individual over time including individuals carrying chronic parasitaemia who are reservoirs for transmission to mosquitoes, due to asymptomatic infection (Gonçalves *et al* 2017). Host genetic factors that have been associated with adaptations in asymptomatic or mild infections, and thus protection against severe malaria, include sickle cell disease (SCD), thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other red blood cell (RBC) genetic anaemia with Mendelian inheritance as well as blood group O of the ABO type (Cooling, 2015; Mendonça *et al,* 2012). Protection against severe malaria due to a significant reduction

in parasite densities is observed in some genotypic variants of these factors. This can result in flawed infected erythrocyte cytoadherence, rosette formation and enhanced infected RBC clearance, leading to decreased parasite invasion ability and growth hinderance (Acquah *et al,* 2020; Archer *et al,* 2018).

1.2 Malaria Control Measures

Due to the significant burden malaria inflicts on global socio-economic and public health, there has been continuous implementation of control measures towards reduction or elimination of morbidity and mortality of the disease over the past decades (Tizifa *et al,* 2018). Prevention of the infection by avoiding bites from mosquitoes carrying the parasites, and prevention of the disease with therapeutic use of antimalarial drugs remains the two major prophylactic strategies for malaria control (Skwarczynski *et al,* 2020). Thus, combination of several measures including the distribution of insecticide-treated bed nets (ITNs), indoor spraying of residual insecticides (IRS) and other vector control strategies; access to early diagnosis such as use of rapid diagnostic tests (RDTs); and more effective antimalarial treatments alongside targeted interventions such as intermittent preventive treatment in pregnancy (IPT) and seasonal malaria chemoprevention (SMC) have been in place by malaria control programmes (Nice *et al,* 2020; Wagman *et al,* 2020; Sy *et al,* 2019; White et al 2011). Development of vaccines for malaria has also increasingly been explored over the past decade (Nureye and Assefa, 2020; Rogerson *et al,* 2020). Current control and elimination campaigns against malaria include the Action and Investment to Defeat Malaria (AIM) by the Roll Back Malaria programme in alliance with the Global Technical Strategy for Malaria 2016 - 2030 (GTS). This Global Malaria Programme of the World Health Organization (WHO) is aimed at reaching reductions

of cases of at least 90% as well as elimination in up to endemic 35 countries by 2030 (Nsereko *et al,* 2020; Smith Gueye *et al,* 2016)

1.2.1 Vaccine Development

The enormous complexity of malaria parasite biology and infection cycle, the diversity of the complicated parasite genomes, as well as the ability to evade the host immune response presents a great challenge in the development of an effective vaccine for the highly virulent *falciparum* malaria (Mahmoudi and Keshavarz, 2018). Significant progress has been achieved generally in the efforts made towards the development of effective vaccines with approaches targeted at transmission-blocking, pre-erythrocytic and erythrocytic stages of the parasite life cycle (Molina-Franky *et al,* 2020; Frimpong *et al,* 2018). Vaccines targeting the erythrocytic stage of the parasite aimed at preventing disease development rather than infection have been explored. These vaccines have been developed based on the antigens expressed in the blood stage of infection including Merozoite Surface Proteins (MSP), Apical Membrane Antigen (AMA-1) and glutamate-rich protein (GLURP) (Skwarczynski *et al,* 2020; Pance, 2020). Approaches including synthetic peptides or recombinant proteins that combined sporozoite antigens with blood stages had varied results that were not promising.

The availability of the parasite genome revealed the new prospect of a wide range of malaria antigen-based vaccines (Penny *et al,* 2020, Hill, 2011). One of the first synthetic peptide-based vaccines developed against malaria based on pre-erythrocytic blood-stage fragments involving circumsporozoite protein is SPf66, which demonstrated partial efficacy in humans in South America but not against *P. falciparum* in African trials or in Thailand (Salamanca *et al,* 2019; Schwartz *et al,*

2012). Amongst the current vaccines in development targeting *P. falciparum* malaria, RTS,S/AS01 is the first vaccine that reached completed Phase III clinical trials in 2014. RTS,S/AS01 manifested a protective efficacy of 36% to 50% in young children aged 5 to 17 months and above in late-stage clinical trials. There is evidence of vaccine efficacy against clinical malaria episodes at a 4-dose scheme administered in 1-month intervals from 5 months and a booster dose at 25 months (Hogan *et al,* 2020; Duffy and Gorres, 2020; Mahmoudi *et al,* 2017). RTS,S is the most advanced pre-erythrocytic vaccine targeting *Plasmodium* sporozoites and liver stages aimed at sporozoite clearance from the skin and bloodstream or hepatocyte invasion blockage, as well as stimulating immune response against infected hepatocytes (Duffy *et al,* 2020; Draper *et al,* 2020). RTS,S includes the fusion of a central tandem repeat C-terminal fragment of circumsporozoite (CSP) protein (R) with T-cell epitopes (T) bound to hepatitis B (HBsAg) surface antigen (S) with S protein and adjuvant AS01/ASO2. However, adjuvant AS01 has displayed higher efficacy than adjuvant ASO2 (Sánchez *et al,* 2020; Neafsy *et al,* 2015).

A favourable outcome presenting a better cost-effective conclusion has been suggested by several clinical data that pre-erythrocytic and transmission-blocking vaccines could be incorporated in one construct thus the necessity for continuous investigation of multi-stage approaches (Yusuf *et al,* 2019). However, reduced immunogenicity and efficacy have been reported in some combination multi-stage malaria vaccine trials emphasising the need for further evaluation of alternative schedules or immunization sites for adequate efficacy (Rampling *et al,* 2018).

1.2.2 Vector Control:

Vector control remains a major essential component of malaria control and elimination programmes and has been an effective approach of preventing transmission and thus successfully impacting malaria morbidity and mortality to malaria and other vector-borne diseases (Wilson *et al,* 2020; Lobo *et al,* 2018). Control interventions targeting mosquito vectors mainly include the use of insecticide treated nets (ITNs) that involve the treatment of bed nets with insecticides, and indoor residual spraying of insecticides (IRS) which involves treatment of household walls with chemical insecticides (Choi *et al,* 2019). Other vector control strategies not as widely applied include larval source management (LSM) as well as host-mediated control such as zooprophylaxis in transmission settings involving zoophilic vectors and use of attractive toxic sugar baits that targets a toxin susceptible vector population. Mosquitoes are killed in the process of feeding on toxic sugar meals that they are attracted to, sprayed on plants or used in bait points (Lobo *et al,* 2018; Tusting *et al,* 2013).

Organochlorines, pyrethroids, carbamates, and organophosphates are the four classes of insecticides recommended for IRS by the Word Health Organisation (Yewhalaw *et al,* 2017). The use of dichloro-diphenyl-trichloroethane (DDT) and synthetic pyrethroids which are safe alternatives to DDT, have been the main active insecticides used in IRS and ITN distribution campaigns over the past two decades (N'Guessan *et al,* 2010). However, substitute insecticides with shorter environmental persistence such as carbamates and organophosphates, which are more expensive, are used in settings where widespread vector resistance to DDTs and pyrethroids is observed, thus making IRS approaches more complex and less cost-effective (Kané *et al,* 2020; Wanjala *et al,* 2015; Kleinschmidt *et al,* 2009). The use of carbamates and

organophosphates for IRS requires repetitive application due their short residual duration, thus resulting in high cost despite their rapid lethal effects. The use of combined organophosphate pirimiphos-methyl (PM) formulation and other recently developed long-lasting formulations which has demonstrated higher efficacy against pyrethroid resistant vectors and has been a countermeasure to address that limitation (Keita *et al,* 2021; Abong'o *et al,* 2020; Protopopoff *et al,* 2018; Ngufor *et al,* 2017; Hamainza *et al,* 2016).

The use of ITNs which targets the vector population that bites indoors and are susceptible to the insecticide-in-use have been observed to be most effective in protecting against late-night and indoor-biting vectors, demonstrating individual and community wide protection (Levitz *et al,* 2018; Lobo *et al,* 2018). The long-lasting insecticidal nets (LLINs) category of ITNs provides greater prolonged effectiveness due to the embodiment of insecticide into the fibers during manufacturing of the net. These LLINs are thus more effective than bed nets that are repetitively treated with insecticides at six-month intervals (Wangdi *et al,* 2018). Pyrethroid treated LLINs, which have pyrethroid as the main active ingredient, are the current standard across major malaria-endemic regions and are reported to have an estimated life span of 3 years and lasting after up to 20 washes (World Health Organisation, 2020; Fuseini *et al,* 2019). Although the independent implementation of LLINs and IRS has resulted in a dramatic reduction of morbidity and mortality, the combination of these two interventions should be explored further as data from large surveys and trials implementing the combination of the two strategies has suggested higher efficiency in incidence reduction (Guerra *et al,* 2020; Loha *et al,* 2019). However, eliminating malaria transmission in many settings, because of operational limitations, increasing resistance to available insecticides and behavioural evolution of mosquitoes to avoid

contact with these interventions, remains a great challenge for these strategies, despite the high efficiency of LLINs and IRS (Killeen *et al,* 2016). Moreover, insecticide resistance has been reported to all four classes of insecticides recommended by the WHO for vector control, with resistance to pyrethroids being the most compromised insecticide in vector resistance (Tangena *et al,* 2020; Fuseini *et al,* 2019; Wanjala *et al,* 2015). One of the major challenges in vector control programmes is the lack of knowledge on geographical distribution and spread of resistance as well as on molecular mechanisms of resistance, which recent developments in sequencing technology is progressively addressing (Clarkson *et al,* 2018). The issues of the negative environmental impact of insecticides has led to the increased development of vector control techniques that are safer and more suitable to the environment such as the use of biorational pesticides. This involves the use of natural or synthetic materials with insecticidal effects that are acquired from animals, plants, bacteria, or minerals on mosquito breeding sites (Ogunah *et al,* 2020).

The mass release of sterilised male mosquitoes is one of the environmentally safe tools that has received great attention. The sterilisation of male mosquitoes known as sterile insect technique (SIT) using radiology or chemicals prevents production of offspring after mating thus leading to a reduction in vector population (Gentile *et al,* 2015). However, limitations reported in this technique include partial sterility during transformation, competition from wild males and insignificant population decrease after successful sterilisation (Khamis *et al,* 2018; Maïga *et al,* 2014; Godfray; 2013). The application of transgenic sterility using genetic manipulation techniques such as CRISPR/Cas9 is another alternate approach. This method aims to introduce genetically edited mosquitoes with a dominant lethal gene that leads to the production of abnormal offspring after mating with wild-type females. Trials of this method have

been reported to lead to local population reduction, and is thus a potentially effective control strategy of insecticide resistant vectors (Simoni *et al,* 2020; Yamamoto *et al,* 2019; Kyrou *et al,* 2018; Catteruccia *et al,* 2009). Limitations of these techniques requiring continued investigation include the applicability in larger scale, and technical challenges such as Cas9-based off-target cleavage in CRISPR-based methods (Kandul *et al,* 2019; Hammond *et al,* 2016).

1.2.3 Antimalarials (Drug Discovery and Development)

The use of antimalarial drugs remains a key aspect in the control and elimination of malaria and can be applied for prevention of malaria using different strategies such as chemoprophylaxis, mass drug administration and intermittent preventive therapy (Cui *et al,* 2015). In combination with vector interventions, the extensive distribution of antimalarials has been highly effective in control of the disease and significantly reducing morbidity and mortality rates in highly endemic regions over the past two decades (Lubis *et al,* 2020; Khamis *et al,* 2018). The antimalarial drugs commonly used are categorised into different chemical classes including the antibiotics, antifolate compounds, quinoline-containing compounds, hydroxynaphthoquinone atovaquone and artemisinin and its derivatives. These antimalarials demonstrate differing modes of action and target different malaria life cycle stages (Deshpande and Kuppast, 2016). Antibiotics that have been in use for malaria chemoprophylaxis or in combination with other quinolines include doxycycline and clindamycin (Dahl and Rosenthal, 2008).

1.2.3.1 *Quinolines and Antifolates*

Several natural and synthesized quinoline compounds have been developed over the past years following the isolation of quinine in 1820, which was the first chemically purified compound effective for malaria treatment belonging to the quinoline class of antimalarials that was sourced from the cinchona tree (Tse *et al,* 2019; Golden *et al,* 2015; Raynes, 1999). The antimalarial activity of quinoline compounds has been reported to be exerted by their interference with heme detoxification by preventing the polymerisation of heme to hemozoin (Herraiz *et al,* 2019; Kapishnikov *et al,* 2019; Sullivan *et al,* 2002). Other quinoline-containing compounds have been developed based on the improvement and modification of quinine, comprising a class of heterocycles with varying lengths and nature of their basic amine side chains. These include 4-aminoquinolines such as chloroquine, amodiaquine and piperaquine, 8-aminoquinolines like primaquine, 4-quinoline methanols such as mefloquine, lumefantrine and quinine and hydroxynaphthoquinones such as atovaquone (Baird, 2019; Nqoro *et al,* 2017; Shreekant and Bhimanna, 2016; Bawa *et al,* 2010;).

The development and use of chloroquine in the 1940s, which is a 4-aminoquinoline drug that has been suggested to be trapped in the parasite food vacuole was a major advancement in treatment of malaria. These drugs were widely used for over 4 decades as the gold standard for malaria treatment due to the minimal host toxicity, high clinical efficacy, ease of use as well as cost effectiveness and simplicity of their synthesis (Kapishnikov *et al,* 2019; Cui *et al,* 2015; Bawa *et al,* 2010; Stocks *et al,* 2002). However, the widespread development of parasite resistance to chloroquine and other quinolines in various endemic regions has hindered that success and led to a significant reduction of their efficacy and use in malaria treatment

(Kumar *et al,* 2015; Rajapakse *et al,* 2015). Resistance to quinoline antimalarial drugs has been reported to be as a result of drug efflux out of the food vacuole caused by point mutations mainly on the genes of the membrane transporters *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance-1 (*pfmdr1*). Multiple single point mutations have been observed at codons of the *pfcrt* and *pfmdr* genes. *Pfmdr1* encodes for a P-glycoprotein homolog that is shown to localize to the membrane of the food vacuole, which is the site of action of a number of drugs (Lawrenson *et al,* 2018; Cui *et al,* 2015).

Another vastly used group of antimalarial drugs used in the treatment of malaria is the antifolates which targets enzymes of the folate pathway essential for survival of the parasites thus leading to decreased formation of pyrimidines, purines, and some amino acids. The two major enzymes inhibited by antifolates are dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) (Saifi *et al,* 2013). Antifolate antimalarials targeting these enzymes have extensively been in use over the past decades. These include sulfadoxine, pyrimethamine and proguanil, which are mostly used as a combination of two inhibitors of different targets or in combination with other classes of antimalarials (Posayapisit *et al,* 2020; Yuthavong *et al,* 2014). Development of resistance to antifolates results from point mutations at several codons in the *pfdhfr* and *pfdhps* genes, which have been reported in various endemic regions. These mutations have been implicated in the development of resistance to the most used antifolate combination sulfadoxine-pyrimethamine (SP), a treatment that was effective against chloroquine resistant malaria (Sugaram *et al,* 2020; Haldar *et al,* 2018; Cui *et al,* 2015; Karema *et al,* 2009; Mkulama *et al,* 2008). SP is still widely used in IPT in pregnancy, SMC in children of sub-Saharan Africa,

chemoprophylaxis and in combination with other antimalarial classes (Amambua-Ngwa *et al,* 2018).

1.2.3.2 Artemisinin

Artemisinin and its derivatives, which are sesquiterpene lactones, currently remain the frontline treatment of malaria for the past decades due to their highly potent activity against parasites that are resistant to previously developed antimalarial drugs (Abood *et al,* 2017). This class of antimalarial compound is characterised by an unusual endoperoxide bridge that exists in the natural extract, artemisinin, which was isolated from the sweet wormwood (*Artemisia annua*) plant which has been used for fever treatments in China, or the semi-synthesised derivatives (Czechowski *et al,* 2020; Xie *et al,* 2020). Artemisinin was discovered to be highly effective in rapidly reducing parasitaemia. The widespread use of this drug in all regions over the past two decades has led to a significant reduction in malaria mortality (Rathmes *et al,* 2020; Shibeshi *et al,* 2020; Lagarce *et al,* 2016). Although a precise mode of action is still being discussed, the antimalarial activity of artemisinin has been reported to include cleavage of the endoperoxide bond of artemisinin from interaction with heme in the parasite food vacuole, leading to drug activation. This results in the alteration of developing intraerythrocytic parasite proteins and lipid molecules by carbon-centred radicals and reactive oxygen species produced by the activated artemisinin compound (Marapana and Cowman, 2020; Shibeshi *et al,* 2020; Xie *et al,* 2020; Hoppe *et al,* 2004). Other potential modes of action that have been identified in recent studies include interaction of haem and PfATP6 which is a Ca2+ transporter, the unfolded protein response (UPR) pathway in which upregulation was shown to associated with artemisinin and linked to reduced parasite development, as well as the lipid kinase

phosphatidylinositol-3-kinase (*Pf*PI3K) (Tse *et al,* 2019; Mbengue *et al,* 2015; Mok *et al,* 2015; Shandilya *et al,* 2013).

Artemisinin derivatives have shown great efficacy as rapidly acting antimalarials and include artemether, artesunate and dihydroartemisinin. Like artemisinin, these derivatives are widely used mostly in combination therapies due to their short half-lives (Rosenthal, 2003). There has been significant reduction in malaria morbidity and mortality, due to the extensive distribution of artemisinin-based combination therapies (ACTs), combined with other control programmes, particularly in Africa (Lubis *et al,* 2020). However, the increasing reports of *P. falciparum* resistance to artemisinin and several partner drugs currently in use in ACTs in several endemic regions poses a global threat. Thus, there is a need for continuous surveillance and a renewed approach to management. As discussed below, various polymorphisms for drug resistance-associated molecular markers in *P. falciparum* genome have been identified. Distinct profiles of these polymorphisms are observed in different endemic regions including molecular markers associated with the resistance to the current frontline antimalarial (ACTs) (Bwire *et al,* 2020; Jiang *et al,* 2020; Yobi *et al,* 2020; Reteng *et al,* 2017;).

1.2.3.3 Antimalarial Resistance

The first resistance to the oldest antimalarial drugs, the quinolines, emerged in South America and Southeast Asia and eventually spread to other highly endemic regions of Africa. Polymorphisms in several transporters including SNPs in *pfmdr1*, *pfcrt*, *pfnhe1* and *pfmrp1* have been associated with decreased sensitivity to quinoline antimalarials which were recommended first line malaria treatment for an extensive period in the past and still are recommended first line treatment in combination with

antibiotics for malaria treatment in early pregnancy and some African countries (Tindall *et al,* 2018; Wootton *et al,* 2002). *Pfcrt* was initially identified as a result of mapping a genetic cross between chloroquine resistant and sensitive strains to a 36kb region of chromosome 7 in which point mutations at *pfcrt* were associated with resistant phenotypes (Fiddock *et al,* 2000). One SNP in *pfcrt* in particular, which is highly polymorphic, that remains the main facilitator of chloroquine resistance is K76T (Tola *et al,* 2020). Individual polymorphisms of *pfmdr1* have been reported to result in opposite effects on different drugs. Reduced sensitivity to chloroquine and amodiaquine has been linked with *pfmdr1* N86Y and D1246Y mutations which are common in Africa. These same mutations have been associated with increased sensitivity to lumefantrine, mefloquine, and artemisinin (Cui *et al,* 2015). However, decline in the frequency of chloroquine resistance alleles has been observed after use of chloroquine for *P. falciparum* was discontinued for extended periods in several long-term studies highlighting the effects of fitness costs (Nwakanma *et al,* 2013; Nsanzabana, *et al,* 2010; Mwai *et al,* 2009).

Although point mutations in *pfdhfr* and *dhps* has been strongly associated with hindering SP treatment success, copy number amplifications of *P. falciparum* guanosine triphosphate cyclohydrolase 1 (*pfgch1*), which is an enzyme responsible for coding a vital enzyme in the folate pathway, has also been implicated in antifolate resistance (Sugaram *et al,* 2020). Mutations in *dhfr* that have been commonly implicated in pyrimethamine resistance include S108N, A16V, N51I, C59R and I164L, with I164L reported to result in fast antifolate resistance spread in Africa. Sulfadoxine resistance has been mainly linked to an A437G mutation in *pfdhps* with additional mutations S436A/F/H, A581G, K540E and A613S/T *pfdhps* mutations facilitating elevated resistance *(*Juma *et al,* 2019). The occurrence of multilocus *Pfdhfr*/*Pfdhps*

mutations between N51I, C59R, S108N, I164L / A437G, K540E, S437A A581G, A613S have been widely linked to hindering SP treatment success, with the quintuple *Pfdhfr/Pfdhps* mutant genotype, N51I, C59R, S108N/A437G, K540E, occurring at a higher prevalence (Pacheco *et al* 2020; Quan *et al,* 2020; Okell *et al,* 2017). The increasing occurrence of these haplotypes renders a threat for malaria treatment efficacy in pregnancy and SMC in which SP remains the frontline chemotherapy in Africa, thus emphasising the need for continuous monitoring and evaluation (Turkiewicz *et al,* 2020; Kayode *et al,* 2021).

Increasing prevalence of resistance to artemisinin, which is manifested by delayed parasite clearance, and partner drugs such as piperaquine and mefloquine have been observed mostly in Southeast Asia where it emerged (Pava *et al,* 2020; Imwong *et al,* 2017). Resistance to artemisinin is associated with mutations in the propeller domain of PfKelch13, which was first identified as mediating protection using in vitro evolution (Witmer *et al,* 2020; Ariey *et al,* 2014). *PfKelch13* protein is suggested to have multiple cellular functions involved in intraerythrocytic growth development of asexual parasites, including haemoglobin endocytosis from the host cell, and oxidative stress and unfolded protein responses. *PfKelch13* is suggested to be involved in ubiquitination of specific substrates by functioning as a substrate adaptor for E3 ubiquitin ligases. This leads to proteasomal-mediated degradation when the transfer of ubiquitin from the ubiquitin-conjugating enzyme (E2) to the protein substrate(s) is catalysed by E3 ligase activity (Goel *et al,* 2021; Birnbaum *et al,* 2020; Saddiqui *et al,* 2020; Wu *et al,* 2019). Artemisinin resistant parasites with polymorphisms in *Pfkelch13* have been dominant across the Greater Mekong Subregion over the past decade and only recently emerged in other parts of the world including Guyana, Papua New Guinea and Rwanda where *Pfkelch13* mutations linked to artemisinin resistance were

reported (Mathieu *et al,* 2020; Pava *et al,* 2020). A *Pfkelch13* mutation A578S, which was the most common occurring *Pfkelch13* mutation detected in Africa, had previously been reported with no demonstrated effect in artemisinin efficacy thus the continued success of artemisinin malaria treatment in Africa for the past decades. However, the recent detection of R561H *Pfkelch13* mutations in Rwandan isolates demonstrating resistance phenotypes poses a significant threat to the extended success of antimalarial chemotherapy in Africa (Uwimana *et al,* 2020).

Selection pressure for artemisinin resistance is facilitated by reduced effectiveness of partner drugs as well as use of monotherapy thus establishing the cause of resistance between partner drugs and artemisinin may pose a challenge in studying artemisinin failure. Re-emergence of piperaquine and mefloquine resistance in southeast Asia has occurred due to reduced ACT efficacy (van der Pluijm *et al,* 2019; Sá *et al,* 2018). To date there have been over 100 identified *PfKelch13* mutations. Different degrees of delayed parasite clearance in patients treated with ACT have been demonstrated by various *PfKelch13* variants with mutually exclusive SNPs leading to amino acid changes at different codons including F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H and C580Y which are the mutations that have been more frequently reported and linked to artemisinin resistance (Mathieu *et al,* 2020; Witmer *et al,* 2020; Xie *et al,* 2020). The most frequently occurring *Pfkelch13* mutation observed especially in Southeast Asia is the C580Y mutation. In contrast to *Pfkelch13* mutations in Southeast Asia, only parasites with African genetic backgrounds have demonstrated artemisinin resistance linked to mutations in *P. falciparum* adaptor protein complex 2 mu (*Pfap2mu*), and ubiquitin-specific protease 1 *(pfubp1)* encoding the ubiquitin hydrolase (Sharma *et al,* 2020; Henriques *et al,* 2014).

The delayed parasite clearance in artemisinin-treated patients is a measure of loss of drug efficacy. However, this assay is not suitable for in vitro use. Furthermore, the standard 72 h drug assay used for other drugs does not reveal the tolerance phenotype of *pfkelch*-mutant parasites. Due to the short half-life of artemisinin, parasites in vivo need only withstand a relatively short period at high drug concentration. Thus, to more accurately reflect this short exposure artemisinin, the current standard *in vitro* assay is the ring-stage survival assay which measures the percentage of early (0 to 3 h post-invasion) ring-stage parasites that are able to survive a single 6-hour pulse of high concentrations of dihydroartemisinin (DHA). The identification of a number of artemisinin resistance-associated genetic loci and proteins have been facilitated by this approach, in combination with genome-wide association studies of resistant field isolates and gene manipulation experiments to validate variants of interest (Siddiqui *et al,* 2020). These approaches have played key roles in the progress of a detailed understanding of particular treatment failures, mechanisms to manage resistance to these drugs, and the development of more improved and effective antimalarial treatments (Cowell and Winzeler 2019; Blasco *et al,* 2017). Due to the variations of resistance polymorphisms of different *P. falciparum* genetic markers for different antimalarials across different regions (figure 1.4), there is need for continued investigation to determine specificity associated with fitness and genetic backgrounds. This will provide further insight into *P. falciparum* resistance mechanisms (Ross and Fidock, 2019). The genetic diversity of *P. falciparum* has been strongly associated with enabling its adaptation to antimalarials thus the continued success as a parasite (Apinjoph *et al,* 2019).

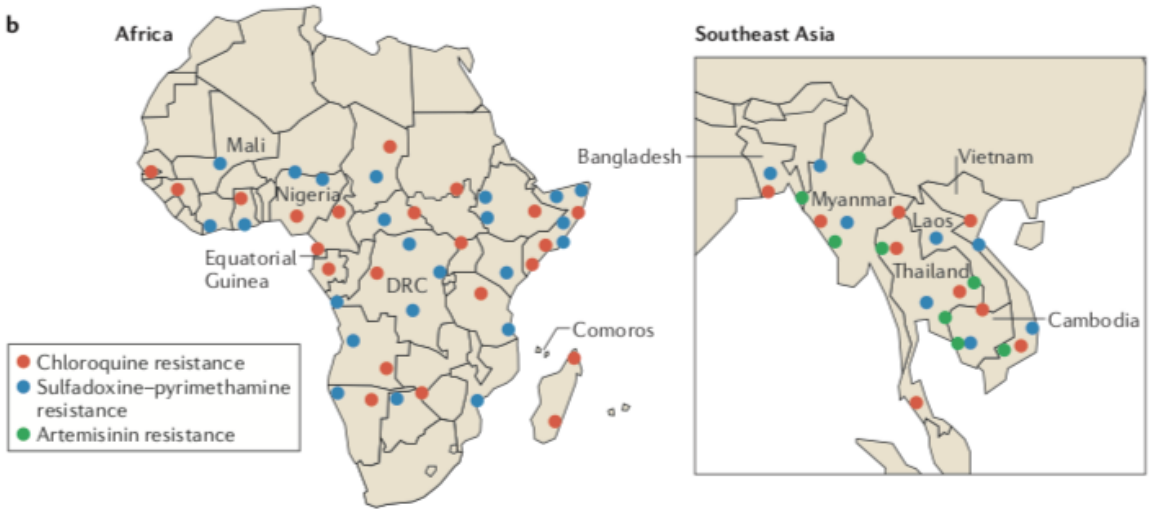

Figure 1.4 Distribution of *Plasmodium falciparum* antimalarial resistance. The distribution of *Plasmodium falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine in Africa and Southeast Asia. (Adapted from Haldar *et al* 2018)

1.3 Genome Editing

Genome editing of malaria parasites has been a new and vital tool for progressive comprehension of genetic mechanisms of *P. falciparum* involved in resistance, as well as the identification of important novel drug targets (Cowell and Winzeler, 2018). In combination with sequencing techniques and the ability to readily culture *P. falciparum,* genome editing is an essential enhancement providing a valuable tool for investigating and validating resistance mutations (Cowell and Winzeler, 2019; Crawford *et al* 2017; Ng *et al* 2016). Various genome engineering methods have been developed for manipulation of the genome of several pathogens which are aimed at making use of DNA repair mechanisms to insert mutations where double-strand breaks (DNA damage) have occurred from exposure to damaging agents (Gopalakrishnan and Kumar, 2013).

Repair of double strand breaks in the majority of eukaryotes can occur by two major pathways as shown in figure 1.5. Homology directed repair (HDR), which is highly accurate and error-free, uses a homologous DNA template from a sister chromatid, a homologous sequence in diploids, a DNA segment like a donor plasmid or an ectopic donor if the double-strand break (DSB) occurs within a repeated sequence, to restore error-free DNA (Lee *et al,* 2014). The alternative DNA repair pathway referred to as non-homologous end joining (NHEJ) is facilitated by rejoining broken ends of the DSB without the use of a DNA template which makes use of DNA ligase IV and Ku70-Ku80 proteins binding to DNA ends, and is prone to error and loss or gain of some nucleotides (Badugu *et al,* 2015; Fleck and Nielsen, 2004). Most of the factors of canonical-NHEJ that are essential for DSB repair in most eukaryotes appear to be absent in the *P. falciparum* genome although there is evidence of DSB repair in *Plasmodium* parasites through an alternative-NHEJ. The prime mechanism

for DSB repair in *P. falciparum* is the HDR pathway which has been shown to have its core genes encoded in the *Plasmodium* genome. In addition, point mutations in genes leading to DNA repair functions linked to antimalarial resistance phenotypes have been revealed by whole genome sequencing, suggesting the increasing chances of new polymorphisms emerging (Mathews *et al,* 2018; Gupta *et al,* 2016).

1.3.1 Genome editing techniques

The introduction of single nucleotide polymorphisms into the *Plasmodium* genome has been important for dissecting major roles in essential biological functions and obtaining a detailed understanding of gene functions. In particular, given the prevalence of SNPs in mediating drug resistance, genome editing has been vital in the aims of understanding *P. falciparum* resistance mechanisms. This requires precise *Plasmodium* genome manipulation which various existing techniques have provided significant insights for are still being perfected to fully eliminate these challenges (Straimer *et al,* 2012).

1.3.1.1 Nuclease based genome editing

Nuclease-mediated genome editing has been successfully applied to several organisms based on previous observations of the binding and cleavage properties of the natural type IIS restriction enzyme, *Fok*I (Bibikova *et al,* 2002; Li *et al,* 1992). These approaches utilise site-specific nucleases to trigger DNA repair mechanisms which have been developed for introduction of SNPs in the *P. falciparum* genome by fusing the FokI nuclease with a designable DNA binding protein (Straimer *et al,* 2012).

Prior to CRISPR technologies, zinc-finger nucleases (ZFN) were the most widely used technique to introduce a double strand break in the genome. These

breaks would stimulate either the NHEJ pathway (donor free) or HDR pathway in the presence of a donor template to alter target DNA as a result of gene disruption leading to targeted gene replacement or targeted mutagenesis, as shown in Figure 1.5. This genome editing technique makes use of the DNA-binding domain of the zinc finger proteins, which can be engineered for recognition of specific DNA sequences, and the FokI nuclease that induces the targeted double strand break (Straimer *et al,* 2012; Caroll, 2011; Miller *et al,* 2007).

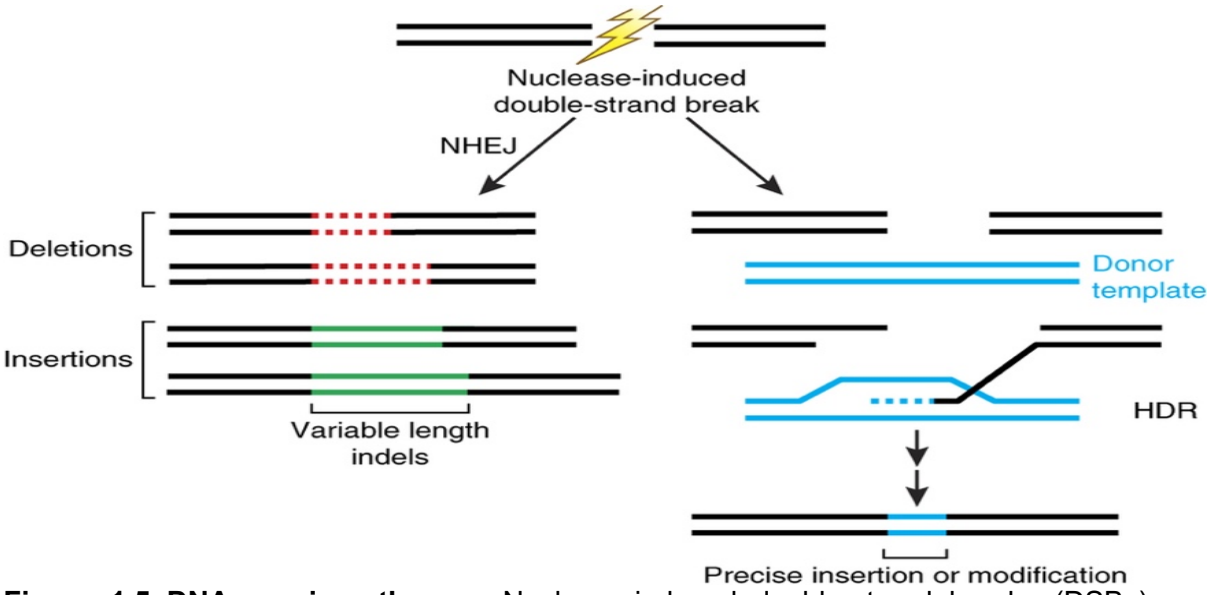

Figure 1.5 DNA repair pathways. Nuclease-induced double-strand breaks (DSBs) are repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). Gene disruption can occur by the introduction of errors during DSB repair to produce variable-length insertion and deletion mutations in the NHEJ pathway. A DNA donor template can be used to introduce point mutations or insertions via the HDR mediated repair pathway (Sander and Joung, 2014)

1.3.1.2 Transcription activator-like effector nuclease-based genome editing

Genome manipulation using transcription activator-like effector nucleases (TALENs) is another nuclease-based genome modification system. TALENs are a less complex system than ZFNs due to the more straightforward array design of the DNA binding element, as well as improved specificity and reduced toxicity. Similar to ZFNs, specific DNA sequences are recognised by TALENS through repeat DNA-binding

domains in the presence of two hypervariable amino acid residues required for target site specificity. These residues are engineered to promote site-directed genome editing by generating manipulative DNA-binding proteins which is complicated by the high similarity of TALE recognition sequences. This, combined with the nature of the highly repetitive DNA-binding domain and large size of the nuclease poses a challenge for its use in genome editing of organisms like *P. falciparum* (Gaj *et al,* 2016; Basu *et al,* 2015; Ma and Lui, 2015;).

1.3.1.3 CRISPR/Cas9 genome editing system

Genome engineering based on the clustered, regularly interspaced, short palindromic repeat (CRISPR)–CRISPR-associated protein (Cas) (CRISPR-Cas9) system is a powerful tool that has been widely and successfully used in genome manipulation of several organisms accelerating vital gene functional studies (Shinzawa *et al,* 2020; Xu *et al,* 2019). This nuclease-based editing mechanism which has also been successfully applied to *P. falciparum,* is mediated by the Cas9 endonuclease, shown in figure 1.6, that is guided by a single guide RNA (sgRNA) to cause double strand breaks in target sites (Lee *et al,* 2019; Lee and Fidock, 2014). The CRISPR/Cas based technique for *P. falciparum* was first reported by Wagner *et al,* (2014) and Ghorbal *et al* (2014) facilitated by the use of a guide RNA (gRNA), donor template and two-plasmid system for Cas9 delivery.

The CRISPR/Cas9 system evolved as a bacterial immune response against invading viruses and plasmids in which integration of foreign DNA within the CRISPR locus occurs. This foreign DNA is then used by the host to guide the direct sequence-specific degradation of related pathogenic DNA aided by the gRNA-Cas9 protein system. A protospacer-adjacent motif (PAM) (typically -NGG for the *Streptococcus*

pyogenes Cas9) is also required immediately following the gRNA site in the target DNA for recognition (Gaj *et al,* 2016).

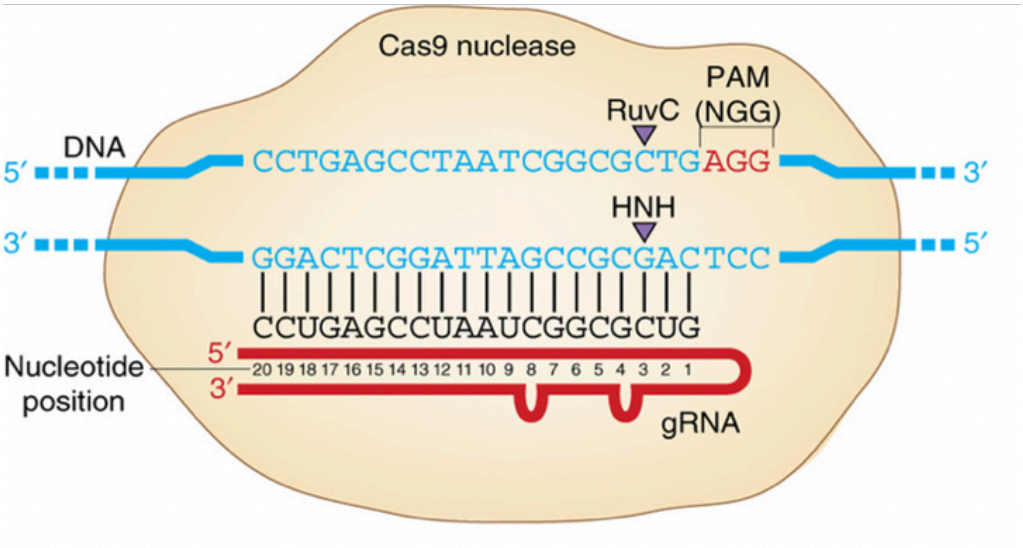

Figure 1.6 Cas9 nuclease. Cas9 nuclease-induced double strand breaks at DNA target sites are directed by complementarity base pairing to the 5′ end of a gRNA. The NGG PAM motif is not included in the guide sequence. (Adapted from Sander and Joung, 2014)

In the recent developments of genome editing in *P. falciparum* with the CRISPR/Cas9 system, the expression of the gRNA and Cas9 is driven by specific promoters, with all components supplied on transfection plasmids. Although modification of the nuclease is not required in the Cas9 system, unlike ZFNs and TALENs, factors to be considered in the experimental design include careful selection and design of the gRNA, which should conform to a 20-nucleotide sequence followed by a (-NGG) protospacer adjacent motif (PAM). Donor design is also crucial in the success of the CRISPR/Cas9 tool for *P. falciparum* editing, which is influenced by a diverse number of factors including length of homology region as well as size and distance of target point mutations to gRNA binding site (Lee *et al,* 2019). In a previous study by Ghorbal *et al* (2014), one of the two plasmids designed harboured the donor sequence and sgRNA cassette with a positive drug-selectable cassette h*dhfr* flanked by homology arms to generate a gene knockout. Another donor for introducing point-

mutations was designed for kelch13, with the desired coding mutation as well as a silent mutation to prevent repeated cleavage of the edited locus. All target sites were edited including *Pfkelch13* mutation C580Y with no off-target activity (Ghorbal *et al,* 2014). The application of the CRISPR/Cas9 tool has greatly accelerated insight into studies of various aspects of *P. falciparum* gene investigations including drug resistance mechanisms and epigenetic studies for pathogenesis (Nasamu *et al,* 2021; Xiao *et al,* 2019; Bryant *et al,* 2017).

1.4 Next Generation Sequencing

The prospect of continuous monitoring and surveillance to track developing drug-resistant parasite populations in near real time has significantly enhanced the genomic surveillance and epidemiology of malaria parasites and has become a powerful tool in determining potential drug resistance threats (Kümpornsin *et al,* 2019). The field of genomics has been greatly enhanced by the availability of *de novo* sequencing platforms such as Next Generation Sequencing (NGS) which have been developed for greater genome coverage with higher throughput enabling the identification of crucial polymorphisms involved in *P. falciparum* drug resistance. For genome-scale sequencing, NGS approaches have a significantly lower cost compared to previous sequencing platforms such as Sanger sequencing. This was one of the widely used sequencing methods, that determined each sequence base with the use of deoxynucleotides (ddNTPs) and polymerase, using capillary sequencing since its development in 1970s (Cowell *et al,* 2018; Wang *et al,* 2012; Sanger and Coulson, 1975).

The NGS method developed by Illumina is characterised by the generation of short reads from DNA sequencing and is now the most widely used NGS technology.

Illumina sequencing, which is based on a cyclic reversible termination chemistry (Figure 1.7) is initiated with library preparation that involves the priming of a DNA template by a complementary sequence of the adapter region. Specific adapters are ligated on each DNA molecule and used as substrates cleaved to the double-stranded DNA (dsDNA) region shearing DNA into short length fragments (Goodwin *et al,* 2016). Cluster generation occurs in a glass-surfaced flow cell containing oligonucleotide sequences complementary to library fragment adapters. The single stranded DNA fragments hybridize to these oligonucleotides and go through repeated amplification cycles in a bridge amplification mechanism. This repeated process leads to generation of billions of clusters from clonal amplification of all fragments, with thousands of template copies generated in each cluster. Sequencing takes place by incorporation of fluorescently labelled nucleotides that contain a terminator sequence. These nucleotides that are labelled with a fluorophore specific to each DNA base are detected by light source excitation allowing parallel sequencing of identical fragments. The base calling process upon which alignment of short reads to a reference genome is performed, depends on emission wavelength and signal intensity. Both forward and reverse strands sequences are produced as end product in the Illumina platform (Del Vecchio *et al,* 2017; Slatko *et al,* 2018).

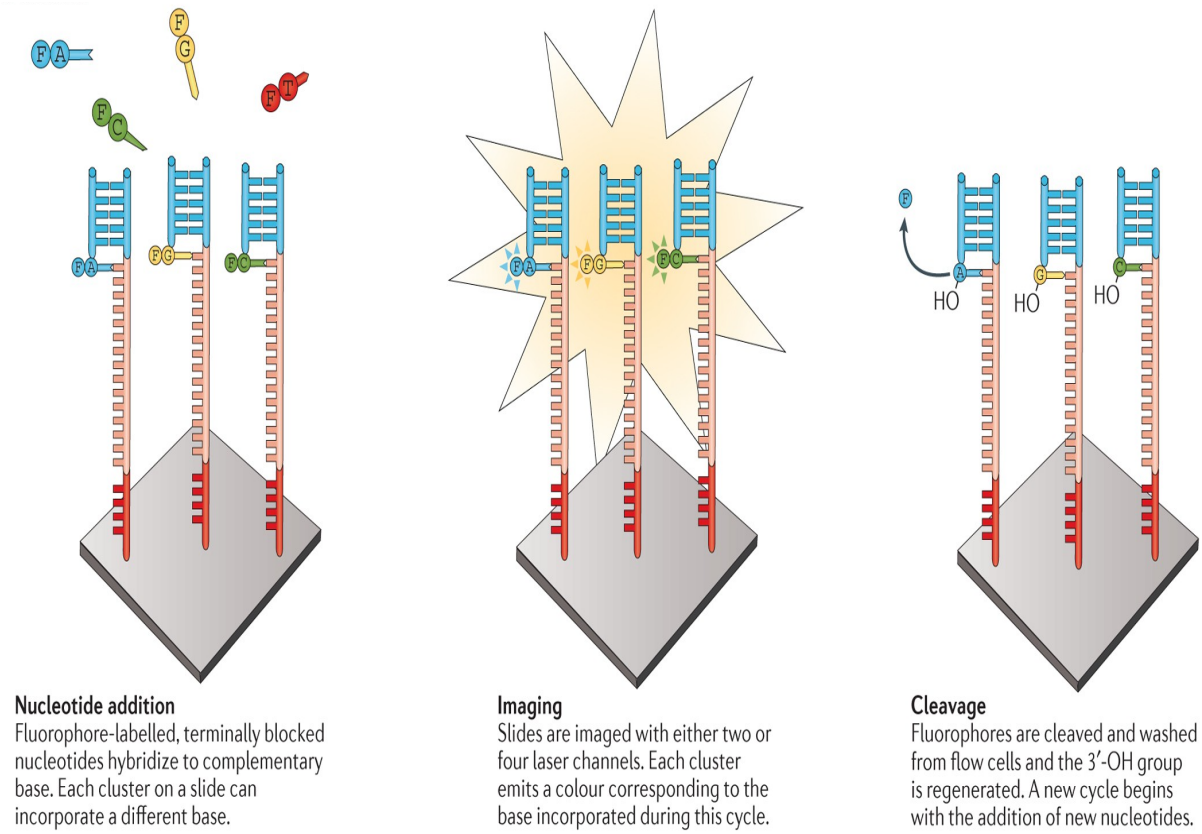

Figure 1.7 The Illumina cyclic reversible termination (CRT) system to determine DNA sequence. Template enrichment is followed by addition of primers, DNA polymerase and modified nucleotides to the flow cell with each nucleotide blocked by a 3′-O-azidomethyl group and labelled with base-specific, cleavable fluorophore (F). Incorporated bases are identified by laser excitation using two or four channels in multiple cycles of nucleotide addition, elongation and cleavage and unincorporated bases are washed away. This repetition of cycles results in determining sequences of the DNA molecules in each cluster. Figure from Goodwin *et al,* 2016.

1.5 Barcode Sequencing (BarSeq)

Barcode Sequencing (BarSeq), which involves the use of genome modified (molecular barcoded) parasites, facilitates high throughput phenotyping of *Plasmodium* parasites by simultaneously assessing multiple uniquely barcoded parasites in a single pool using Illumina NGS. The BarSeq approach has been applied in *P. berghei* to probe gene essentiality and phenotype growth measurements of gene knockouts (KO). Using a reverse genetics approach, pJazz-based *Plasmodium* Genetic Modification (PlasmoGEM) KO vectors were constructed with unique gene-

specific 11 base pair molecular barcodes and annealing sites for binding of Illumina indexing primers (Schwach *et al,* 2015; Bushell *et al,* 2017). A high proportion of essential *P. berghei* genes and potential antimalarial drug targets were identified in this study by simultaneously phenotyping 2578 mutants using the BarSeq technique (Bushell *et al,* 2017).

BarSeq and Illumina NGS was used by Carrasquilla *et al*. (2020) as part of a study to accurately quantify the multiplicity of vector uptake in *P. falciparum* transfections in which unique *P. falciparum* transfection vectors were tagged with barcodes derived from the PlasmoGEM resource vectors (Carrasquilla *et al,* 2020). As part of M. Carrasquilla's thesis work, unique barcodes were also inserted into a pseudogene (*Pfrh3*) of *P. falciparum* parasites using CRISPR/Cas9-based genome editing, to allow growth measurements in a similar approach adapted from the previously developed *P. berghei* BarSeq technique (Carrasquilla, 2019). These studies highlight the potential of the combination of genome modification tools and amplicon sequencing by NGS that has significantly enhanced insights in investigations on the *Plasmodium* genome. These continuous advances are providing phenotypic, molecular and genomic information on *P. falciparum* that collectively are highly useful for malaria elimination programs (Nag *et al,* 2017).

1.6 Project Aims

My project is aimed at investigating:

I. How barcode tagging and sequencing approaches could be used to examine how genetic background of parasites affect fitness and resistance.

II. The impact of *Pfkelch13* mutations on parasite resistance and fitness.

III. Potential antimalarial activity of Human keap1 inhibitors on *Plasmodium falciparum*