Chapter 1: Introduction

1.1 Malaria: history, epidemiology, and biology

Malaria is a parasitic infectious disease that has plagued humans, amongst other species, for millennia. Despite this long history and global efforts to eradicate the disease, 435,000 thousand people died of complications caused by malaria in 2017 and an estimated 3.2 billion people—roughly half of the people on earth—are at risk of infection (World Health Organization, 2018). Though these statistics might suggest otherwise, malaria is a *treatable* disease that can be cured. However, one of the greatest obstacles to malaria elimination is the ability of the malaria parasite, *Plasmodium*, to develop drug resistance and evade death by antimalarial chemotherapy (Menard and Dondorp, 2017). This thesis aims to provide insight into the biological mechanisms of drug resistance in the malaria parasite. With a focus on malaria in Southeast Asia, this work combines clinical and functional genetic studies to elucidate molecular modes of resistance to the widely-used antimalarial drug, piperaquine.

1.1.1 History of malaria

Malaria is caused by a unicellular protozoan parasite of the genus, *Plasmodium*. The parasite is from a larger group of eukaryotic organisms in the phylum, *Apicomplexa*. *Plasmodium* are some of the most promiscuous parasites on our planet, with over a hundred *Plasmodium* species known to infect a diverse array of mammals, reptiles, and birds (Levine, 1988, Schlagenhauf, 2004). The malaria species that infect humans today are believed to have originated as zoonotic diseases that were transmitted from non-human primates to humans in Africa and spread with human migrations throughout the world (Carter and Mendis, 2002, Bruce-Chwatt, 1965). Today, more species of malaria infect primates than any other mammalian orders (Cox, 2010, Garnham, 1996). Six of those *Plasmodium* species infect humans: *P. falciparum*, *P. vivax*, *P. ovale* (recent work has distinguished two distinct species of *P. ovale* that infect humans (Sutherland et al., 2010)), *P. malariae*, and *P. knowlesi*. Though exact dates of transmission to humans is unknown, evidence of malaria-like symptoms have been present in human civilizations for centuries (Carter and Mendis, 2002) and more recently, detection of *Plasmodium* antigens in remains from antiquity have provided further affirmation of written accounts (Miller et al., 1994, Abbott, 2001).

Early descriptions of malaria can be found in writings and artifacts from civilizations in Mesopotamia, India, China, Egypt, Greece, and Rome (Bruce-Chwatt, 1988). Over 2,000 years ago in India, the *Atharvaveda* scripts described the periodic fevers of what was very likely malaria and bestowed upon it the title "king of diseases" (Kaur and Singh, 2017). The oldest book of medicine from China, the *Nei Ching* or "Canon of Medicine", describes both the fevers and enlarged spleens of what was likely malaria-infected individuals. In 400 BC, Hippocrates was one of the first to write, in detail, about the paroxysmal fevers of malaria and associated malaria with the air—as were most ailments attributed at that time. Diseases that were then invisible to the naked eye were believed to be caused by "miasma," a poisonous vapor in the air, often believed to emanate from swamps and decaying matter. Hippocrates associated certain environments and seasons with fevers that he described as "tertian" (*P. falciparum*/P. *vivax*/*P. ovale,* for which fevers recur every third day), "quartan" (*P. malariae*, fevers return every fourth day), or "intermittent." He also recorded the strong association of splenomegaly with the disease (Bruce-Chwatt, 1988). This almost ethereal association of malaria with the air infiltrated civilizations for centuries, much like an actual noxious substance would, earning the disease its name, "*mal'aria*" or "bad air" amongst the citizens of Rome in the 1600s (Hempelmann and Krafts, 2013). Though incorrectly attributing malaria to vapors from foul-smelling swamp waters, they astutely connected the importance of stagnant water and time of year to the disease (Cunha and Cunha, 2008).

It was not until centuries later during the Golden Age of Microbiology in the 1800s, that the capabilities for identifying the causal agent of malaria emerged (Maloy and Schaechter, 2006). The long-searched for culprit of the disease was discovered in 1880 by French army doctor, Charles Louis Alphonse Laveran while stationed in Algeria, during the Franco-Prussian War (Bruce-Chwatt, 1981). In a fresh blood sample from an afflicted soldier, Laveran identified live *Plasmodium* parasites on a microscope slide by noticing moving filaments and pigment-containing cells. Laveran named the organism, *Oscillaria malariae* (Bruce-Chwatt, 1981). In 1885, two Italian scientists, Ettore Marchiafava and Angelo Celli, created a new genus for the malaria parasite called, *Plasmodium* (McFadden, 2012, Marchiafava, 1885). The nomenclature change was based on Marchiafava and Celli's observations that the parasites, when inside of erythrocytes, or red blood cells (RBCs), had multiple-nuclei and an ameboid-like shape that resembled a slime-mold of the same name, plasmodium (McFadden, 2012, Marchiafava, 1885). During this same time period, a cohort of Italian scientists led by Camillo Golgi, Marchiafava, Celli, Grassi, among others, deduced

that the *Plasmodium* genus was made of distinct species that caused different forms of malaria (Cox, 2010).

Despite these insights into the biology of *Plasmodium*, it took over a decade from when the parasite was first discovered to determine how it was transmitted from person-to-person. In the early 1890s, Patrick Manson hypothesized that malaria was transmitted by mosquitoes (Manson, 1894). This theory was later confirmed in 1897 by Sir Ronald Ross (Cox, 2010, Manson, 1898). Under the mentorship of Manson, Ross had observed pigmented bodies (what we now know as oocysts) in the midgut of an *Anopheles* mosquito that had bitten a malaria-infected patient (Ross, 1897). It should be noted that at the same time, Grassi and colleagues had also documented the *Plasmodium* life cycle in the mosquito and demonstrated that the *Anopheles* mosquito was the only vector for the parasite (Cox, 2010).

1.1.2 Malaria today: global burden and epidemiology

When Laveran, Ross, and the other influential researchers mentioned above made their discoveries in the late 19th century, malaria existed on every continent except Antarctica (**Figure 1.1**). As can be seen on the map in Figure 1.1, malaria extended through both temperate and tropical regions, spreading as far north as Russia. Interventions by mankind from 1900-2002 substantially reduced the number of countries at risk of malaria from around 140 to 88, shrinking the land distribution of *Plasmodium* endemicity by half (Hay et al., 2004, Murray et al., 2012).

This reduction was made possible through national and global efforts in malaria endemic regions. Such strategies combined multidisciplinary approaches to reduce the malaria burden including, antimalarial drugs, insecticides, insecticide-treated bed nets, malaria surveillance tools, improved sanitation, and many other measures. This work has been achieved and sustained in the 21st century by many organizations and programs, including the Centers for Disease Control and Prevention (CDC) in the United States, the World Health Organization (WHO), the United Nations (UN), and the Bill & Melinda Gates Foundation, to name a few (World Health Organization, 2018). In spite of these efforts, the risk of malaria infection remains in approximately 100 countries and territories.

According to the most recent Malaria Report from the World Health Organization, malaria occurs in 87 countries with the majority of cases caused by *P. falciparum* and *P. vivax* (**Figure 1.2**) (World Health Organization, 2018). As seen in the maps in Figure 1.2, *P.*

falciparum and *P. vivax* have widespread global distributions with distinct regions of prevalence, with *P. falciparum* predominating in Sub-Saharan Africa and Southeast Asia (SEA), while *P. vivax* is most prominent in South America, SEA, and the Western Pacific. *P. falciparum* is the most dangerous malaria species and causes the highest rates of morbidity and mortality due to its ability to progress to severe malaria. *P. vivax* is sometimes referred to as "benign" malaria because it is often asymptomatic and progression to severe malaria is less common (Carter and Mendis, 2002, Price et al., 2007). However, benign is misleading because *P. vivax* also contributes to morbidity and mortality, though to a lesser extent than *P. falciparum* (Price et al., 2007). Furthermore, *P. vivax* and *P. ovale* have dormant liver stages that can relapse in infected individuals months or even years after initial infection. The less prevalent *P. malariae* and *P. ovale* have been identified in all malaria endemic regions, but their incidence rates are lower than *P. falciparum* and *P. vivax*, although studies suggest that it is likely these species are underreported due to missed detection, misdiagnosis, or co-infection with *P. falciparum* or *P. vivax* (Mueller et al., 2007, Doctor et al., 2016). *P. knowlesi*, a zoonotic parasite in macaque monkeys that also infects humans, has a less clearly delineated geographical distribution, but has been demonstrated in patients throughout SEA, with incidence rates recently reported on the rise in Malaysia (Barber et al., 2017, Cooper et al., 2019). Similar to *P. malariae* and *P. vivax*, it is hypothesized that *P. knowlesi* infections are underreported due to misdiagnosis as one of the other species (Shearer et al., 2016). It should also be acknowledged that *P. knowlesi* infections are currently being reported in countries that are making strides in eliminating the human malarias, so further studies are warranted and its prevalence should not be overlooked (Shearer et al., 2016).

From an epidemiological perspective, there were 219 million malaria cases in 2017 and 435,000 deaths (World Health Organization, 2018). The largest proportion of infections (92%) and deaths (93%) were in Sub-Saharan Africa. *P. falciparum* represented 99.7% of cases in Africa and 62.8% of malaria cases in SEA (World Health Organization, 2018). *P. vivax* was responsible for the majority of malaria infections outside of Africa, contributing to 74.1% of cases in the Americas and 37.2% of cases in SEA (World Health Organization, 2018). All individuals in malaria endemic regions are at risk of infection, but the severity and likelihood of illness is greatly increased for children under the age of five, pregnant women, and immunocompromised individuals. Children accounted for over half of the malaria deaths (61%) in 2017 (World Health Organization, 2018). These statistics show that this disease from antiquity is still very much a disease of the present.
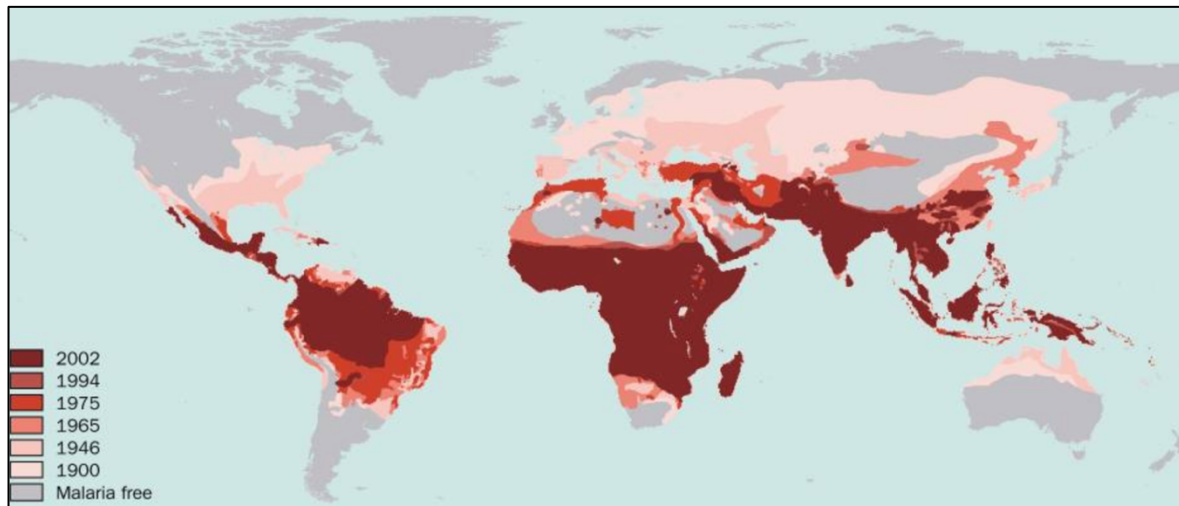
Figure 1. 1. The global distribution of malaria in the 20th century. The maps show the risk of malaria from *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* from circa 1900 to 2002, as estimated by Hay *et al.* (Hay et al., 2004). The figure has been reprinted from Hay *et al.* (2004) with permission from Elsevier.
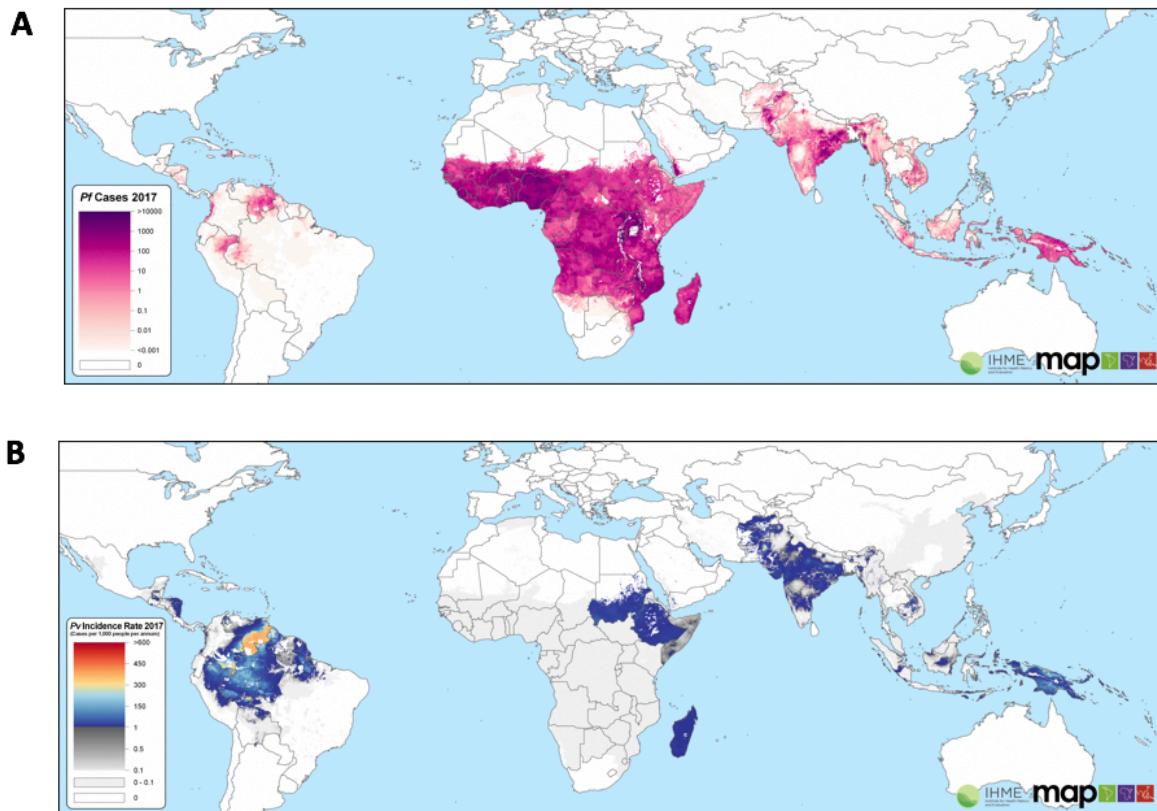

Figure 1. 2. The global incidence of P. falciparum and P. vivax malaria in 2017. A.) Incidence of *P. falciparum* malaria in all age groups in 2017. **B**.) Incidence of *P. vivax* malaria in all age groups in 2017. The maps are reprinted from The Malaria Atlas Project (2019) and are available in the public domain under the Creative Commons Attribution 3.0 Unported License.

Malaria disproportionately affects populations living in extreme poverty. The disease is both a cause of and result of poverty, and the cycle continues to repeat itself (Teklehaimanot and Mejia, 2008, Gallup and Sachs, 2001). For example in 2017, five countries with some of the

widest poverty gaps—Nigeria, the Democratic Republic of the Congo, Mozambique, India, and Uganda—comprised almost half of the total number of malaria cases globally (World Health Organization, 2018). This inextricable link between malaria and poverty has long been acknowledged (Teklehaimanot and Mejia, 2008, Feachem et al., 2019), but the steps for unravelling the two requires multidisciplinary approaches that remain complex. Economic, social, and political factors, amongst many other dynamics combined with ecological conditions in tropical regions, favors the continued transmission of malaria. Though progress has been made through the aid of the aforementioned programs (CDC, WHO, UN, Gates Foundation) the current World Malaria Report shows that no significant progress in reducing the malaria burden was made within the last three years from 2015-2017 (World Health Organization, 2018).

In spite of these issues, all hope for malaria elimination (reduction of the disease to zero new incidences in a distinct region) and eradication (permanent removal of disease pathogen) should not be quelled. There has been a considerable reduction of the malaria burden since 2010 and although overall global statistics from 2015-2017 have stalled, country specific advancements are numerable. Sri Lanka eradicated malaria in 2016. In 2017, Rwanda reported 430,000 less malaria cases than in 2016 and India had a 24% decrease in malaria infections. Furthermore, 46 countries had less than 10,000 cases of malaria in 2017 (World Health Organization, 2018). Continued reduction of the malaria burden and eventual eradication is possible in this century. Experts believe eradication could be possible by 2050 and have put forth the slogan, "malaria eradication within a generation" (Feachem et al., 2019).

In addition to the social, economic, and political factors that must be addressed, continued scientific advancements are imperative for the goals of eradication to be realized. Although malaria remains a curable disease, drug resistance and the potential for it to develop threatens the efficacy of the limited number of antimalarial drugs currently available. As endemic countries strive to reduce the burden caused by malaria, it is necessary to understand the mechanisms of drug resistance, which is the focus of this dissertation. Drug resistance has been reported in both *P. falciparum* and *P. vivax* malaria (Bloland, 2001, Cui et al., 2015, Blasco et al., 2017, Price et al., 2009, Dayananda et al., 2018). Due to the global burden of *P. falciparum* malaria and its resistance to currently available drugs in SEA, *P. falciparum* malaria is the focus of this dissertation. Investigation of the mechanisms of drug resistance in *P. falciparum* provides both fundamental insight into parasite biology and also into the

mechanisms through which resistance can develop. For these studies to be possible, it is necessary to understand both the life cycle and pathogenesis of *P. falciparum*.

1.1.3 *P. falciparum* life cycle

The life cycle of *P. falciparum* consists of three phases (**Figure 1.3**): a sexual stage in the mosquito vector (sporogonic cycle), an asexual stage in the liver of the human host (exo-erythrocytic cycle), and an asexual stage in the blood of the human host (intraerythrocytic cycle) (Cowman et al., 2016, Miller et al., 2013). The parasite is an obligate intracellular pathogen in liver hepatocytes and erythrocytes, which facilitates the parasite's ability to avoid detection by the host's immune system.

The life cycle begins when a *P. falciparum*-infected female *Anopheles* spp. mosquito takes a bloodmeal from a human host. Male mosquitoes do not bite, so are thus not able to spread malaria. When the female mosquito bites an individual, the contents of the mosquito's saliva, including anticoagulants and *P. falciparum* sporozoites, are injected into the human host's blood. The sporozoites enter the bloodstream and migrate to the liver (30-60 minutes) where they invade hepatocytes and take ~6 days to mature into schizonts that contain ~40,000 merozoites per hepatocyte (Miller et al., 2013, Phillips et al., 2017). This stage is clinically silent and the infected individual does not present with any symptoms. During *P. vivax* and *P. ovale* infections, it is during this exo-erythrocytic stage that some sporozoites differentiate to hypnozoites which can remain dormant in the liver for weeks to years before maturing to merozoites (Miller et al., 2013, Cowman et al., 2016).
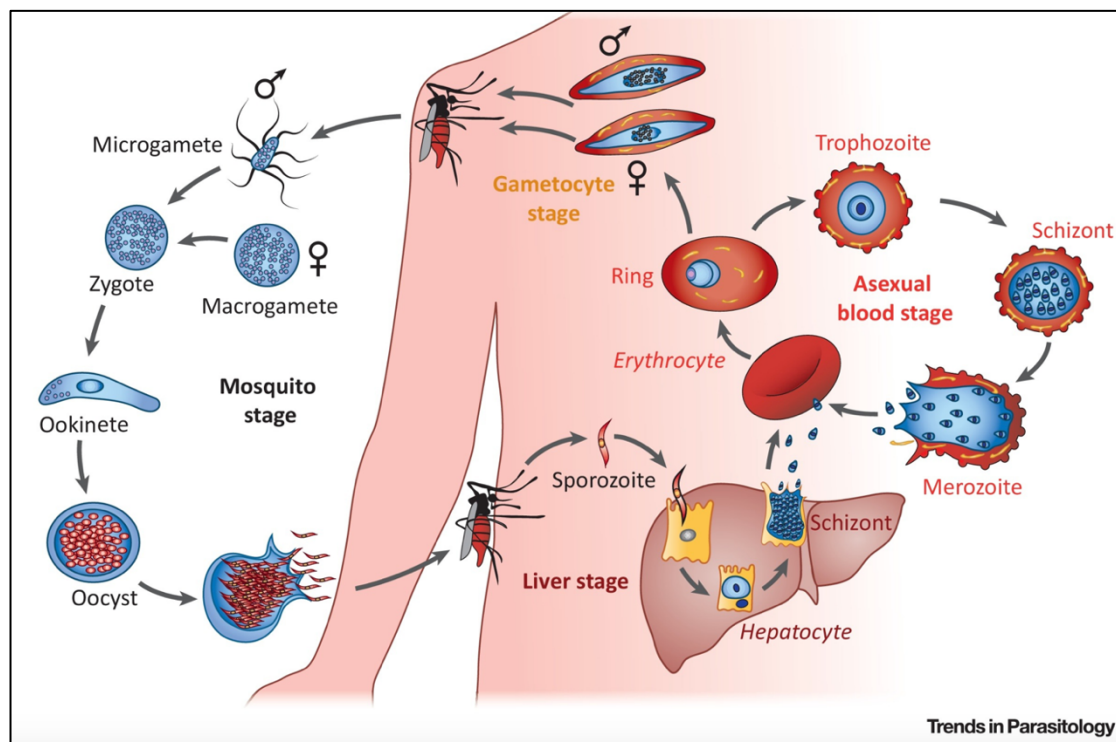
Figure 1. 3. The life cycle of *P. falciparum*. The life cycle begins when an infected female *Anopheles* mosquito bites a human host and sporozoites are inoculated into the host and migrate to the liver (liver/exo-erythrocytic stage) where they invade hepatocytes and form liver schizonts. When the liver schizonts rupture, they release merozoites that invade erythrocytes to start the asexual blood (intraerythrocytic) stage. When merozoites egress from the ruptured schizonts, they can reinvade erythrocytes to propagate the asexual cycle, or differentiate to sexual stage gametocytes. As gametocytes mature, they differentiate into male and female gametocytes that are taken up by a mosquito during a blood meal. Once inside the mosquito midgut (sporogonic/mosquito stage), the male and female gametes fuse to form a zygote that becomes an ookinete and then develops into an oocyst. When the matured oocyst ruptures, it releases sporozoites that migrate to the mosquito salivary glands to be transferred to a human host when the mosquito takes its next blood meal to repeat the cycle. The figure is reprinted from Maier *et al*. (2019) (Maier et al., 2019) with permission from Elsevier.

The intraerythrocytic stage commences when an infected hepatocyte bursts and releases the hepatic merozoites into the bloodstream where they invade erythrocytes. Merozoite invasion into the erythrocyte takes about 1-2 minutes (Weiss et al., 2015). The intraerythrocytic cycle is when clinical symptoms present and is marked by three distinct forms of parasite growth: ring, trophozoite, and schizont (**Figures 1.3-1.4**) (Bannister and Mitchell, 2003, Bannister et al., 2000). Upon invading the host erythrocyte, the parasite enters the ring stage and begins to grow within the host cell over the course of 24 hours. The stage name is based on how the early ring stage parasite looks on a Giemsa-stained blood smear, with the nucleus off to one side and the disc-like cytoplasm adjacent (**Figure 1.4**). During the ring stage, the parasite begins to digest the host cell hemoglobin to obtain nutrients in the form of amino acids. Hemoglobin digestion enables the parasite to have space to grow (Bannister and Mitchell,

2003, Bannister et al., 2000, Cowman et al., 2016) and provides an osmotic balance within the erythrocyte (Lew et al., 2003). Following the ring stage the parasite enters the stage of highest metabolic activity, trophozoite (~24-36 hours), and continues to feed off of the host cell hemoglobin. As trophozoites mature and enter the schizont stage (~36-48 hours), they undergo asexual replication to yield ~16-32 daughter merozoites. When the schizont has fully matured after ~48 hours, the merozoites egress, or rupture, from the host erythrocyte (**Figure 1.4**). This process destroys the erythrocyte as the merozoites are released into the bloodstream where they can invade a new erythrocyte to continue the intraerythrocytic cycle (Cowman and Crabb, 2006, Cowman et al., 2016). This intraerythrocytic asexual cycle will continue until it is cleared by the host's immune system or an antimalarial drug, or until it causes severe disease and death of the host.
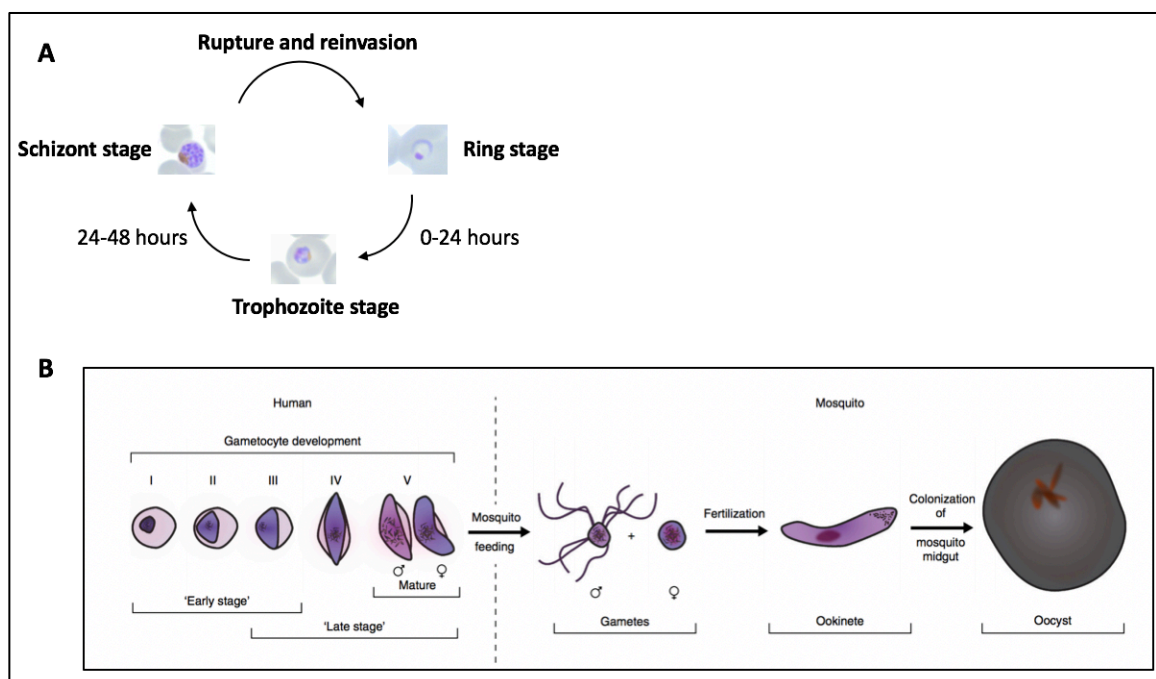

Figure 1. 4. The asexual and sexual intraerythrocytic life cycles of *P. falciparum*. A.) The 48-hour life cycle of *P. falciparum* asexual parasites as observed in Giemsa-stained blood smears. **B**.) A schematic of the sexual stages of *P. falciparum* gametocytes, including progression from sexual stage gametocytes in the human host to the mosquito vector. The image in part B was modified from Delves *et al.* (Delves et al., 2016) and reprinted with permission from Springer Nature Publishing.

During the asexual intraerythrocytic stages, a portion of merozoites will commit to the sexual stage by undergoing a process called gametocytogenesis (Baker, 2010, Ngotho et al., 2019). The time at which this developmental conversion occurs is the topic of much debate and remains to be fully elucidated, but studies have shown it is regulated by the transcription factor, AP2-G (Kafsack et al., 2014, Sinha et al., 2014). It was previously shown that following an increase in AP2-G expression, parasites complete an additional asexual life

cycle as committed parasites before reinvading as gametocytes in the next cycle (Baker, 2010). Recent studies have shown in addition to this pathway, parasites can undergo a developmental switch within the same asexual life cycle that increased AP2-G expression occurs (Bancells et al., 2019). Once committed to sexual development, gametocytogenesis consists of five stages (I-V), with the earliest stage (stage I) morphologically indistinguishable from asexual parasites (**Figure 1.4**) (Ngotho et al., 2019). Gametocytes require ~12 days to develop and the mature stages are identifiable by their "falciform" shape, from which falciparum earned its name (**Figure 1.4**). During development, the gametocytes are sequestered in the bone marrow to escape splenic clearance (Joice et al., 2014). In the final maturation stages, the gametocytes differentiate into haploid male (microgamete) or female (macrogamete) forms, which can remain in circulation for several days (Smalley and Sinden, 1977). The male and female gametocytes are the infective forms that are taken up by the mosquito during a blood meal (Ngotho et al., 2019). Once inside the mosquito midgut, the male gametocyte undergoes a process called exflagellation (induction) by which it becomes motile and fuses with the female macrogametocyte to form a diploid zygote (Phillips et al., 2017). In ~24 hours the zygote becomes an ookinete which enters the mosquito midgut epithelium and develops into an oocyst. When the oocyst is fully matured (after ~2 weeks), it releases thousands of haploid, motile sporozoites that travel to the mosquito's salivary glands where they can be transferred by mosquito bite to the next host to restart the cycle (**Figure 1.3**) (Barillas-Mury and Kumar, 2005). Given that the *Anopheles* adult lifespan is 2-3 weeks in nature, this development cycle enables just enough time for the *P. falciparum* life cycle to be propagated (Clements, 1992, Centers for Disease Control, 2018).

The intraerythrocytic stage of *P. falciparum* malaria is the focus of this study. As will be discussed in later sections of this introduction, asexual *P. falciparum* isolates can be cultivated and maintained *in vitro* using human erythrocytes (Trager and Jensen, 1976). This enables the parasite to be studied using molecular biology techniques and tools.

1.1.4 Pathogenesis of *P. falciparum* malaria: asymptomatic, uncomplicated, severe disease

A malaria infection can be categorized as asymptomatic, uncomplicated, or severe. As described by the name, asymptomatic infections do not present with symptoms, due to the host's immunity or other factors. However the infection can still be spread to mosquitoes because the host has circulating parasites in their blood (Chen et al., 2016). Uncomplicated

malaria is characterized by the classic malaria symptoms (described in the next section), but with no signs of severe disease. All species of *Plasmodium* can cause asymptomatic infections and uncomplicated malaria (Phillips et al., 2017). Severe malaria includes the symptoms of uncomplicated malaria in addition to multi-organ complications (Wassmer and Grau, 2017). Severe malaria is predominantly caused by *P. falciparum* but *P. vivax* and *P. knowlesi* can also cause severe disease (Phillips et al., 2017).

1.1.4.1 Uncomplicated P. falciparum malaria

The severity of *Plasmodium* spp. infection varies greatly depending on the species and a multitude of host factors, including inherited factors of resistance (such as sickle hemoglobin trait), age, prior exposure, and immunity levels (Bruce-Chwatt, 1985). In general, uncomplicated malaria is infamously recognized for its classic symptoms: acute fevers, sweats, headache, myalgia, fatigue, nausea, and vomiting. The symptoms are notorious for their paroxysmal, or sudden, onset.

The classic fevers observed in malaria infections correspond to the intraerythrocytic life cycle of the *Plasmodium* species. There are no clinical symptoms associated with the exoerythrocytic stage (liver stage), the sexual stage (gametocytogenesis), or when sporozoites are injected into the host bloodstream, aside from mild inflammation at the mosquito bite site (Bruce-Chwatt, 1985). In *P. falciparum*, *P. vivax*, and *P. ovale* infections, the intraerythrocytic life cycle is ~48 hours, which corresponds to fevers every third day (tertian). *P. malaria* has a 72 hour lifecycle with fevers every fourth day (quartan) and *P. knowlesi* has a 24 hour life cycle with fevers spiking daily (quotidian) (Bruce-Chwatt, 1985). The febrile episodes are the body's response to the rupture of infected erythrocytes. Although the *P. falciparum* life cycle is ~48 hours, the fevers are often irregular and do not adhere to the periodicity observed with respect to infection by the other *Plasmodium* species (Bartoloni and Zammarchi, 2012). All symptoms are also non-specific and could be caused by a plethora of other infectious diseases present in malaria endemic regions (for example, dengue, chikungunya, bacterial septicaemia and typhoid). The physical findings are also nonspecific and can include splenomegaly, hepatomegaly, jaundice, and abdominal pain. Therefore, malaria should be diagnosed by a laboratory test, particularly microscopy (Mathison and Pritt, 2017).

The gold standard for malaria diagnosis is to create a blood film, or smear, to directly examine patient blood for the presence of parasites (Mathison and Pritt, 2017). Blood smears

entail spreading a drop of malaria infected blood on a glass microscopy slide. The slides are then fixed in methanol and stained with a Giemsa solution. A component of Giemsa binds to the phosphate groups of DNA while other components in the Giemsa solution stain the nucleus and cytoplasm differently so they can be visualized by light microscopy. Since erythrocytes do not contain DNA, the only positively stained cells on a patient blood smear are white blood cells and malaria-infected erythrocytes. The microscopy examination can be performed immediately upon request and by any trained microscopist. As part of malaria control and elimination efforts, many countries have standardized training and protocols for performing microscopy-based malaria diagnoses. Figure 1.5 shows an example of a handbook for performing diagnostic malaria microscopy in Cambodia, for which the guidelines have been translated from English to Khmer, the official language.

Rapid diagnostic tests (RDTs) are another way to detect malaria infections quickly. These tests detect malaria-specific antigens in minutes, such as histidine-rich protein 2 (HRP2) (Wellems and Howard, 1986, Wellems and Howard, 1995), by using a drop of patient blood (Rock et al., 1987, Beadle et al., 1994, Shiff et al., 1993). Because RDTs can have false negatives, they should always be combined with microscopy. Additionally, multiple studies within the last three years have noted *P. falciparum* infections with deleted *HRP2/3* genes (Mathison and Pritt, 2017, World Health Organization, 2018, World Health Organization, 2017a), necessitating the need for microscopy to be jointly performed alongside RDTs and also demonstrating a need for new diagnostic tests to be developed.

There is also the possibility that malaria infections are undetectable by microscopy, as often seen with asymptomatic malaria infections. Many studies have developed various PCR, quantitative (qPCR), and droplet digital PCR (ddPCR) methods to detect lower limits of parasitemia (Tham et al., 1999, Lee et al., 2002, Koepfli et al., 2016). However, these methods do not offer the rapid results that RDTs and microscopy can provide and they also require more advanced laboratory equipment and materials that may not be available in the field. Advancements in diagnostic tests would greatly aid malaria elimination efforts and it will be interesting to see how the field changes in the next decade as countries push for malaria eradication.
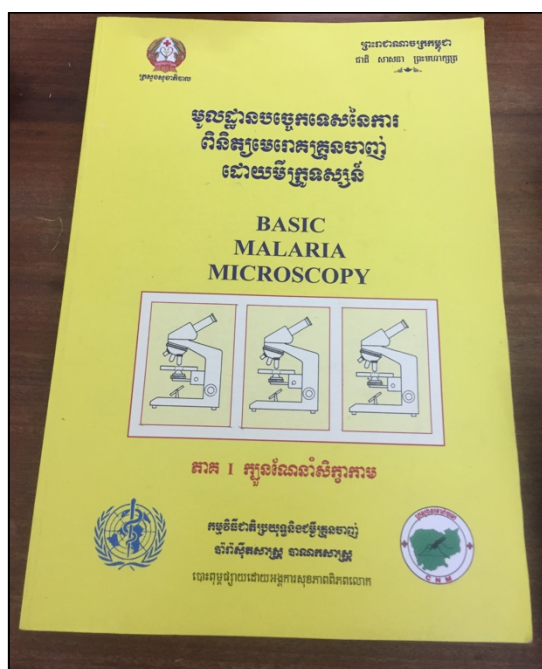
Figure 1. 5. Microscopy-based malaria diagnosis is the gold standard in all malaria endemic countries. This is a microscopy book that is used to train microscopists in Pursat Province, Cambodia. The *P. falciparum* isolates used in this dissertation were obtained from field sites in Cambodia and the photograph was taken during a field site visit to Pursat Province.

1.1.4.2 Severe *P. falciparum* malaria

P. falciparum is responsible for most cases of severe malaria, which often results in death. Young children are the population most impacted by severe disease but adults can progress to severe malaria as well. The pathophysiology of severe malaria is complex and not fully understood, but in general it is characterized by three coinciding conditions: severe anemia, metabolic acidosis, and cerebral malaria (Miller et al., 2013). Within the last two decades, research has also demonstrated that severe malaria results in multi-organ damage not limited to the brain and includes other organs such as the kidneys and pulmonary system (Miller et al., 2002, Wassmer and Grau, 2017, Wassmer et al., 2015).

One of the major causes of *P. falciparum* pathogenesis in severe malaria is its ability to cause infected erythrocytes to adhere to the endothelial lining of blood vessels, including those in the brain which results in cerebral malaria (Wassmer and Grau, 2017, Wassmer et al., 2015). This sequestration of infected erythrocytes drastically affects blood perfusion throughout the body. The effects of this cause different clinical presentation in children and adults, with complications in children predominantly resulting in cerebral malaria, seizures, hypoglycemia, and severe anemia. In adults, there is often kidney failure, jaundice, and pulmonary edema (Wassmer et al., 2015). Metabolic acidosis is a key symptom in both children and adults (Miller et al., 2002). Severe malaria is caused by the asexual

intraerythrocytic stages of the parasite and death often occurs within 48 hours after presentation of the disease (White, 2004). This means the parasites present at the onset of severe malaria symptoms will ultimately contribute to death, so preventing their maturation and reinvasion is vital.

In the context of severe malaria, it is important that the drugs are stage-specific to prevent the ring stages from progressing and the parasitemia from increasing. In the context of uncomplicated malaria, it is important to lower parasitemia as fast as possible, and prevent the likelihood that the disease will become severe (White, 2004). The way this is achieved is chemotherapy.

1.2 Antimalarial chemotherapy

The current frontline treatments for uncomplicated *P. falciparum* malaria worldwide are artemisinin combination therapies (ACTs) (World Health Organization, 2018, World Health Organization, 2015). ACTs combine a potent short-acting artemisinin derivative with a long-acting partner drug with a different mechanism of action. Severe malaria is treated with intramuscular (IM) or intravenous (IV) artesunate, an artemisinin derivative, for at least 24 hours followed by standard course of an ACT (World Health Organization, 2018).

P. falciparum has demonstrated resistance to nearly every antimalarial drug it has come in contact with and presently, decreased susceptibility of the parasite to both artemisinin and several commonly used partner drugs has been reported in multiple countries in SEA (Amaratunga et al., 2016, Dondorp et al., 2009, Denis et al., 2006, van der Pluijm et al., 2019, Blasco et al., 2017). This is of great concern as there are a limited number of drugs available to treat uncomplicated and severe malaria. In order to put antimalarial drug resistance into perspective, it is first necessary to delve into the history of antimalarial drug therapies.

1.2.1. Brief history of widely used antimalarial drugs: from monotherapies to combination therapies

1.2.1.1 Quinine

Extracted from the bitter bark of a cinchona tree, quinine was the first treatment discovered for malaria in the 1600s (**Figure 1.6**) (Tse et al., 2019, Butler et al., 2010, Arrow, 2004). The compound responsible for the bark's antimalarial action was first discovered by French

chemists, Joseph Pelletier and Jean Biename Caventou in 1820 and the structure of this compound from nature has inspired many synthetic treatments for malaria (Arrow, 2004).

Early anecdotal observations of quinine resistance were recorded in 1884 and 1910 (Talisuna et al., 2004). In the 1960s, quinine resistance was reported in human volunteers in SEA and Brazil, and later associated with treatment failures in SEA in the 1980s-90s (Peters, 1970, Peters, 1987, Pukrittayakamee et al., 1994). More recently, treatment failures were observed in Uganda when comparing quinine to the ACT, artemether-lumefantrine (Achan et al., 2009). In spite of intermittent periods of resistance, quinine is still an effective treatment for malaria in many regions and can still be used as a "drug of last resort" to treat severe malaria when an artemisinin derivative is unavailable (Achan et al., 2011, Schlitzer, 2007, Bloland, 2001). One reason for the continued efficacy of quinine is likely due to decreased use of the drug as newer drugs with higher therapeutic efficacy and less toxicities have become available (Achan et al., 2011). Quinine has severe side effects, both at standard therapeutic levels and when overdosed, which include tinnitus, blurred vision, confusion, vertigo, skin rashes, cardiotoxicity, nausea, and many additional symptoms referred to broadly as cinchonism (Achan et al., 2011). The mechanism of quinine action remains to be fully elucidated, but it is postulated to exert its antiplasmodial effects by accumulating in the parasite's digestive vacuole and interfering in heme detoxification (**Figure 1.7**) (Blasco et al., 2017, Greenwood et al., 2008).

Quinine remained the only available drug to treat malaria until the 20th century (Talisuna et al., 2004, Tse et al., 2019). This changed after the First World War when Germany made a push to develop synthetic antimalarial drugs after suffering huge losses to malaria during the war because of their inability to access quinine (Butler et al., 2010). The process to find quinine analogues that would be toxic to the malaria parasite began years before the war with the dye, methylene blue. Based on the compound's similarity to quinine and it's toxic effects on malaria parasites, it was used as a structural basis for synthesizing quinine-like compounds. (Presently, methylene blue is back in the spotlight and being considered in various combinations for malaria treatment (Tse et al., 2019). Shortly after the war in the early 1930s, mepacrine (also called quinacrine) and plasmochin (also called pamaquine) were the first synthetic antimalarial compounds synthesized as quinine analogues (Schlitzer, 2007). Plasmochin was not widely used due to toxicities, but mepacrine was used during the Second World War as an antimalarial prophylactic (Tse et al., 2019). Mepacrine also has severe side effects, including psychosis and yellow staining of the skin and eyes (Tse et al.,

2019). Both plasmochin and mepacrine are no longer used for malaria prophylaxis or treatment, however the aminoquinoline-based structures of both plasmochin and mepacrine led to two of the most commonly used antimalarial drugs: primaquine and chloroquine. Modifications of the plasmochin structure led to primaquine, and mepacrine helped to inspire the discovery of chloroquine (Schlitzer, 2007).

Chloroquine (originally called resochin) was synthesized in 1934 by the group of German chemists employed to synthesize a new quinine-like compound (**Figure 1.6**), but it was believed to be too toxic. Sontochin (3-methylchloroquine) followed after chloroquine and it was believed to have fewer side effects, so it was given to German troops during the Second World War. During the war, the Allies acquired sontochin and when Americans tried to increase the drug's efficacy, they synthesized what they thought was a new drug, chloroquine. However, upon comparing resochin and chloroquine, they realized they were the same drug (Arrow, 2004). Similar to quinine's proposed mechanism of action, chloroquine inhibits heme detoxification (Wellems and Plowe, 2001). Chloroquine has also been shown to accumulate in the parasite digestive vacuole (**Figure 1.7**) (Ridley, 1998, Sullivan et al., 1996).

1.2.1.2 Chloroquine
The deployment of chloroquine for the treatment of malaria had an enormous impact on the eradication of malaria in many countries in the first half of the 20th century. Chloroquine's potential was fully realized in the 1950s-1960s during WHO's Global Malaria Eradication Program (GMEP) (Wellems and Plowe, 2001, Croft, 1999). The GMEP (1955-1969) was immensely successful in eliminating malaria in multiple countries by combining the affordable chloroquine with the deployment of the insecticide, dichlorodiphenyltrichloroethane (DDT), and malaria case surveillance. The program eliminated malaria in North America, Europe, the Caribbean, parts of Asia, and Central America (Croft, 1999). However, many African countries were not included in the GMEP (the program did not involve these countries due to logistical/"technical" challenges) and thus no successes were observed in Africa (World Health Organization, 2001, Alonso et al., 2011). The GMEP ended in 1969 due to the emergence of both chloroquine resistance and insecticide resistance combined with the lack of organization for the necessary infrastructure and surveillance measures within individual countries—demonstrating that a single approach cannot be used globally long-term (Talisuna et al., 2004, Alonso et al., 2011).

Chloroquine resistance emerged from four independent locations (Wellems and Plowe, 2001, Talisuna et al., 2004). The first was in SEA on the Thai-Cambodian border in 1957 (Harinasuta et al., 1965). Around the same time period, another foci was reported in South America in Colombia and Venezuela (Payne, 1987). The last two emergences were in Papua New Guinea (1970s) and in Africa beginning in Kenya and Tanzania in 1978 (Peters, 1987, Talisuna et al., 2004). From these locations, chloroquine-resistant *P. falciparum* spread throughout endemic areas.

1.2.1.3 Antifolates: sulfadoxine/pyrimethamine (SP), proguanil
A combination of antifolate drugs were also used to treat malaria in response to the reduced efficacy of chloroquine. These drugs function by blocking steps in the folate synthesis pathway in bacteria, protozoa, and fungi. The pyrimidine analogue, proguanil (synthesized in 1945), was a product of the push to generate new drugs after the Second World War (Tse et al., 2019, Arrow, 2004). In the 1950s-1960s, pyrimethamine and sulfadoxine were also developed. When the drugs were introduced as monotherapies, it was quickly realized that they could not be used alone. Sulfadoxine and pyrimethamine (SP) were then combined to reduce the likelihood of drug resistance, but resistance continued to develop rapidly. In 1967, SP was introduced in Thailand and resistance was documented in the same year (Wernsdorfer and Payne, 1991). Today, SP is not used to treat malaria unless it is combined with an artemisinin derivative, most commonly with artesunate. SP is also used successfully as intermittent preventive treatment in pregnancy (IPTp) (World Health Organization, 2018, World Health Organization, 2015). IPTp is a public health measure designed to prevent malaria in pregnant women by administering antimalarial drugs during their pregnancy. It is recommended by WHO that pregnant women in moderate to high transmission areas in Africa receive IPTp at the beginning of their second trimester until the end of their pregnancy.

With widespread chloroquine resistance and the unreliable efficacy of SP, the pressure to find new antimalarial drugs was greatly increased (again at a time of war) during the Vietnam War, when all sides were subjected to the malaria scourge. During this time period, the US Army Medical Research and Development Command, the Walter Reed Army Institute of Research (WRAIR), and WHO sought to discover new drugs as well as promote research into parasitology and malariology, for which efforts had died down after the GMEP (Alonso et al., 2011). Concurrently, the Chinese government, which was supporting the North Vietnamese in the war, launched Project 523 recruiting hundreds of chemists, scientists,

pharmacologists, doctors, and other biomedical experts to discover new antimalarial drugs (Butler et al., 2010). It was through this project that the next drug to revolutionize the malaria field was discovered: artemisinin (Tu, 2011).

1.2.1.4 Artemisinin

Artemisinin was discovered by Tu Youyou in 1972 as part of the efforts of Project 523. Tu extracted the compound from the *Artemisia annua* plant, commonly referred to as sweet wormwood or *qinghao* in Chinese (Tu, 2011). The plant had been used by Chinese herbalists for thousands of years as a treatment for a variety of maladies and around 300 AD, Ge Hong published the use of *qinghao* for fevers in the book, *A Handbook of Prescriptions for Emergencies* (Tu, 2011). Similar to quinine, another natural compound that had been known for its antipyretic properties, artemisinin was re-"discovered" and chemically isolated for the treatment of malaria. In contrast to quinine, artemisinins have few side effects and are well tolerated (White, 2008). Artemisinins are the fastest-acting, most potent malarial drugs available. The compounds are active against all erythrocytic asexual stages of parasite development and have also been shown to kill sexual stage gametocytes (Skinner et al., 1996, Adjalley et al., 2011), but they have no effect on liver stages (Meister et al., 2011). The mechanism of action of artemisinin is the topic of continued debate and remains a complex, to-be-continued story (Meshnick, 2002, Cui and Su, 2009). Artemisinins are prodrugs and one theory is when artemisinin is activated (some theories believe it is activated by heme in the erythrocyte (**Figure 1.7**) (Wang et al., 2015, Meshnick, 2002), it generates toxic free radicals or reactive oxygen species (ROS) that cause damage to any nearby cellular targets (Tilley et al., 2016, Blasco et al., 2017). Additional studies have also suggested that activated artemisinins have many targets they can bind resulting of alkylation of both lipids and proteins (Wang et al., 2015). Thus, artemisinin likely has many ways through which it exerts its antiparasitic effects.

Early clinical trials by Tu and colleagues in the 1970s showed that artemisinin was very effective at clearing parasites, however some recrudescences were observed (Cui and Su, 2009). Recrudescence means that the malaria infection has persisted at an undetectable level and when it reoccurs at a detectable level, it is termed a recrudescent infection. In spite of the recrudescences, the potency of artemisinin prompted the development of several artemisinin derivatives that are still recommended for malaria treatment today: dihydroartemisinin (which is the active metabolite of artemisinin), artemether, and artesunate (World Health Organization, 2015).

The short half-lives of artemisinin derivatives (~1 hour) are believed to be one of the main reasons why recrudescences are observed (Cui and Su, 2009). Because the Chinese scientists were aware of the pitfalls of artemisinin monotherapy early on, they performed multiple efficacy studies to optimize treatment regimens. In comparing 3-, 5-, and 7-day artemisinin monotherapy efficacy studies, Li *et al*. (Li et al., 1984) found that a 7-day course of artemisinin was most effective with only 5% treatment failures (Cui and Su, 2009, Li et al., 1984). Acknowledging that administering artemisinin monotherapy over the course of one week was neither feasible for patient compliance nor cost-efficient, Li and colleagues also tested artemisinin combination therapies (Li et al., 1984). Li *et al*. found the combination therapy artesunate-piperaquine to be most successful and after further optimization, artesunate was replaced with dihydroartemisinin and the two drugs, dihydroartemisinin-piperaquine, were co-formulated and first produced in Vietnam as CV8 in 1997 (Cui and Su, 2009). After slight changes of the drug dosages based on WHO recommendations, the combination therapy was marketed as Artekin® (Cui and Su, 2009). These early findings in the 1980s/90s set the stage for the current antimalarial regimens and combination therapies used today.

1.2.2.1 Combination therapies in malaria: ACTs

Combination therapies are common treatment regimens for many diseases, including tuberculosis, HIV, cancer, and autoimmune diseases (Ascierto and Marincola, 2011). The justification for combination therapies is that by using two or more drugs with different mechanisms of action, resistance is less likely to develop. Another potential advantage of a combination therapy is that the course of the disease is likely to be lessened in duration and/or severity as a result of more rapidly killing/inhibiting the causal agent. One of the classic examples of a disease in which multidrug therapies are standard course is tuberculosis. As early as the 1940s/50s, multiple drugs were used (and needed) to effectively cure patients infected with *Mycobacterium tuberculosis*, the causal agent of tuberculosis (Kerantzas and Jacobs, 2017).

If combination therapies were common in tuberculosis, why did it take decades after the emergence of chloroquine resistance for the malaria field to use combination therapies for the treatment of malaria? Perhaps it was the lack of available antimalarial drugs or the toxicities (quinine) that some drugs cause when used alone. Or, maybe it was the concern that using multiple drugs might cause the development of resistance to all currently available drugs? As seen in the case of tuberculosis, the disease is treated with four drugs, and multidrug

resistance to all four drugs, termed excessively drug-resistant tuberculosis (XDR-TB) has emerged and become a significant global health concern (Johnston et al., 2009). Alternatively, perhaps the cost of multidrug therapies was the prohibitive barrier. Although SP, described above, was a combination of two drugs, it was not considered a combination therapy in the true sense of the regimen, for SP combined drugs with similar mechanisms of action. Of note, several studies also combined mefloquine with SP in the 1980s when SP was largely failing (Khim et al., 2005). However, the first regulated and recommended combination therapies for the treatment of malaria were ACTs.

In 2001, WHO recommended ACTs as the first-line treatment for uncomplicated *P. falciparum* malaria (World Health Organization, 2001). Currently, there are five ACTs recommended by WHO: artemether + lumefantrine; artesunate + mefloquine; artesunate + amodiaquine; dihydroartemisinin + piperaquine; and artesunate + sulfadoxine/pyrimethamine. A sixth ACT, artesunate + pyronaridine, has received positive reviews (Pryce and Hine, 2019) and is under consideration for being recommended by WHO (World Health Organization, 2018).

The rationale behind ACTs is that the potent artemisinin compound is responsible for clearing most parasites in the first several days of treatment while the partner drug, which has a different mechanism of action and a longer half-life, clears any remaining parasites. All ACTs are administered over a 3-day treatment course. This is designed to promote treatment efficacy and patient compliance as well as reduce the risk of resistance (World Health Organization, 2018, World Health Organization, 2015, World Health Organization, 2001). As mentioned above, the mode of action of artemisinin is still unknown but it has been suggested that the parasite rapidly kills parasites by actively damaging nearby proteins and other targets within the parasite (Wang et al., 2015). Similar to artemisinin, the exact mechanism of the partner drugs is unknown, but all are posited to target hemoglobin degradation or the detoxification of heme in the parasite digestive vacuole (Greenwood et al., 2008, Blasco et al., 2017) (**Figure 1.7**). This is not entirely surprising based on the chemical structures of the drugs. Piperaquine (PPQ) and amodiaquine (AQ) are 4-aminoquinilones, like chloroquine. Lumefantrine (LUM) and mefloquine (MQ) are aryl amino alcohols, like quinine (**Figure 1.6**). With the 3-day treatment course, any parasites that survive treatment and reinvade after 48 hours would still be exposed to a maximal dose of artemisinin on day 3 as well as to the partner drugs. After the artemisinin derivative has been rapidly cleared from the patient's system, the partner drugs are still at maximal concentration clear any additional

parasites over the next couple of days to weeks depending on the drug. The half-lives of the recommended partner drugs range from ~4 days to over 20 days. Mefloquine and piperaquine have the longest half-lives around ~21-28 days (Tarning et al., 2008), the half-life of amodiaquine is ~7 days (Stepniewska et al., 2009), lumefantrine is ~5 days, and sulfadoxine and pyrimethamine half-lives are ~4 to ~8 days, respectively.
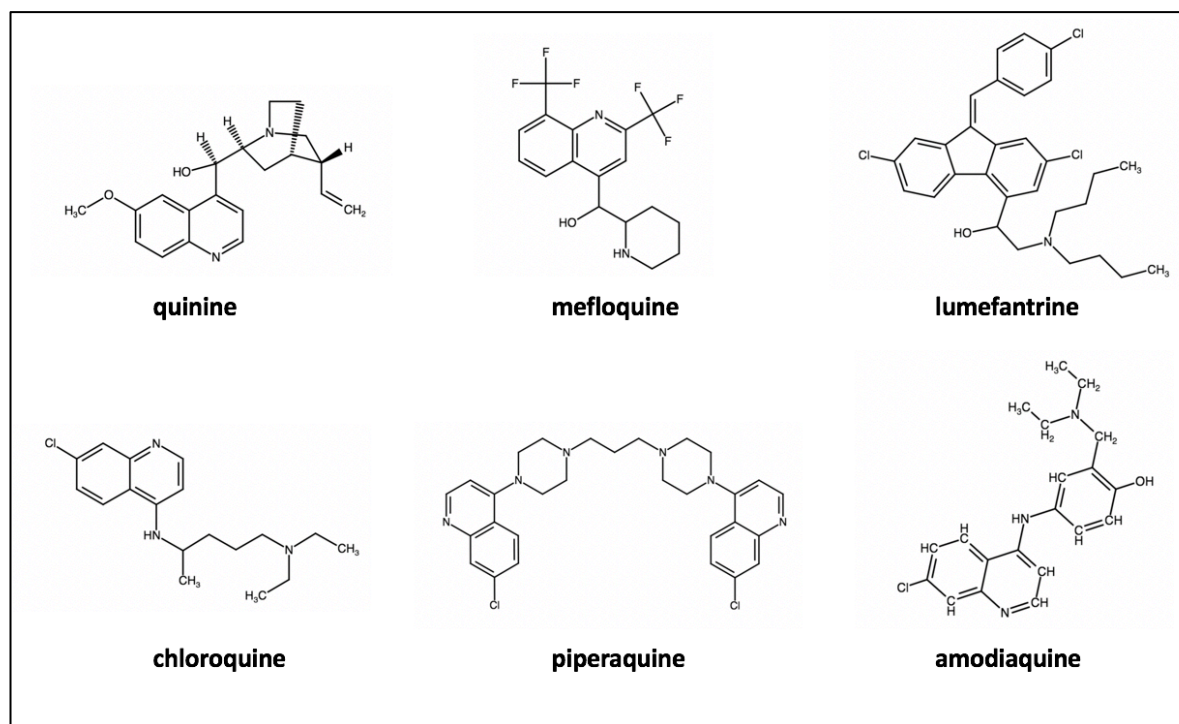

Figure 1. 6. Structural similarities of antimalarial drugs.

1.2.2.2 ACTs: dihydroartemisinin-piperaquine

The combination therapy that is the focus of this study is dihydroartemisinin-piperaquine (DHA-PPQ). As mentioned above, DHA-PPQ was first co-formulated by Li *et al*. in the late 1980s/90s (Cui and Su, 2009), however piperaquine was first synthesized in the 1960s (Davis et al., 2005). Piperaquine is a bisquinoline drug that was synthesized independently in China at the Shanghai Research Institute of Pharmaceutical Industry and in France at Rhone-Poulenc. Structurally, it resembles two linked chloroquine molecules (**Figure 1.6**). In China, it was used as frontline antimalarial monotherapy and as prophylaxis starting in 1978. However, by the 1980s piperaquine-resistant parasites emerged, so the monotherapy was discontinued (Davis et al., 2005). It was not until the studies by Li *et al.* with CV8 that piperaquine re-emerged as an antimalarial drug contender in combination therapies (Davis et al., 2005).
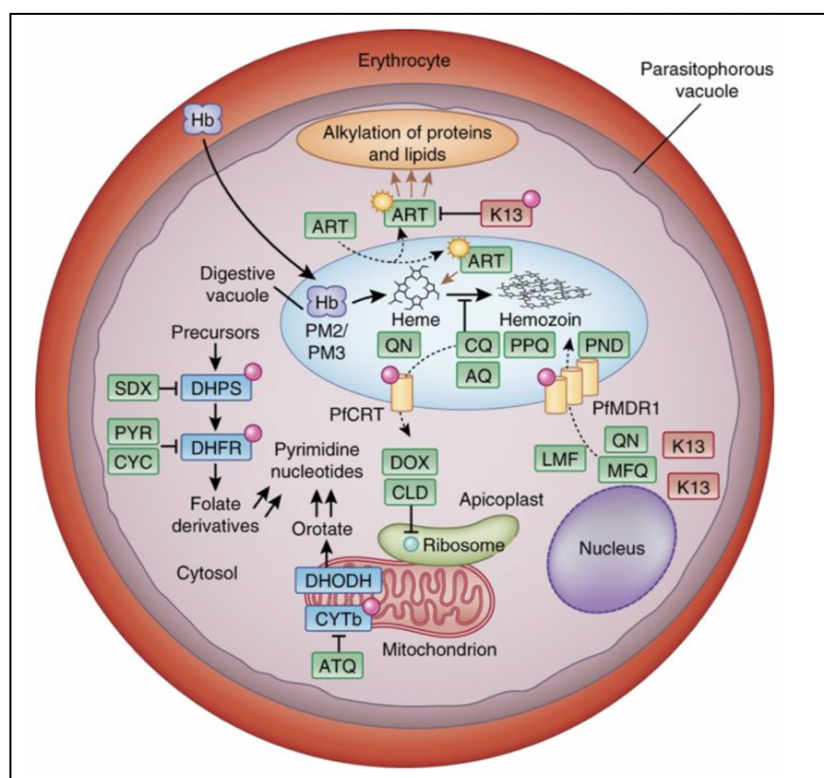
Figure 1. 7. Antimalarial drugs depicted with their proposed mechanism of action in the erythrocytic (asexual) blood stage of *P. falciparum*. The process of hemoglobin degradation is depicted in the parasite digestive vacuole (light blue) and common antimalarial drugs (green) are depicted near their proposed site of action. Of note, chloroquine (CQ) and piperaquine (PPQ) are shown interfering with heme detoxification. PfCRT (yellow) is shown on the digestive vacuole membrane with a mutation (red circle) that enables the efflux of CQ. PfMDR1 (yellow) is also shown on the digestive vacuole membrane (with increased copies) transporting mefloquine (MFQ) into the vacuole. Artemisinin (ART) is depicted in its proposed mode of action that causes oxidative stress and alkylation of proteins and lipids. This figure was reprinted from Blasco *et al*. (Blasco et al., 2017) with permission from Springer Nature Publishing.

Since their implementation, ACTs have been responsible for the immense decline of *P. falciparum* malaria mortality and transmission worldwide (World Health Organization, 2018, World Health Organization, 2015). However, over the last decade, both artemisinin resistance and resistance to two commonly used partner drugs, mefloquine and piperaquine, has been reported in SEA (Blasco et al., 2017, van der Pluijm et al., 2019, Wongsrichanalai and Meshnick, 2008). Western Cambodia and the region along the Thai-Cambodian border are the sites where ACT treatment failures were first reported (Noedl et al., 2008). SEA has long been regarded one of the prominent foci of drug-resistant malaria. As detailed above, resistance to many of the former frontline therapies (quinine, chloroquine, SP) emerged and spread throughout SEA, with all three treatment failures materializing first on the Thai-Cambodian border. Unlike the development of chloroquine resistance, which emerged in multiple independent locations (in South America at the same time as SEA), ACT treatment

failures are contained in SEA, at least for the time being. These failures severely threaten the disease's ability to be qualified as "treatable," for few other drugs are available as viable options. In order to understand the current setting of ACT resistance in SEA, it is necessary to understand the history of drug resistance in Cambodia and the genetic landscape through which parasites have developed resistance.

1.3 Drug-resistance in *P. falciparum*

1.3.1 ACT failures: emergence of artemisinin and partner drug resistance in Cambodia

In 2001, Cambodia was one of the first countries to implement ACTs with the inclusion of artesunate-mefloquine (AS-MQ) in their nationwide treatment guidelines (**Figure 1.8**) (World Health Organization, 2001). Less than four years after AS-MQ implementation, therapeutic efficacy studies reported >10% failure rates from 2001-2004 (Denis et al., 2006, Wongsrichanalai and Meshnick, 2008). Mefloquine was used as a monotherapy in Cambodia in the early 1990s (**Figure 1.8**) and was stopped due to resistance in the early 2000s, shortly before AS-MQ was introduced (Wongsrichanalai and Meshnick, 2008, Lim et al., 2009). It is therefore not entirely surprising that the combination treatment eventually failed. This caused a shift from AS-MQ to DHA-PPQ therapy in 2008 and DHA-PPQ was implemented nationwide in 2010 (**Figure 1.8**). By 2013, DHA-PPQ showed significant failure rates, specifically in Western Cambodia where rates were nearly 50% (Amaratunga et al., 2016, Leang et al., 2015, Saunders et al., 2014, Spring et al., 2015). This prompted an efficacy study by Amaratunga *et al.* in 2014-2015 to determine if AS-MQ could be reintroduced in Cambodia (Amaratunga et al., 2019). This study showed that AS-MQ was highly effective with recrudescence reported in only 1 patient out of 296 (Amaratunga et al., 2019). The continued reports of DHA-PPQ failures (van der Pluijm et al., 2019) and the demonstration that parasites returned to AS-MQ susceptibility prompted the reintroduction of AS-MQ and the use of triple-ACTs (TACTs) in Cambodia from 2015 to the present day (World Health Organization, 2017b). The triple therapy includes DHA-PPQ + mefloquine and has been shown to be efficacious in patients with negligible toxicities (van der Pluijm et al., 2019).
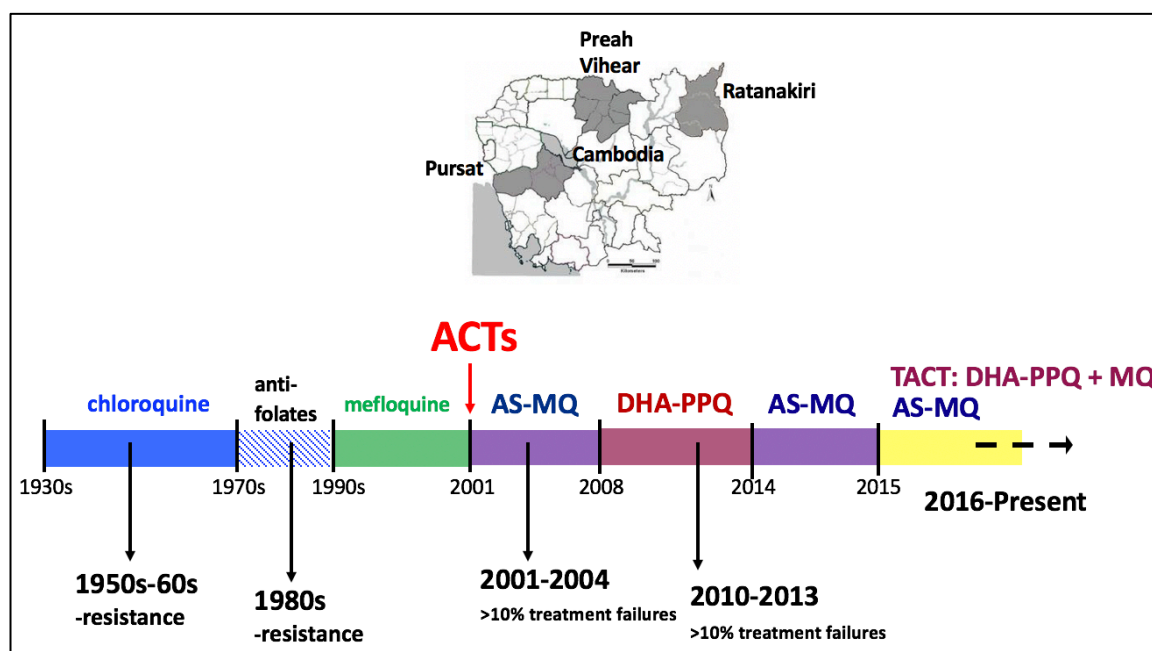
Figure 1. 8. Timeline of antimalarial drug use for the treatment of *P. falciparum* malaria in Cambodia. The period of time each drug has been used is indicated in color on the timeline. ACTs (red arrow) were introduced in 2001 in Cambodia. The approximate time widespread resistance developed (chloroquine and SP) or >10% treatment failures were first reported (AS-MQ (Denis et al., 2006) and DHA-PPQ (Amaratunga et al., 2016, Leang et al., 2013)) is indicated by the black arrows. Currently both triple ACTs (TACT) consisting of DHA-PPQ + MQ and AS-MQ are being used to treat uncomplicated *P. falciparum* malaria. A map of Cambodia is shown above the timeline and the shaded regions indicate three provinces where drug efficacy studies have been performed: from West to East, Pursat, Preah Vihear, and Ratanakiri. This dissertation has used parasite isolates from these three provinces and full details for the clinical sites will be discussed in Chapter 2 (General methodology).

In order to understand how piperaquine resistance emerged and continues to spread in SEA, it is necessary to identify and characterize genetic changes in parasite populations that promote increased survival to piperaquine.

1.3.2 Assessing drug-resistance in *P. falciparum*

Antimalarial drug resistance is defined as the ability of parasites to survive and/or multiply in the clinical setting despite treatment with the appropriate medication and dose. This definition necessarily includes that the parasite must have been exposed to the drug for the duration of time needed for the drug to exert its full antimalarial properties, ensuring drug compliance and proper absorption (taken with a fatty meal, etc.). This verifies that resistance is due to parasite adaptations and not due to the inability of the drug to "gain access to the parasite" (Bloland, 2001, Bruce-Chwatt, 1986). In such *in vivo* drug efficacy studies, true resistance is confirmed only after both remaining parasitemia and adequate serum drug concentrations are documented (Talisuna et al., 2004, Bloland, 2001).

Apart from the clinical setting, general ways to study and measure drug resistance include: surrogate *in vitro* assays, animal models, and detection of molecular markers (for example, by PCR assays or genomic studies) (Bloland, 2001). Surrogate *in vitro* assays test the effect of compounds on the parasites outside of the human host in cell culture, usually by a measure of survival. In *Plasmodium*, this is most easily performed with *P. falciparum* and *P. knowlesi*, since they have been adapted to grow in cell culture (Trager and Jensen, 1976, Moon et al., 2013). *In vitro* tests enable direct parasite biology to be assessed in response to specific drugs in a controlled environment outside of a host, however some notable disadvantages are also inherent because they are outside of the host. For example, if a prodrug needs to be cleaved within the host to its active metabolite (such as proguanil), it cannot be tested unless the compound is available and stable in its active form (as is the case with DHA). There is also no way to test the effects that host immunity may have on infection in *in vitro* settings. Nevertheless, *in vitro* work has provided much insight into the complex biology of the malaria parasite. Animal models (such as mice or non-human primates) can support both parasite biology studies and transmission studies under *in vivo* conditions. Lastly, molecular genomics studies encompass many techniques and approaches to enable molecular markers of resistance to be identified. In turn, they provide fundamental insight for highlighting the genetic candidates and pathways that *in vitro* studies can investigate. Candidate molecular markers can consist of any genetic variation including, single nucleotide polymorphisms (SNPs), copy number variations, insertions or deletions in the genome, and other such variations that are associated with the resistant-phenotype. It is then up to functional studies to verify if these markers are causal determinants of resistance.

1.3.3 The genetic basis of resistance

Drug resistance is the result of spontaneous and rare genetic mutations that alter genes directly or change the expression of genes (White, 2004). Sometimes a single genetic event is enough to produce a resistant phenotype, other times multiple genes may be involved. Furthermore, the resistance may be associated with the genetic background (epistasis) (White, 2004). *P. falciparum* has an approximate spontaneous mutation rate of $1.0–9.7 \times 10^{-9}$ mutations per base pair per generation in asexual culture (Bopp et al., 2013). Considering an average malaria infection can present with around $\sim 10^{12}$ parasites in the blood stream (which is about 2% parasitemia in the bloodstream), it is likely that random mutations will occur during several asexual life cycles (Cowell and Winzeler, 2019, White, 2004). These mutation rates are in the absence of selective drug pressure and are thought to be independent of the drug used (White, 2004). However, in the presence of drug pressure, mutations that confer

both a survival and fitness advantage would be favored. ACT partner drugs with long half-lives, such as mefloquine and piperaquine offer a favorable selective environment because they remain in the bloodstream at suboptimal levels for weeks post initial infection.

There are many ways that parasites can develop resistance to antimalarial drugs. In general, two of the main mechanisms through which resistance is achieved are by: decreased availability of a drug at its target site (frequently due to mutations in transporter genes) and/or modification of the drug target (Paloque et al., 2016).

1.3.4 The parasite digestive vacuole and antimalarial drugs

Many antimalarial compounds, specifically ACT partner drugs, are believed to exert their effects in the parasite digestive vacuole by disrupting a crucial pathway: hemoglobin degradation (**Figure 1.7**). The digestive vacuole, located inside the parasite's cytosol and sometimes referred to as the food vacuole, is a membrane-bound organelle with an acidic pH. The digestive vacuole is the site of hemoglobin degradation (Wunderlich et al., 2012), during which 60-80% of the host cell hemoglobin is digested (Wunderlich et al., 2012). The parasite uses the degraded amino acids as a source of nutrients, since they have limited capabilities for *de novo* amino acid synthesis and exogenous uptake of amino acids (Goldberg et al., 1990).

Hemoglobin is the major protein in all erythrocytes that is responsible for carrying oxygenated blood throughout the body. Hemoglobin consists of four globin chains each containing a heme group, which consists of an iron ion in a heterocyclic ring surrounded by pyrrole molecules, called a porphyrin. In adults, hemoglobin A (HbA) is the most common form of hemoglobin and consists of two alpha globin chains and two beta globin chains. In SEA, hemoglobin E (HbE) is a very common variant and consists of two alpha globin chains and two mutant beta globin chains (Munkongdee et al., 2016). In Africa, hemoglobin S (HbS) consists of two alpha globin chains and two beta globin chains with a different mutation that results in sickle cell anemia. Individuals who are heterozygous for the sickle cell trait have protection from malaria infection (Weatherall and Clegg, 2001). In contrast, some studies have shown that HbE does not provide protective advantage against infection with *P. falciparum* malaria in studies in Thailand (Lithanatudom et al., 2016, Carter and Mendis, 2002) and in Cambodia (personal communication with Dr. Chanaki Amaratunga) where parasites may invade and develop normally in HbE erythrocytes. It is worth mentioning that some studies conducted in Thailand have found that it may be more difficult

for *P. falciparum* to invade HbE erythrocytes *in vitro* (Chotivanich et al., 2002). The authors suggest that although HbE does not prevent infection, it could prevent high parasitemias, however further *in vivo* and *in vitro* studies are necessary to further validate these findings.

When the parasite breaks down hemoglobin, heme is released as a toxic byproduct. To prevent oxidative damage from the reactive iron-heme groups, enzymes in the digestive vacuole convert (detoxify) the reactive heme to inert hemozoin crystals (Wunderlich et al., 2012). The hemozoin is what accounts for the characteristic brown pigment in malaria parasites that can be seen in the digestive vacuole when viewed by light microscopy. (This is the same pigment that Laveran and Ross distinguished when identifying *Plasmodium* in the blood and mosquitoes, respectively.) There are many different enzyme families that facilitate hemoglobin degradation and heme detoxification. Four plasmepsin enzymes (plasmepsins 1-4) are aspartic proteases that localize to the digestive vacuole and digest hemoglobin. Plasmepsins 1 and 2 initiate hemoglobin digestion and plasmepsins 3 and 4 aid in degradation after initial cleavage (Banerjee et al., 2002, Goldberg et al., 1991, Francis et al., 1997). Falcilysins and falcipains, cysteine proteases and metalloproteases, respectively, function downstream of the plasmepsins to further degrade hemoglobin (Subramanian et al., 2009, Eggleson et al., 1999). Another enzyme, heme detoxification protein (HDP) helps to generate inert hemozoin crystals from heme (Jani et al., 2008, Nakatani et al., 2014). Studies have also suggested a role for a glutathione-dependent process of heme degradation (Ginsburg et al., 1998). These are just a few examples of the various proteins and often overlapping processes that take place to achieve hemoglobin metabolism in the digestive vacuole. Such overlapping and redundant functions of many hemoglobin-degrading proteins may explain why many genes encoding these enzymes can be knocked out (Moura et al., 2009, Omara-Opyene et al., 2004, Bonilla et al., 2007) without compromising parasite viability.

Multiple transporters and ion pumps are also located on the membrane of the digestive vacuole. Two transporters that function in drug resistance are the chloroquine resistance transporter, PfCRT and the multidrug resistance protein 1 (PfMDR1). PfCRT has been shown to efflux substrates from the digestive vacuole into the parasite cytosol, while PfMDR1 has been shown to import substrates from the cytosol into the digestive vacuole (Bray et al., 2005, Ecker et al., 2012, Valderramos and Fidock, 2006, Reiling and Rohrbach, 2015).

In order to understand the mechanisms of drug resistance and prevent the emergence and spread of resistance, it is important to discover the genetic determinants of resistance and their corresponding markers.

1.4 Genetic background and mechanisms of resistance in SEA

1.4.1 Chloroquine

Chloroquine is a weak base and its mode of action is believed to be through its localization to the parasite's acidic digestive vacuole, where it disrupts heme detoxification (Wellems and Plowe, 2001). When chloroquine is inside the acidic vacuole, it becomes protonated and builds up inside the organelle (Homewood et al., 1972, Yayon et al., 1984). Once inside the digestive vacuole, it disrupts heme detoxification and results in parasite death due to the build-up of its own toxic byproduct (**Figure 1.7**) (Wellems and Plowe, 2001).

The mechanism of chloroquine resistance is through the parasite's ability to prevent the accumulation of chloroquine in the digestive vacuole. This is accomplished via the transporter, PfCRT (Wellems et al., 1991). PfCRT is a highly polymorphic protein with 10 transmembrane domains consisting of 424 amino acids located on chromosome 7 (PF3D7_0709000). Mutations in the *pfcrt* gene, specifically K76T, were found to play a role in chloroquine resistance *in vitro* and were also associated with clinical resistance in field studies across endemic regions (Fidock et al., 2000, Carlton et al., 2001, Bray et al., 2005, Picot et al., 2009). The switch from the positively charged lysine residue to the uncharged threonine residue enables protonated-chloroquine to be effluxed from the digestive vacuole (Lehane and Kirk, 2008, Martin and Kirk, 2004, Martin et al., 2009). The PfCRT K76T mutation is found in almost all chloroquine-resistant isolates, regardless of geographic location.

Mutations in the multidrug transporter, PfMDR1, have also been associated with decreased susceptibility to chloroquine. PfMDR1 is a transporter in the ATP-binding cassette (ABC) transporter family consisting of 1419 amino acids on chromosome 5 (PF3D7_0523000) encoding a predicted 12 transmembrane domain protein. The homologous protein in mammalian cells is often referred to as P-glycoprotein (P-gp). Several studies have found an N86Y mutation in *pfmdr1* that, when jointly present with PfCRT mutations, decreases parasite susceptibility to chloroquine (Foote et al., 1990, Babiker et al., 2001, Sa et al., 2009). Molecular studies have found that editing the *pfmdr1* N86Y mutation into chloroquine-resistant parasites decreases the parasites susceptibility to chloroquine (Veiga et al., 2016a).

However, additional work has shown that the N86Y mutation alone is not enough to confer chloroquine resistance (Djimde et al., 2001, Valderramos and Fidock, 2006) and further work is needed to assess the role of the *pfmdr1-N86Y* mutation in modulating chloroquine resistance in the field.

Recent studies have found that the PfCRT K76T mutation has reverted to a majority of wildtype after removal of chloroquine pressure in some countries in Africa (Mohammed et al., 2013, Mwanza et al., 2016) and China (Wang et al., 2005). However, the mutation remains at some level nearly everywhere (SEA, South America, and Africa). Residual persistence at low levels could be due to the continued use of chloroquine for symptomatic relief or for the treatment of presumed *P. vivax*. An alternative reason could be that the mutation is fixed in a population, because the sensitive parasites without the mutation did not survive (Cowell and Winzeler, 2019). Notably, studies by Pelleau *et al.* in French Guiana have found that in spite of the fixed PfCRT K76T allele, parasites are again chloroquine susceptible and they have identified a PfCRT C350R mutation that associates with the chloroquine-sensitive phenotype (Pelleau et al., 2015). This study also found that the PfCRT C350R mutation decreased parasite susceptibility to piperaquine *in vitro*. They hypothesized that this mutation could be due to increased pressure from piperaquine, as DHA-PPQ is available in the region (Pelleau et al., 2015).

In the context of chloroquine-resistance, mutations in PfCRT and PfMDR1 have both served as molecular markers of potential resistance, with PfCRT having the causal role in resistance. It was on this genetic background that mefloquine was introduced as a monotherapy and later as a partner of ACTs in Cambodia.

1.4.2 Mefloquine: monotherapy and ACT

Mefloquine is an aryl amino alcohol that was synthesized as a derivative of quinine at the Walter Reed Army Institute of Research (WRAIR) shortly after the Vietnam War during the push to discover new antimalarials in the 1960s-1970s (Arrow, 2004). Five years after its introduction in SEA, mefloquine resistance was reported in Thailand in the early 1980s (Nosten et al., 1991, Boudreau et al., 1982).

Resistance to mefloquine has been shown to be the result of a *pfmdr1* copy number variation (**Figure 1.7**). Rather than a point mutation as observed in chloroquine resistance, mefloquine resistance results from increased copies of *pfmdr1* both in the field and *in vitro* (Oduola et al.,

1988a, Cowman et al., 1994, Reed et al., 2000, Lim et al., 2009, Price et al., 2004). Furthermore, increasing the number of *pfmdr1*copies, increases the level of resistance *in vitro* (Sidhu et al., 2006). The *pfmdr1* amplification may also result in cross-resistance to the partner drug, lumefantrine, which is another aryl amino alcohol. Some studies have found an association of artemether-lumefantrine treatment failures and increased *pfmdr1* copy numbers (Price et al., 2006). However, increased *pfmdr1* copy numbers are infrequent in African countries, where artemether-lumefantrine is often used (Cheeseman et al., 2016).

As widespread AS-MQ treatment failures occurred throughout SEA and countries were switching to DHA-PPQ, the list of molecular markers of drug resistance continued to grow. In addition to monitoring PfCRT mutations, studies began tracking PfMDR1 mutations and copy numbers.

1.4.3 DHA-PPQ: piperaquine resistance in concert with decreased artemisinin susceptibility

In 2008, decreased susceptibility of parasites to artemisinin *in vivo* was reported in Western Cambodia (Dondorp et al., 2009, Noedl et al., 2008). Over the next couple of years, decreased artemisinin susceptibility was documented at other sites in Cambodia, Thailand, Vietnam, Myanmar, Laos, and China (Fairhurst and Dondorp, 2016).

As briefly mentioned in the previous sections of this introduction, both the mechanism of action of artemisinin and the mechanism of artemisinin resistance have sparked continued debate and the discussion is ongoing. Multiple genome-wide association studies (GWAS) have been conducted to uncover molecular markers that associate with decreased artemisinin susceptibility *in vivo* and *in vitro* (Cheeseman et al., 2012, Takala-Harrison et al., 2013). Work by Ariey *et al*. identified mutations in the propeller domain of a kelch protein (K13) that associated with artemisinin resistance *in vivo* and *in vitro* (Ariey et al., 2014). The Tracking Resistance to Artemisinin Collaboration (TRAC) study further validated these studies in a multi-country collaboration that confirmed the association of several K13 mutations and decreased artemisinin susceptibility *in vivo* (Ashley et al., 2014). Genome editing studies also found an association between the K13 mutations and artemisinin resistance *in vitro*, as measured by a "ring-stage assay" that involves a short pulse of DHA on early ring stage parasites (Ghorbal et al., 2014, Straimer et al., 2015, Witkowski et al., 2013). Previous studies identified five K13 mutations that associated with the artemisinin-resistant phenotype (*K13-C580Y*; *K13-R539T*; *K13-Y493H*; *K13-I542T*; and *K13-P553L*) (Miotto et

al., 2015) and a more recent study by the Worldwide Antimalarial Research Network (WWARN) identified 20 *K13* mutations (WWARN, 2019).

The K13 protein (PF3D7_1343700) is postulated to serve a role in the parasite's response to oxidative stress through multiple protein-protein interactions (Ariey and Menard, 2019). The *P. falciparum* K13 protein is homologous to the human KLHL12 and KLHL2 proteins and Keap1, which function in ubiquitin-based degradation of proteins and oxidative stress responses, respectively (Dhanoa et al., 2013, Itoh et al., 1999). Recent findings have also raised the intriguing postulation that *P. falciparum* gametocytogenesis may be improved by certain K13 mutations, and thus promote transmission to mosquitoes as a parasite survival mechanism (Ashley et al., 2014, Lozano et al., 2018).

It should be emphasized there are multiple studies that have observed artemisinin-resistant phenotypes in the absence of K13 mutations, suggesting additional molecular determinants of resistance exist, and thus further work is necessary to determine if and how these may impact artemisinin resistance (Demas et al., 2018, Sa et al., 2018, Sutherland, 2017, Su et al., 2019).

1.4.4 Piperaquine resistance

Less than four years after the reports of decreased artemisinin susceptibility, DHA-PPQ treatment failures were reported in Western Cambodia (Spring et al., 2015, Amaratunga et al., 2016). Most recently, clinical studies have demonstrated very high treatment failures with an average of 50% treatment failures in parts of Cambodia, Vietnam, and Thailand (van der Pluijm et al., 2019).

Multiple GWAS studies were performed to identify potential molecular markers of piperaquine resistance. A GWAS of 297 Cambodian isolates revealed single-nucleotide polymorphisms (SNPs) on chromosome 13 in genes that encode a putative exonuclease (*exo-E415G*) (PF3D7_1362500) and a putative mitochondrial carrier protein (*mcp-N252D*) (PF3D7_1368700) that associate with reduced PPQ susceptibility *in vitro* and DHA-PPQ failures in patients (Amato et al., 2017). Additionally, a copy number variation (CNV) in the *plasmepsin 2* (*PM2*) and *plasmepsin 3* (*PM3*) genes on chromosome 14 associated with reduced susceptibility to piperaquine (Witkowski et al., 2017). Specifically, increased copies of *PM2-3* are associated with increased parasite survival under piperaquine pressure. Little is known about the putative exonuclease protein and mitochondrial carrier proteins on chromosome 13. However, as described above, previous work has determined a role for PM2

and PM3 in hemoglobin degradation in the parasite's digestive vacuole (Banerjee et al., 2002).

More recent studies have also demonstrated a role for mutations in the familiar transporter, PfCRT. Drug pressure experiments conducted over eight years ago identified a PfCRT-C101F mutation and a deamplification of *pfmdr1* in parasites that had been pressured with piperaquine *in vitro*. Genome editing of the C101F mutation in parasites decreased parasite susceptibility to piperaquine, but increased sensitivity to chloroquine (Dhingra et al., 2017). Another recent study identified three mutations in culture-adapted isolates from Cambodia that exhibited *ex vivo* piperaquine resistance: PfCRT H97Y; PfCRT M343L; and PfCRT G353V (Duru et al., 2015). Genome editing of these three mutations and an additional mutation observed in patient isolates, PfCRT F145I, was found to confer piperaquine resistance *in vitro* (Ross et al., 2018a). Additional GWAS studies have continued to find these mutations associated with piperaquine failures in patients and have also reported two new mutations associated with PPQ-resistance, PfCRT T93S and PfCRT I218F (Hamilton et al., 2019). These piperaquine resistance-associated mutations are all on the PfCRT K76T genetic background and the parasites still demonstrated reduced chloroquine susceptibility in *ex vivo* and *in vitro* survival assays (Amaratunga et al., 2016).

The piperaquine-resistant parasites isolated from Cambodia have single copies of *pfmdr1*, indicating that the parasite population reverted back to a single copy of *pfmdr1* after the removal of mefloquine pressure from the region. One hypothesis is that piperaquine gains access to the digestive vacuole through PfMDR1 (Witkowski et al., 2017), so reducing *pfmdr1* expression could reduce the amount of piperaquine in the digestive vacuole. It is also possible that the *pfmdr1* copy number expansion results in a fitness cost, so in the absence of mefloquine pressure, there is no need (and is metabolically disadvantageous) for the parasites to maintain it.

It is hypothesized that increased tolerance to artemisinin facilitated the emergence of piperaquine-resistant parasites. This is further supported by the genetic background of the parasites, as the majority piperaquine-resistant parasites have K13-C580Y mutations (Amaratunga et al., 2016, van der Pluijm et al., 2019). Out of 2465 parasite genomes, 82% of parasites that highly associated with the piperaquine-resistant phenotype were K13-C580Y, 5.4% were Y493H, and 5.3% were R539T (Hamilton et al., 2019).

Several studies have hypothesized that piperaquine (like chloroquine and the other ACT partner drugs) interferes with heme detoxification and/or hemoglobin degradation. This is supported by transmission electron microscopy (TEM) studies by Sachanonta *et al.* that show distended digestive vacuoles with undigested hemoglobin and fewer hemozoin crystals than the untreated control after treatment with piperaquine (**Figure 1.9**) (Sachanonta et al., 2011). It is possible that increased *PM2-3* copy numbers could enable the parasite to circumvent any inhibition caused by the drug. In a similar fashion to the mechanism of chloroquine resistance, the PfCRT mutations could also enable efflux of piperaquine from the digestive vacuole.
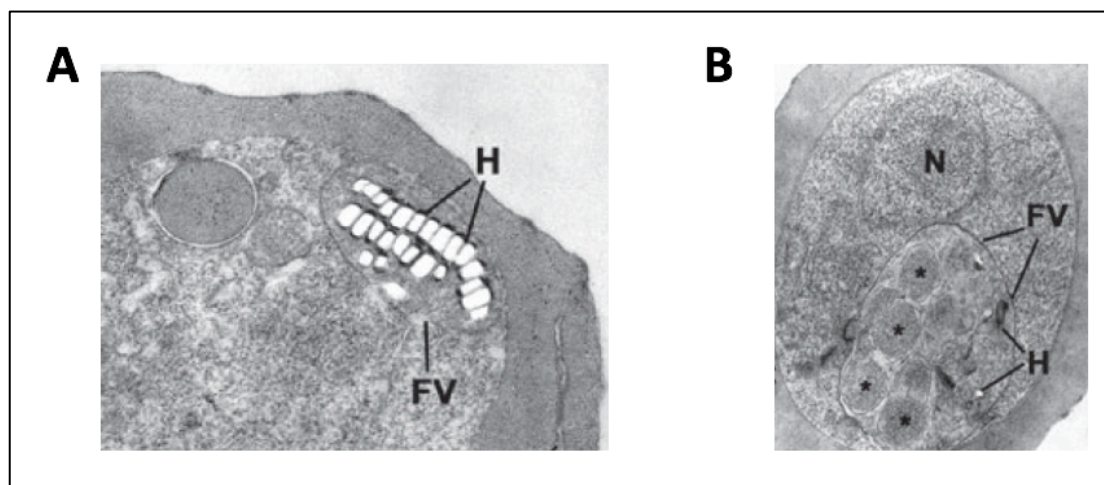

Figure 1. 9. Transmission electron microscopy of *P. falciparum* infected erythrocytes. A.) Low-power TEM of the control sample (no drug) in the trophozoite stage **B.)** Low-power TEM of treated sample after 4-hour exposure to piperaquine in the trophozoite stage. The asterisks (*) indicate undigested hemoglobin in the distended digestive vacuole. Abbreviations indicate: nuclease (N), food vacuole (FV), and hemozoin crystal (H). The images in this figure were modified and reproduced from Sachanonta *et al.* (Sachanonta et al., 2011) with permission from Taylor & Francis Publishing.

1.5 Specific aims

The purpose of my thesis work has been to elucidate the molecular mechanisms of piperaquine resistance. This dissertation has utilized molecular genomics, *in vitro* gene editing, and drug-pressure experiments to provide new insight into piperaquine resistance in *P. falciparum*.

In the first research chapter (Chapter 3), I sought to design a high-throughput assay that could be utilized for analyzing copy number amplifications of the hemoglobin degrading proteases, *plasmepsin 2* and *plasmepsin 3*, using low quantities of genomic DNA. I developed methods to detect and monitor *plasmepsin 2-3* copy numbers using both traditional PCR and qPCR methods. I then examined the role of the *PM2-3* amplification in piperaquine resistance by generating and transfecting a plasmid for overexpressing the *PM2-3* enzymes.

In the second research chapter (Chapter 4), I aimed to assess the effects of the putative *exonuclease* (PF3D7_1362500) and *mitochondrial carrier protein* (PF3D7_1368700) SNPs (*exo-E415G* and *mcp-N252D*) on susceptibility to piperaquine and other commonly used antimalarial drugs. I used CRISPR-Cas9 genome editing to introduce the wildtype and mutant SNPs into the lab strain Dd2 and evaluate the phenotypes of piperaquine response in these transformants.

In the final research chapter (Chapter 5), I aimed to select for a piperaquine-resistant phenotype *in vitro* using a potential hypermutator *P. falciparum* parasite line. After generating piperaquine-resistant parasites, I used whole genome sequencing to compare genetic differences between the parental parasites and the piperaquine-pressured parasites. Through this analysis, I identify gene candidates that may play a role in the piperaquine-resistant phenotype.