Universidade do Minho
Escola de Medicina

Miguel João Ferreira da Silva

Defining molecular pathways in antimalarial drug resistance

junho de 2022

Defining molecular pathways in antimalarial drug resistance
Miguel João Ferreira da Silva

UMinho | 2022

Universidade do Minho
Escola de Medicina

Miguel João Ferreira da Silva

Defining molecular pathways in antimalarial drug resistance

Tese de Doutoramento
Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Maria Isabel Mendes Veiga
e do
Doutor Pedro Eduardo Mendes Ferreira

junho de 2022

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

ACKNOWLEDGMENTS

I would like to thank all the people that supported me along these years, particularly to the people from the malaria laboratory group. Carla for the support, work discussions, for always being ready and glad to help in everything and of course the friendship and all the moments of fun and distraction. Vitória, also known as “cérebro de ervilha” for all the laboratory help, work discussions, for listening to my complains and for the friendship. Miguel Teixeira for the endless talks during lunch, after work at the café and being a great lab mate and Pedrosa for joining us in our lunch talks. The rest of the people that crossed my path in this group, such as Lúcia and Pinheirinho.

My appreciation to all my supervisors, Isabel Veiga, Pedro Fereira and Pedro Gil for believing in my capabilities to conduct the PhD and for all the support over these years. My thanks also to all the people from the ICVS institute and MIRD domain that gave feedback or help when needed.

To my family for the much-needed support. To all my friends, specially to Nuno and Margarida, for being available anytime I needed. To Petra my best friend and companion for all the support as you made this possible to be achieved.

FINANCIAL SUPPORT

The work presented in this thesis was performed in the Life and Health Sciences Research Institute (ICVS), University of Minho. Financial support was provided by grants from the SFRH/BD/129769/2017, "Starting" Mérieux Research Grant 2016 and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) 2014, ICVS Scientific Microscopy Platform, member of the national infrastructure PPBI - Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122, by the projects NORTE-01-0145-FEDER-000039 and NORTE-01-0145-FEDER-085468, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and by National funds, through the Foundation for Science and Technology (FCT) - project UIDB/50026/2020 and UIDP/50026/2020.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Definir vias moleculares envolvidas em resistência a antimaláricos

A malária é uma das doenças mais mortíferas do mundo e uma grave ameaça ao progresso das áreas afectadas. O *Plasmodium falciparum* é um dos parasitas causadores da doença e tem uma elevada capacidade de desenvolver resistência aos fármacos, tornando ineficiente a maioria das terapias. Apenas as terapias de combinação à base de artemisinina resistem como tratamentos eficazes, no entanto estudos recentes apontam para uma redução da sua eficácia. Consequentemente, é necessário identificar os principais marcadores de resistência e compreender a evolução desta. Também é importante compreender e identificar os mecanismos biológicos do parasita para contornar os mecanismos de resistência ou identificar novos alvos eficazes para fármacos.

Nesta tese, amostras de ensaios clínicos provinientes de África foram analisadas para marcadores de resistência à piperaquina e foram explorados os padrões transcripcionais do *pfk13*, o gene mais predicativo da tolerância à artemisinina. Duplicações do gene *plasmepsin 2*, um marcador associado à resistência à piperaquina no sudeste asiático, foram encontradas em África, indicando uma possível presença de parasitas com mutações favoráveis à emergência da resistência à piperaquina. A análise da resposta transcripcional do *pfk13* após tratamento com arteméter-lumefantrina revelou uma correlação entre *downregulation* do *pfk13* e um maior tempo de eliminação de parasitas. Portanto, a resposta transcripcional é um possível mecanismo de tolerância à artemisinina.

Para explorar o mecanismo de resistência da piperaquina, este trabalho gerou estirpes de *P. falciparum* geneticamente modificadas com duplicação da *plasmepsina 2* e *plasmepsina 3-1*. Estes parasitas revelam maior resistência a elevadas concentrações de piperaquina, fenótipo que foi aumentado através da utilização de bloqueadores químicos dos transportadores PfMDR1 e PfCRT. Estes dados sugerem um fenótipo multigénico e uma interacção de múltiplos sistemas presentes no vacúolo digestivo do parasita.

Este trabalho também explorou o papel da proteína transportadora citoplasmática PfMRP1, não tendo impacto particular para os fármacos antimaláricos comummente utilizados. Contudo, foi observada resistência a antifolatos num fenótipo incompatível com a anteriormente proposta funcionalidade de exportador. A acumulação de moléculas de antifolatos fluorescentes foi diminuida em parasitas com o gene *pfmrp1* interrompido. Através de uma análise filogenética demonstramos que o *pfmrp1* encontra-se distante de outros transportadores da mesma classe de vários organismos. Por conseguinte, este trabalho propõem a PfMRP1 como uma proteína importadora.

Palavras-chave: Edição genómica, malaria, plasmepsinas, resistência fármacos, transportadores

SUMMARY

Defining molecular pathways in antimalarial drug resistance

Malaria is one of the deadliest diseases in the world and a severe threat to the progress of affected areas. *Plasmodium falciparum* is the parasitic species responsible for most severe malaria cases. This parasite has a high capacity to develop drug resistance. Only artemisinin-based combination therapies still endure as effective treatments, but concerning evidence piles up of decreased efficacy. Accordingly, efforts need to be done to identify key markers of resistance and understand the evolution of resistance in the field. Moreover, the elucidation of the biological mechanisms of the parasite is important to identify and bypass mechanisms of drug resistance or identify new effective drug targets.

Field samples from African clinical trials, the continent most ravaged by malaria, were analyzed for piperaquine resistance markers and explored the transcriptional patterns of *pfk13*, the most predicative gene of artemisinin tolerance. *Plasmepsin 2* gene duplication, a marker associated with piperaquine resistance in Southeast Asia, was detected in Africa, indicating possible presence of background favorable for emergence of piperaquine resistance. Analysis of *pfk13* transcriptional response after artemether-lumefantrine revealed a correlation of *pfk13* down-regulation and longer parasite clearance time. Therefore, transcriptional response may be a mechanism of artemisinin tolerance, which is in accordance with recent studies of *pfk13* mutations generating resistance through decreased protein expression.

To explore the piperaquine mechanism of resistance, this work generated edited *P. falciparum* strains with duplicated *plasmepsin 2* and *plasmepsin 3-1* genes. These lines displayed increased resistance to high piperaquine concentrations, an effect that was enhanced by using chemical blockers of PfMDR1 and PfCRT transporters. The data suggest a multigenic phenotype and an interplay of multiple systems present at the digestive vacuole of the parasite. Moreover, these data clarify the likely evolution of piperaquine resistance and together with epidemiological data indicate that an initial selection of *plasmepsins* duplications and *pfmdr1* single copy number could have generated a favorable background for *pfcrt* mutations to arise and give high-grade resistance to piperaquine.

Transporter proteins are key for resistance phenotypes. This work explored the role of the cytoplasmic transporter protein PfMRP1 finding no impact for commonly used antimalarial drugs. However, resistance to antifolate compounds was observed in a phenotype incompatible with the previously proposed exporter functionality. Accumulation of fluorescent antifolate molecules was reduced in *pfmrp1* disrupted parasites. Phylogenetic analysis corroborates unusual function with *pfmrp1* distant from transporters of the same class of multiple organisms. Therefore, in this work, PfMRP1 is proposed as an importer protein.

Keywords: Drug resistance, genome editing, malaria, plasmepsins, transporter proteins

TABLE OF CONTENTS

ABBREVIATIONS

ABC – ATP-binding cassette

ABCD4 – ABC Subfamily D Member 4

ACT – Artemisinin-based combination therapy

AM-LF – Artemether-lumefantrine

AS-AQ – Artesunate-amodiaquine

AS-MQ – Artesunate-mefloquine

AS-PY – Artesunate-pyrimetamine

AS-SP – Artesunate-sulfadoxine-pyrimethamine

bp – Base pair

bsd – Blasticidin S deaminase gene

BSD – Blasticidin S HCl

cDNA – Complementary DNA

CNV – Copy number variation

DHA-PQ – Dihydroartemisinin-piperaquine

DHF – 7,8-dihydrofolate

DHFR – Dihydrofolate reductase

DHFS – Dihydrofolate synthase

DHPS – Dihydropteroate synthase

DNA – Deoxyribonucleic acid

D-PBS – Dulbecco's phosphate buffered saline

dTMP – Deoxythymidine monophosphate

DTT – Dichlorodiphenylchloroethane

DV – Digestive vacuole

F-MTX – Fluorescein methotrexate

GCH1 – GTP-cyclohydrolase 1

GFP – Green fluorescent protein

gRNA – Guide RNA

GSH – Reduced glutathione

GSSG – Oxidized glutathione

GTP – Guanosine-5'-triphosphate

hDHFR – Human dihydrofolate reductase

HIV – Human immunodeficiency viruses

hMRP – Human multidrug resistance protein

IC_{50} – Half maximal inhibitory concentration

kb – Kilobase

KO – Knockout

LB – Lysogeny-broth

MCM – Malaria culture medium

MRP – Multidrug resistance-associated protein

NBD – Nucleotide binding domain

pABA – para-amino benzoic acid

PBS – Phosphate-buffered saline

PC – Parasite clearance

PCR – Polymerase chain reaction

PfCAM – *P. falciparum* calmodulin

PfCRT – *P. falciparum* chloroquine resistance transporter

PfFT – *P. falciparum* folate transporter

PfK13 – *P. falciparum* kelch 13 protein

PfMDR – *P. falciparum* multidrug resistance protein

PfMRP – *P. falciparum* multidrug resistance-associated protein

PfPM2 – *P. falciparum* plasmepsin 2

PfPM3-1 – *P. falciparum* plasmepsin 3-1

P-gp – P-glycoprotein

RNA – Ribonucleic acid

rpm – Rotations per minute

RT – Room temperature

SBP – Substrate binding protein

SDM – Site directed mutagenesis

sgRNA – Single-guide RNA

SHMT – Serine hydroxymethyltransferase

SLI – Selection linked integrase

SNP – Single nucleotide polymorphism

SOB – Super optimal broth

SP – Sulfadoxine-pyrimethamine

TAE – Tris-acetate-EDTA

TALEN – Transcription activator-life effector nuclease

TMD – Transmembrane domain

TS – Thymidylate synthase

UV – Ultraviolet

WHO – World Health Organization

WT – Wild-type

FIGURE INDEX

TABLE INDEX

1. INTRODUCTION

1.1 Malaria disease and brief history

Malaria is an old disease with an ancient story that has affected humans over millennia. Evidence of parasites responsible for malaria was found in mosquitoes preserved in amber stones approximately over 30 to 100 million years old. These parasites evolved with multiple hosts to eventually cause human disease, which is probably derived from gorilla malaria (Cox, 2010; Liu et al., 2010). The first known reference to malaria is mentioned as repeated paroxysmal fevers and can be found dated over 3000 years old in the Chinese *Nei Ching*. Moreover, periodic fevers are referred in ancient Sumerian, Egyptian, and Hindu texts (Cunha and Cunha, 2008). In ancient Greece, Hippocrates was the first, as far as written recordings survived, to clearly identify different types of malaria depending on the periodicity of the fevers and also to relate the significance with splenomegaly and seasonality (Cunha and Cunha, 2008). Over the centuries, the disease was described as affecting the ones in proximity with swamps and was wrongfully believed to be the result of foul air from still water. This assumption gave the name to the disease, which is derived from the Italian "mala" plus "aria" and translates to bad air. Despite malaria continuing to be a widespread and prevalent disease over history, the causative agent was only identified by Alphonse Laveran in 1880. Later, the vector of transmission of avian malaria was identified by Ronald Ross in 1897 and the human vector by a team of Italian scientists in 1897 (Cox, 2010).

Malaria is caused by a parasitic infection with protozoans from the genus *Plasmodium*, which are transmitted between human hosts through the bites of infected female *Anopheles* mosquitoes. A wide range of *Plasmodium* species can infect multiple organisms ranging from mammals, birds and reptiles. From this variety, traditionally only four species are documented to cause disease in humans: *P. falciparum, P. malariae, P. ovale spp. (curtisi* and *wallikeri)* and *P. vivax.* More recently, simian species were also shown to infect humans. The latest species identified was *P. cynomolgi*, firstly identified as able to infect the human host *in vitro* but recently identified in patients (Hartmeyer et al., 2019). Moreover, simian species might have been responsible for malaria outbreaks, and have been misidentified as traditional infections due to the lack of available molecular typing techniques. One outbreak in Brazil caused by *P. simium* infection was initially misidentified as a *P. vivax* outbreak (Brasil et al., 2017). The primary host of *P. knowlesi* are the new world monkeys, but human infection with this species has also been reported sporadically in Asia, probably due to zoonotic transmission (Cox-Singh et al., 2008; Luchavez et al., 2008). *P. falciparum* and *P. vivax* are the most common parasites associated with human

disease. *P. vivax* has a wider global distribution due to being able to sustain a broader range of conditions, while *P. falciparum* is more impactful in the African continent (Howes et al., 2016; World Health Organization, 2020). *P. falciparum* is also responsible for the most severe disease state and most of the mortality associated with malaria and the herein focus of this dissertation.

1.2 Malaria epidemiology

In current times, malaria is a major cause of disease and was responsible for more than 600 thousand deaths in 2020 (World Health Organization, 2021). Malaria cases were estimated at 241 million in 2020, with most cases, around 95%, occurring in the African region (World Health Organization, 2021). Concomitantly, most of the deaths, around 94%, occur in Africa where the disease still remains a major cause of children death, particularly in sub-Saharan Africa causing a death every two minutes (Figure 1) (World Health Organization, 2021). Children are the most vulnerable group to malaria accounting for nearly 77% of all malaria deaths (World Health Organization, 2021). Moreover, pregnant women and children, the groups most affected by malaria, suffer a high toll of health consequences due to the impact of moderate and severe anemia (World Health Organization, 2021).

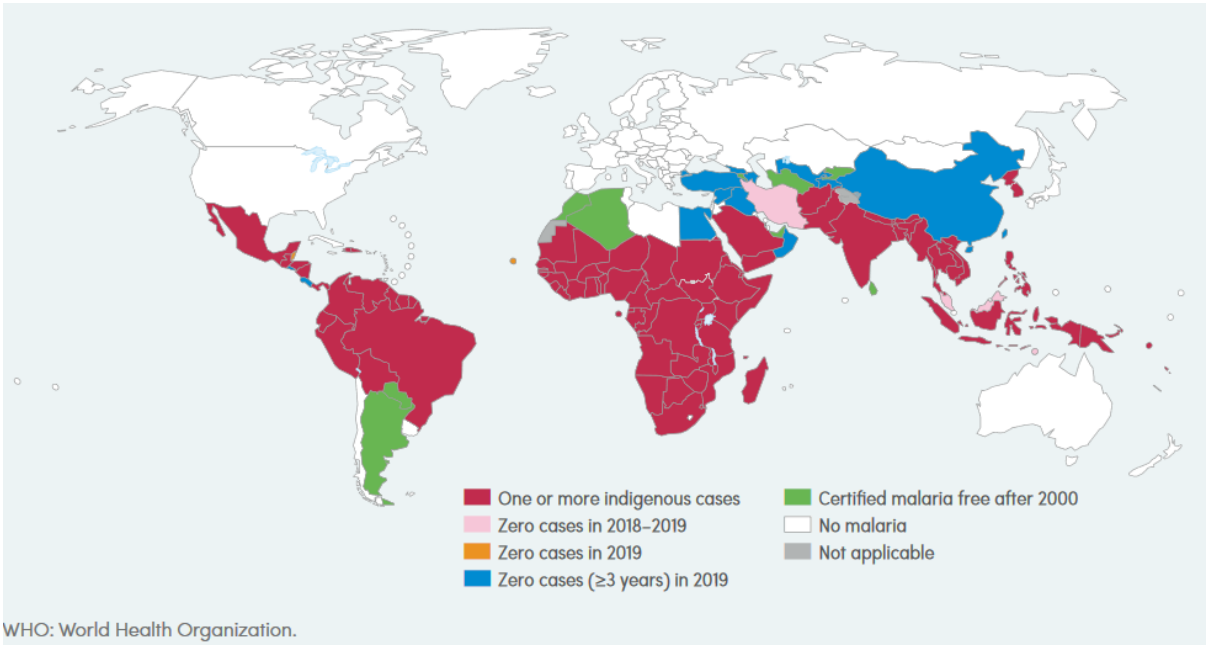


Figure 1. Malaria endemicity in 2019 compared to 2000. Depicts the evolution of progress towards malaria elimination, with countries certified free of malaria and more achieving zero cases, in recent years. Reproduced from WHO, World Malaria Report 2020 under creative commons license CC BY-NC-SA 3.0 IGO (https://creativecommons.org/licenses/by-nc-sa/3.0/igo/) (World Health Organization, 2020).

Despite these numbers, great progress against malaria has been achieved, with a decline of deaths attributed to malaria of approximately 50% and a reduction of 20% in the global cases of malaria since 2000 (Figure 2). However, despite the early century decline of malaria incidence, the change slowed drastically and hit a plateau since 2014, with only Southeast Asia recording progress in this area, while Africa and America having stagnant or even increased incidence of malaria (Figure 2). Similarly, deaths have been steadily on decline but recently hit a plateau in Africa, of about 380 thousand per year, since 2016 (World Health Organization, 2020). In 2020, due to the COVID-19 pandemic there was an increase in the malaria global number of cases and deaths of about 10%. This highlights the fragile state of disease

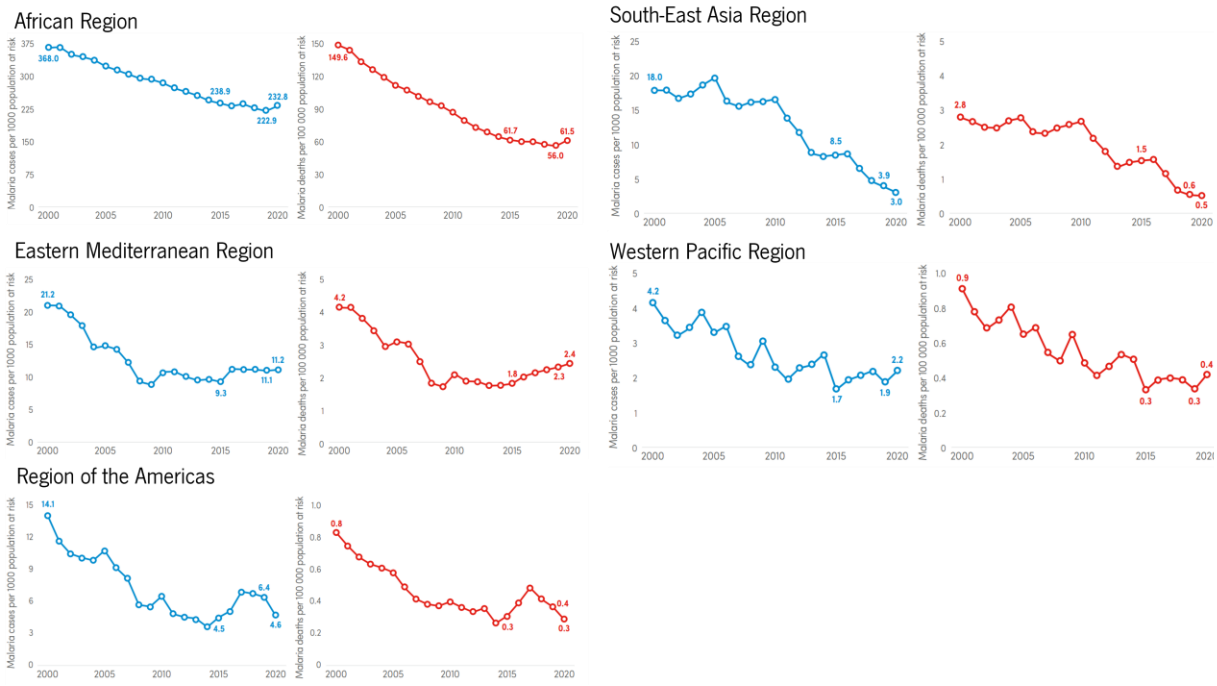


Figure 2. Malaria incidence and mortality trends in different world regions. a) Malaria case incidence rate (cases per 1000 population at risk) and **b**) mortality rate (deaths per 100000 population at risk). Adapted from WHO, World Malaria Report 2020 under creative commons license CC BY-NC-SA 3.0 IGO (https://creativecommons.org/licenses/by-nc-sa/3.0/igo/) (World Health Organization, 2020).

control and that any disruption can significantly increase malaria burden (World Health Organization, 2021). The observed decline in malaria incidence and deaths is mainly attributed to the introduction of artemisinin-based combination therapies (ACTs), as well as long-lasting insecticide-treated nets and indoor insecticide spraying. Due to disease fighting efforts, malaria is no longer the leading cause of death among children in sub-Saharan Africa. Nonetheless, malaria is still a major threat to economic and social development in these regions. In 2020, US$ 3.3 billion were invested in malaria control and elimination efforts, of which US$ 1.1 billion came from the government of malaria endemic countries. Malaria cost nearly US$ 300 million just for patient case management (World Health Organization, 2021). The disease thrives mainly in tropical and subtropical areas where the climatic conditions for propagation are optimal

and coincide with countries where poverty is prevalent (Figure 1). Malaria is a preventable and treatable disease, being already eradicated from Europe and North America, and with more countries achieving malaria free status in recent years (Figure 1). However, poverty greatly extends the malaria impact by hindering the availability of treatment, preventive measures and making treatment compliance harder. As such, malaria creates a vicious cycle in these regions as the disease causes a burden to social and economic development and thrives under these conditions.

In spite of the malaria health, social and economic burden, due to the investment and elimination efforts that have been pursued in this century, malaria elimination prospects are widening, with an increase of 17, in 2010, to 27, in 2018, countries reporting fewer than 100 indigenous cases, an indicator of in reach elimination (World Health Organization, 2020). Furthermore, more countries have been awarded World Health Organization (WHO) certification of elimination in 2018 and 2019, and more are on pace to achieve the target of elimination after 2020 (Figure 1).

1.3 *Plasmodium falciparum*

1.3.1 Life cycle

Plasmodium falciparum has a complex life cycle alternating between two hosts: the *Anopheles* mosquito, where the sexual stage of development occurs; and the human host characterized by a pronounced asexual multiplication and sexual differentiation (Miller et al., 2002). A schematic of the life cycle is presented on Figure 3 and described below.

On the human host, the infection starts when an infected *Anopheles* female mosquito while taking a blood meal injects sporozoites into the bloodstream (Yamauchi et al., 2007). Sporozoites first linger in the bite site, engaging in random forward gliding motion until a few sporozoites penetrate the blood vessels and migrate through the bloodstream to the liver, where they readily invade the hepatocytes (Amino et al., 2008; Miller et al., 2002; Mota et al., 2002; Vaughan and Kappe, 2017; Yamauchi et al., 2007).

In the liver stage, which takes approximately 1 to 2 weeks, the sporozoites undergo an asexual maturation process into multinucleated cells, known as schizonts, that produce thousands of mononucleated merozoites (Vaughan and Kappe, 2017). In this stage, a single successful sporozoite can generate more than 90000 mononucleated merozoites (Vaughan and Kappe, 2017). The merozoites eventually are released from the hepatocytes into the bloodstream (Vaughan and Kappe, 2017).

In the bloodstream, the merozoites invade the erythrocytes and start an intraerythrocytic replication cycle, of approximately 48 hours for *P. falciparum* (Cowman et al., 2012). The merozoites, in the erythrocytes, develop into different morphological forms passing from ring (early trophozoites), late trophozoites and schizonts, which contain multiple merozoites (Bannister et al., 2000). The latter phase of this cycle is characterized by intense metabolic activity and DNA replication, as each individual parasite is capable to generate between 5 and 40 merozoites (Bannister et al., 2000). The infected erythrocytes ultimately lyse releasing the merozoites ready to infect new erythrocytes. This cyclic and synchronized rupture of the erythrocytes causes the clinical manifestations of malaria, and in susceptible subjects each cycle might lead to an increase of several folds in parasitemia.

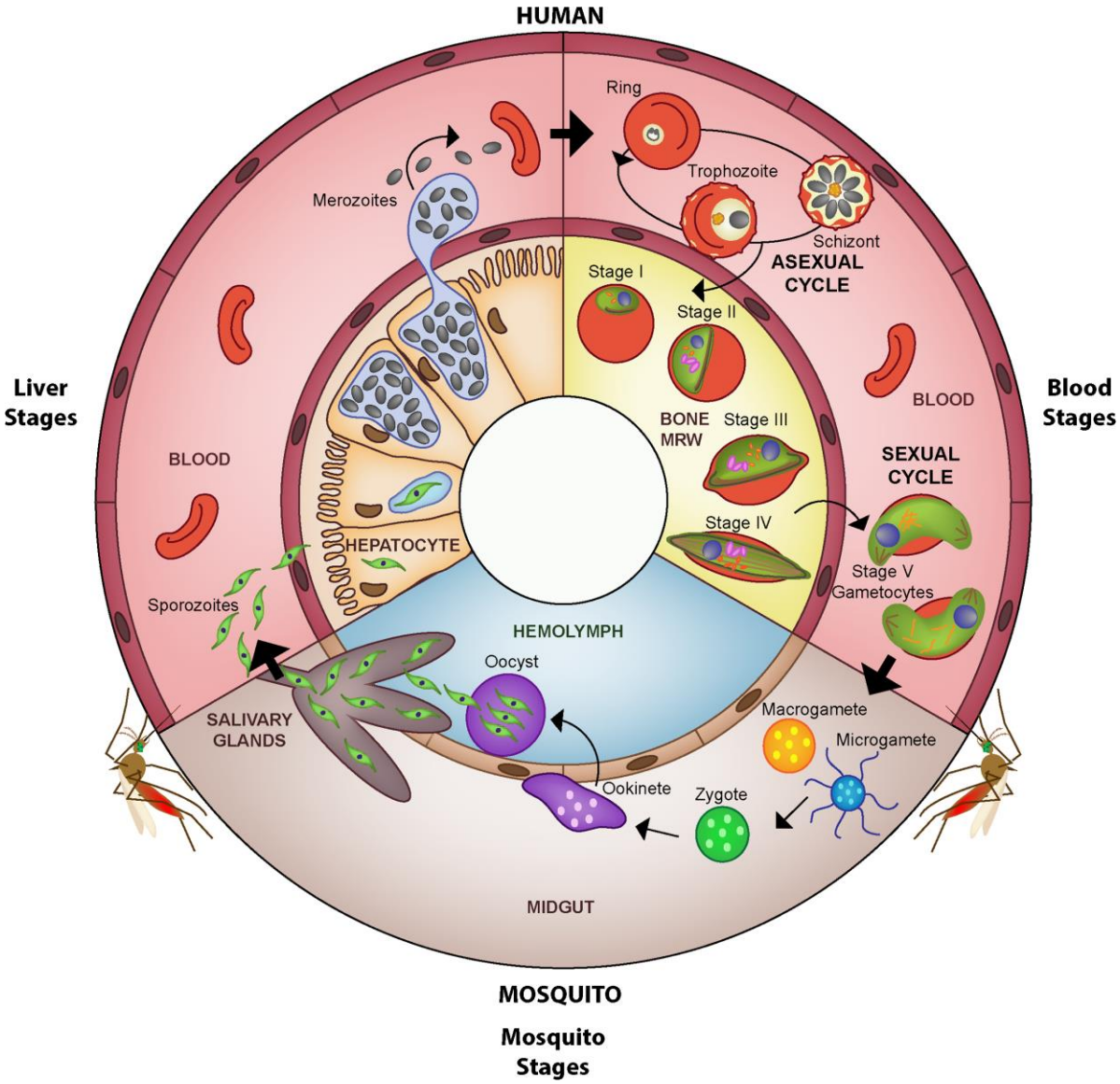


Figure 3. *Plasmodium falciparum* life cycle. Description of the life cycle is presented in the text main body. Reproduced under creative commons license CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/) (Nilsson et al., 2015)

A minority of the merozoites do not undergo the intraerythrocytic replication cycle but instead differentiate into gametocytes (Josling and Llinás, 2015; Meibalan and Marti, 2017; Miller et al., 2002). Gametocytes are retained in the bone marrow before being reintroduced circulating in the bloodstream, after maturation. This stage does not cause symptoms but can be taken by a mosquito during a blood meal (Joice et al., 2014). In the mosquito mid-gut, the microgametes (male) and macrogametes (female) fuse to form the zygote, the diploid stage of the parasite. The zygote becomes motile and further develops into an ookinete that penetrates the gut wall, where the ookinete differentiates into an oocyst (Aly et al., 2009). Through meiosis, the oocyst becomes haploid and through mitotic division produces multiple sporozoites. After maturation, the oocyst ruptures releasing the sporozoites, which migrate to the mosquito salivary glands and are ready to infect a new human host and re-starting the parasite life cycle (Aly et al., 2009).

All *Plasmodium* parasites that infect humans have a similar cycle, with minor differences in developmental stages. The major difference, that also helps distinguish between infections, is the duration of the intra-erythrocytic cycle. This cycle difference defines quotidian malaria of *P. knowlesi* with a 24-hour cycle, tertian malaria of *P. falciparum*, *P. vivax* and *P. ovale*, with a 48-hour cycle, and quartan malaria of *P. malariae* with a 72-hour cycle (Vadivelan and Dutta, 2014). Furthermore, certain species, like *P. vivax* and *P. ovale,* are known to have a hypnozoite stage, which is a dormant form of the parasite. This hypnozoite can remain quiescent for several months, or even years, and are responsible for relapses of the disease (Merrick, 2021).

1.3.2 Genome

In 1996, a global effort to sequence the *P. falciparum* genome started envisioning that the sequence would unravel new venues for tackling the disease, as understanding the genome is vital for adaptation of current treatments and for the discovery of new drugs and vaccine targets (Mu et al., 2007). The parasite genome of the 3D7 strain was sequenced in 2002 and is composed of 14 chromosomes, an overall size of 22.8 megabases and approximately 5500 genes (Gardner et al., 2002). These efforts coupled with recent advantages in sequencing technology further propelled the sequencing of multiple parasite strains, including clinical isolates. The parasite genome is highly rich in AT regions, which comprise near 80% of the parasite genome (Gardner et al., 2002). The unusually high content of AT nucleotides is a major obstacle to genetic manipulation.

The complex life cycle of *P. falciparum*, where the parasite is subjected to various hosts and environments together with the long longevity of this species, estimated to be more than 100000 years

old, suggest a close co-evolution and adaptation to the human host (Crompton et al., 2014). *P. falciparum* can constantly change genetic expression, as the parasite requires specialized protein expression to thrive in several environments (Florens et al., 2002). Accordingly, only 6% of expressed parasite proteins are found in common to sporozoite, merozoite, trophozoite and gametocyte stages (Florens et al., 2002). In the same way, *P. falciparum* has intense variations in gene expression, as 80% of the open-reading frames have variations in transcript abundance during the intraerythrocytic stage, with very little of the densely packed genome being inactive or redundant (Bozdech et al., 2003; Chappell et al., 2020). The transcriptome analysis of *P. falciparum* further indicates that the majority of genes are likely just induced once per cycle at the required time (Bozdech et al., 2003). This coordinated gene regulation produces a cascade of gene expression, from genes required for general processes to specific genes involved in erythrocyte invasion (Bozdech et al., 2003). The rapid variation of genetic expression can help the parasite to evade the immune system by changing the parasite antigens displayed on the surface at any given time point. Moreover, even though the transcriptome appears conserved among strains, differences in gene expression, between strains, occur mainly in surface antigens and have been one of the major obstacles to the creation of effective vaccines (Bozdech et al., 2003; Llinás et al., 2006; Nogueira et al., 2001; Tonkin-Hill et al., 2018).

Genomic variation between *P. falciparum* strains is also a result of the long parasite evolution with the hosts, and allows parasites to overcome chemotherapeutic agents, vaccines development and vector control measures. Thousands of polymorphisms have been identified, which demonstrate rich diversity in these malaria parasites (Mu et al., 2007; Ribacke et al., 2007; Volkman et al., 2007). This variability has been linked to drug evasion phenotypes. These polymorphisms can be used to identify events of selection to understand *P. falciparum* biology, either long-term selection of environmental adaption or recent events of drug induced selection (Park et al., 2012; Volkman et al., 2007).

The complexity of life environments and rapid genetic variation contribute to the parasite success, and all these factors contribute to the capacity of the parasite to avoid drug action and increase difficulties in vaccine development efforts (Hoo et al., 2019; Moser et al., 2020; Park et al., 2012; Volkman et al., 2007).

1.4 Malaria control

Malaria control and elimination programs act by targeting the *P. falciparum* propagation. As the life cycle is complex and spans more than a single host, these efforts need to tackle the parasite at multiple

life stages, either by targeting the mosquito vector, or controlling propagation of parasites in the human host or the transmission between hosts. The mosquito is essential to malaria transmission and thus control or elimination of the mosquito vector is considered a vital tool in fighting malaria. The WHO in 1955 launched an eradication program with emphasis on vector control using residual spraying with the insecticide dichlorodiphenyltrichloroethane (DTT) (Nájera et al., 2011). These successful initial efforts were soon met with a significant drawback, as the massive use of the insecticide led to emergence of resistance and the eradication program failed and was abandoned (Nájera et al., 2011). Nowadays, vector control targets mainly on limiting propagation and reducing contact with the human host by using long-lasting insecticide treated bed nets and indoor residual spraying. However, these approaches also have the challenge of resistance emergence (World Health Organization, 2021). Novel approaches are being developed in order to try to render the mosquito resistant to the parasite by using genetically engineered symbiotic bacteria (Wang et al., 2017), and by using antimalarials with multistage activity capable to block mosquito *P. falciparum* transmission (Paton et al., 2019).

The fight against malaria propagation also occurs in the human host with efforts to stop transmission to humans by vaccine development and to achieve a rapid and early diagnostic. This includes resolving the malaria symptomatic stage but also eliminating asymptomatic gametocytes that are responsible for propagation into the mosquito (Butterworth et al., 2013; World Health Organization, 2020). Substantial progress has been made in vaccine development recently (Duffy and Patrick Gorres, 2020; Molina-Franky et al., 2020). Despite that, the major hurdles for effective vaccine deployment are linked to the complexity of factors that govern the immune response to the parasite. Moreover, the choice of antigen is problematic due to the life cycle that spans two hosts and the several environments within the host at different life stages, including multiple paths of cell invasion and ability of the parasite to rapidly modify gene expression (Molina-Franky et al., 2020). Furthermore, whole organism vaccines or vaccines based on a single parasite variant are insufficient to cover the wide parasite diversity (Molina-Franky et al., 2020). While, until now, no vaccine has managed to surpass all these obstacles and achieve high effectiveness, there are ongoing implementation programs in some African countries and the future landscape looks promising with ongoing trials that might reveal further vaccine prospects (Duffy and Patrick Gorres, 2020; Molina-Franky et al., 2020; PATH, 2020). In October 2021, a new hope emerged with the first WHO recommended malaria vaccine, the RTS,S/AS01, for use in children at risk (World Health Organization, 2021). Results from phase 3 clinical trials showed that a four dose regimen of this vaccine had 36% efficacy, over a 4-year follow-up, for children aged of 5 to 17 months (Laurens, 2019). Even though the

vaccine efficacy is modest, the malaria burden is so high that even a modest efficacy provides a substantial public health benefit, particularly in areas of intense transmission.

Malaria treatment is also fundamental for the disease control and truly important to save lives after disease onset. Treatment is performed via administration of chemical compounds with antiparasitic properties in the erythrocyte cell stage of infection, in order to resolve the symptomatic disease stage responsible for the mortality associated with malaria (World Health Organization, 2020). Malaria symptoms are mainly episodes of fever and chills, that occur due to the release of pyrogenic products when the merozoites burst from the erythrocytes. In the most complicated cases, the immune response cannot contain the infection and parasites may grow exponentially and the symptoms exacerbate each cycle as progressively more parasites exist in the circulation, leading to multiple consequences for the organism that can ultimately result in death (Buck and Finnigan, 2020). As chemotherapy is a fundamental tool in the treatment of the disease and helpful for disease control, compounds that have capacity to eliminate both the erythrocyte stages and gametocytes from the organism are highly desirable to reduce propagation (Ngotho et al., 2019). One of the major problems of malaria chemotherapeutic treatment is the fast emergence and spread of resistance to antimalarial drugs. This phenomenon has affected virtually every drug introduced to treat malaria and is a central factor associated with the failure to eradicate the disease (Antony and Parija, 2016; Conrad and Rosenthal, 2019; Cowell and Winzeler, 2019).

1.5 Antimalarial drugs

Malaria is a treatable and preventable disease, provided rapid diagnosis and effective treatment with antimalarial drugs.

The choice of therapy depends on the species of malaria parasite involved, the severity of the disease and the status of drug resistance in the region. As of now, there are three major classes of antimalarial drugs: quinolines, folate antagonists and artemisinin compounds, which will be further discussed. Antibiotics are also sporadically used and new compounds are being developed, which are out of scope of this thesis and will not be explored in detail.

1.5.1 Quinoline drugs

The history of chemotherapy in the treatment of malaria dates back to the 17th century, with the use of natural extracts from the *Cinchona bark* tree in the treatment of fevers (Institute of Medicine (US)

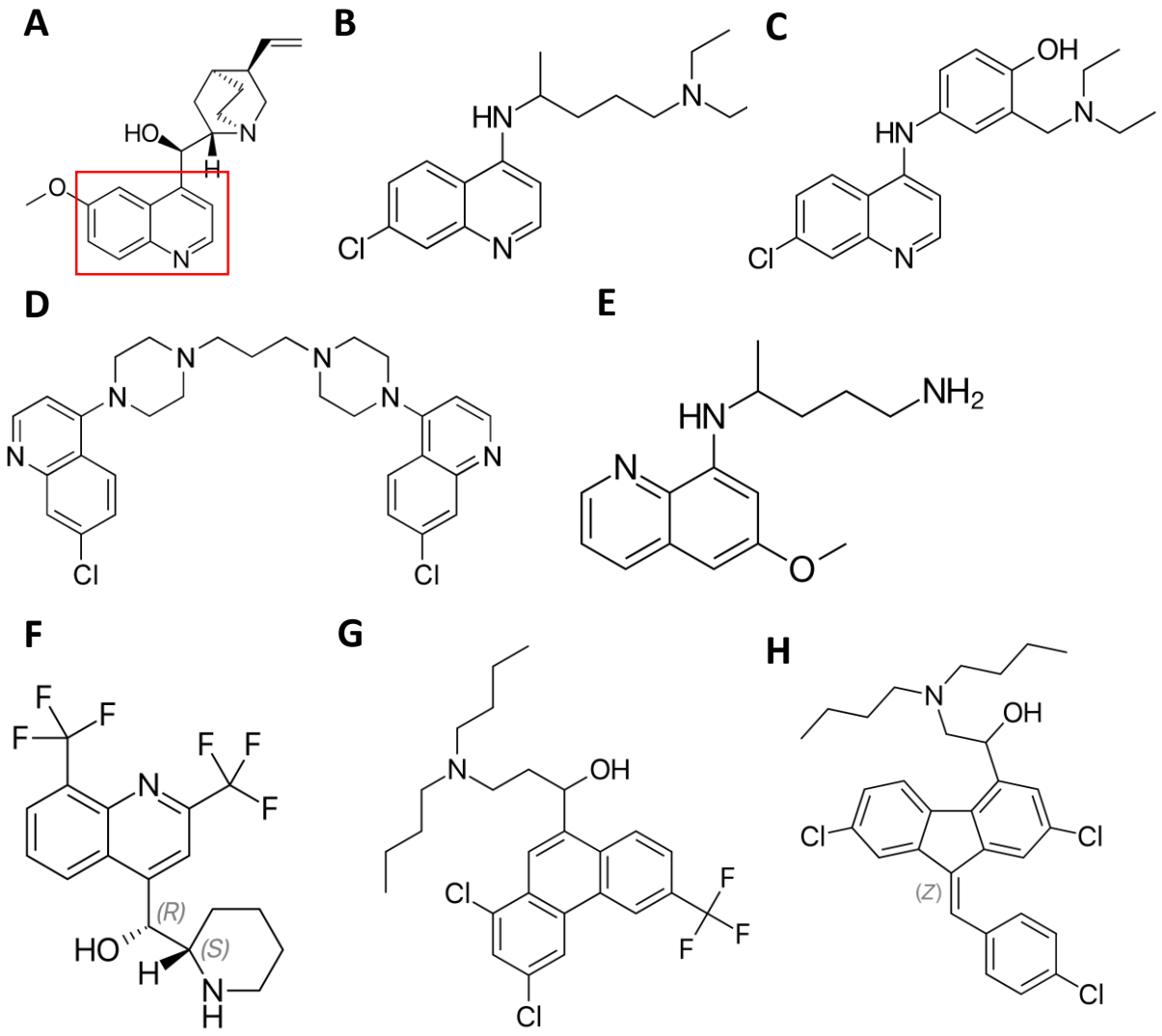


Figure 4. Structure of quinoline family of antimalarials. A – Quinine; **B –** Chloroquine; **C –** Amodiaquine; **D –** Piperaquine; **E –** Primaquine; **F –** Mefloquine; **G –** Halofantrine; **H –** Lumefantrine. The red rectangle highlights the quinoline ring.

Committee on the Economics of Antimalarial Drugs et al., 2004; Renslo, 2013). Later, in 1820 the active compound was isolated, by Pelletier and Caventou, from these extracts and was named quinine. The superiority of the pure alkaloid compared to bark extracts led to a quick adoption in Europe (Bray et al., 2005; Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs et al., 2004; Renslo, 2013). The availability of the pure compound allowed to determine effective dosages precisely and so was created the first chemotherapeutic, in the modern sense (Renslo, 2013). Quinine was used for the treatment of malaria until the 1930s. Moreover, quinine served as a base structure for the synthesis of related compounds including chloroquine, amodiaquine, piperaquine, primaquine and mefloquine (Figure 4). These compounds constitute the family of quinoline antimalarials and have in common the presence of a quinoline ring system. Halofantrine and lumefantrine are also based on the quinoline ring but have

less related rings systems (Figure 4). Chloroquine resulted from efforts during the World Wars to devise an alternative formulation, because of restrictions to the quinine supply chain (Kitchen et al., 2006). Furthermore, challenges for the administration of quinine, particularly poor tolerability and poor compliance due to complex dosing regimens, led to the adoption of more efficacious derivatives (Achan et al., 2011). Emerging after the World Wars, chloroquine together with the insecticide DTT were used as the two principal weapons in the WHO global eradication malaria campaign (Renslo, 2013). Unfortunately, resistance emerged and became widespread rendering the eradication of malaria unsuccessful, despite that chloroquine was a breakthrough to reduce the impact of malaria (Roux et al., 2021).

Quinoline-based antimalarials can be distinguished into two main subclasses: the aminoquinolines – chloroquine, amodiaquine and primaquine – and the arylaminoalcohols – piperaquine, mefloquine, quinine, halofantrine and lumefantrine (Figure 4). The targets of most quinolines remain elusive, albeit due to having related structures these compounds may have similar mechanisms and act on similar pathways. The most well-studied compound in this drug class is chloroquine and the proposed mechanism of action is on the parasite heme detoxification systems (Combrinck et al., 2013). *P. falciparum* during the intraerythrocytic cycle, mainly on trophozoite stage, takes large amounts of hemoglobin into the digestive vacuole (DV), as a source of amino acids (Wellems and Plowe, 2001). Hemoglobin digestion leads to the release of reactive heme, which is toxic for the parasite (Combrinck et al., 2013; Wellems and Plowe, 2001). For the parasite to survive, heme detoxification must be undertaken by polymerization into inert crystals known as hemozoin. Chloroquine is a weak base and therefore is deprotonated at physiological pH. Consequently, chloroquine accumulates in the acidic DV environment by becoming di-protonated (Olliaro, 2001). Chloroquine in the DV binds to the heme group, resultant from the consumption of hemoglobin, disrupting the detoxification process that leads to the polymerization towards the innocuous hemozoin (Combrinck et al., 2013; Kapishnikov et al., 2019; Olliaro, 2001). The free heme group is highly reactive and appears to diffuse out of the DV (Wellems and Plowe, 2001) and thus becoming toxic and lethal to the parasite (Combrinck et al., 2013; Olliaro, 2001; Wellems and Plowe, 2001). The mefloquine, piperaquine, halofantrine, quinine and lumefantrine (the arylaminoalcohols) also appear to inhibit the detoxification of the heme group (Combrinck et al., 2013; de Villiers et al., 2008; Dhingra et al., 2017), however they may also target additional processes on the parasite (Combrinck et al., 2013). Particularly, mefloquine was demonstrated to be a protein synthesis inhibitor (Wong et al., 2017). Additionally, these compounds appear to also inhibit hemoglobin proteolysis (Dhingra et al., 2017).

1.5.2 Antifolate drugs

The antifolates are another important class of drugs that have been used in the fight against malaria. These drugs are a result of supply chain problems during the World War II that propelled development of synthetic antimalarials, such as the antifolates proguanil and chlorproguanil. Proguanil was used as prophylactic treatment in Southeast Asian and South America in the 1950s, which increased pressure for drug resistance that soon emerged (Edeson and Field, 1950; Jones et al., 1948; Maegraith et al., 1946, 1945; Robertson et al., 1952; Walker and Lopez-Antunano, 1968). Soon after, pyrimethamine was developed with increased prophylactic and therapeutic capabilities (Archibald, 1951; Goodwin, 1952; Powell et al., 1963; Vincke and Lips, 1952). However, resistance to pyrimethamine was soon evident in multiple areas (Clyde and Shute, 1954; Jones, 1954; Powell et al., 1964; Rollo, 1955; Young et al., 1963). Sulfonamide drugs were initially tested in the 1930s, but due to more effective antimalarials were not widely adopted (Coggeshall et al., 1941; Hill and Goodwin, 1937; Niven, 1938). New improvements in half-lives and toxicity propelled renewed interest in the 1960s, particularly sulfadoxine (Laing, 1965). This was in part due to studies demonstrating combination of sulfadoxine with pyrimethamine to be more effective (Chin et al., 1966; Greenberg and Richeson, 1950; Harinasuta et al., 1967; Hurly, 1959; Laing, 1970, 1968). With drug resistance to chloroquine spreading, sulfadoxine-pyrimethamine (SP) became the natural alternative. However, indicators of the long-term effectiveness of the combination were not optimal with reports reporting of clinical failures (Bunnag et al., 1980; Miller et al., 1986; Spencer, 1985). The main used drugs of this class are sulfadoxine and pyrimethamine, mainly used in combination. Other folate drugs have also been deployed such as sulfamethoxazole, proguanil, chlorproguanil, trimethoprim and dapsone (Amukoye et al., 1997; Blanshard and Hine, 2021; Mutabingwa et al., 2001; Thera et al., 2005).

Folate metabolism is critical in the majority of species and has been explored in the fight against malaria following the discovery and introduction of antifolate drugs to combat bacterial infections (Hyde, 2005). These compounds target the parasite folate biosynthesis pathway by binding and inhibiting key enzymes in this process (Figure 5). This pathway is essential for many cellular mechanisms, including DNA and protein synthesis, which are required in large scale, particularly in the late stages of the intraerythrocytic cycle where mitotic processes are rampant. The folate pathway is necessary to provide cofactors essential for metabolic reactions, which involve the transfer of one-carbon units. Folate are one-carbon donors in purine biosynthesis and methionine production (Gritzmacher and Reese, 1984; Gutteridge and Trigg, 1971; Heinberg and Kirkman, 2015; Newbold et al., 1982; Schellenberg and Coatney, 1961; Triglia and Cowman, 1999). To note that the term folate is usually used generically to

encompass metabolites that carry the folate moiety, which are a series of close derivatives that vary in the degree of oxidation of the pterin ring, the nature of substituents on the 5 and 10 positions of the molecule, and the number of glutamate residues attached to the para-aminobenzoate moiety that links the pterin ring to these glutamates (Hyde, 2005).

Malaria parasites can synthesize folate *de novo* and scavenge from the environment (Figure 5) (Ferone, 1977; Hyde, 2005; Krungkrai et al., 1989). Humans are unable to synthesize folate *de novo*, due to absence of several key enzymes in the folate synthesis pathway, instead relying on the diet to get necessary folate (Nzila et al., 2005). Therefore, drugs targeting this pathway are attractive against many pathogens since compounds are less likely to interact with human pathways. *De novo* folate biosynthesis starts with conversion of GTP to a dihydroneopterin triphosphate by GTP-cyclohydrolase 1 (GCH1) (Figure 5). This enzyme is present in the human host catalyzing tetrahydrobiopterin, a cofactor important for neurotransmission and nitric oxide production, making an unviable target. Dihydropteroate synthase (DHPS) is responsible for combining the pteridine precursor with para-amino benzoic acid (pABA) to form dihydropteroate (Figure 5). The antimalarial sulfadoxine is a structural analog of pABA and targets by inhibition of the DHPS (Nzila et al., 2000). Despite DHPS seemingly being an essential enzyme,

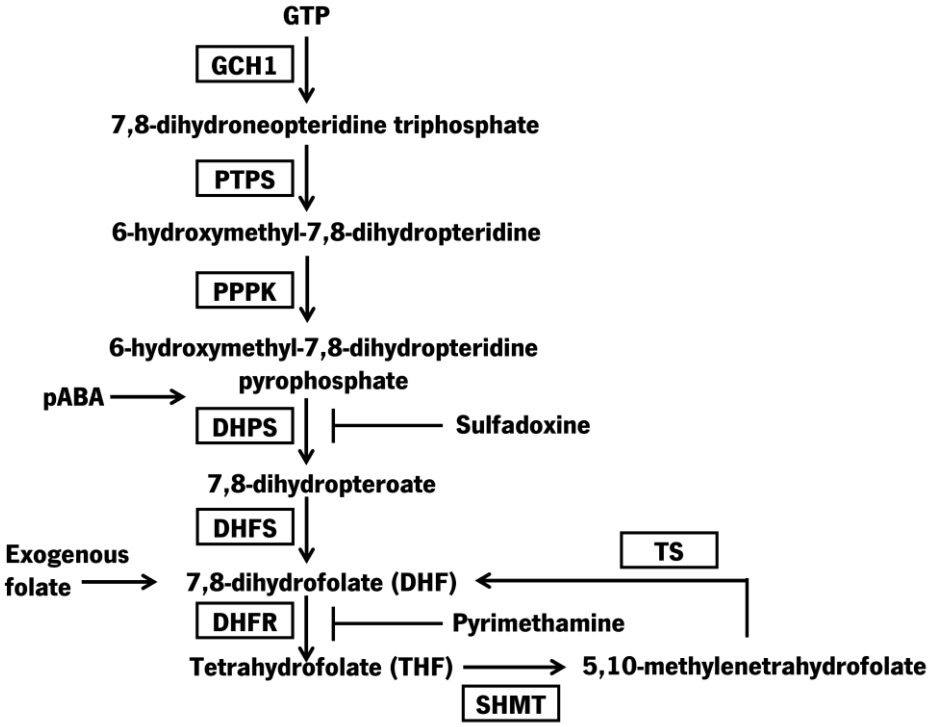


Figure 5. Folate biosynthesis pathway. The parasite can *de novo* synthesize folate or salvage exogenous folate. Para-aminobenzoic acid (pABA) can be used by the parasite dihydropteroate synthase (DHPS) for folate synthesis. Enzymes are in boxes and substracts in plaintext. GTP-cyclohydrolase (GCH1); pyruvolytetrahydropterin synthase (PTPS); hydroxymethyldihydropterin pyrophosphokinase (PPPK, also referred as HPPK); dihydropteroate synthase (DHPS); dihydrofolate synthase (DHFS); dihydrofolate reductase (DHFR). Sulfadoxine and pyrimethamine are drugs that inhibit DHPS and DHFR respectively.

scavenging of exogenous folate from the environment enables many *P. falciparum* strains to survive even high concentrations of sulfadoxine, making the compound not viable for single use in therapy (Figure 5) (Heinberg and Kirkman, 2015; Wang et al., 2004). Dihydrofolate synthase (DHFS) catalyzes the final reaction of the *de novo* pathway converting 7,8-dihydropteroate to 7,8-dihydrofolate (DHF) (Figure 5) (Heinberg and Kirkman, 2015). DHF can then be converted into folate derivatives by dihydrofolate reductase (DHFR), serine hydroxymethyltransferase (SHMT), and thymidylate synthase (TS) (Figure 5) (Heinberg and Kirkman, 2015). DHFR is expressed as a bifunctional protein with TS, and the complex is involved both in folate *de novo* synthesis and salvage pathways, in contrast to upstream enzymes, making an attractive drug target. Pyrimethamine targets the dihydrofolate reductase (DHFR) enzyme, inhibiting both the salvage and *de novo* folate synthesis pathways (Figure 5) (Sridaran et al., 2010). Inhibition of these enzymes leads to decreased production of tetrahydrofolate, an important co-factor necessary for the production of folate byproducts such as deoxythymidine monophosphate (dTMP) and methionine (Sridaran et al., 2010). This inhibition severely hinders the parasite lifecycle (Sridaran et al., 2010).

While *Plasmodium* parasites can obtain folate both through exogenous uptake and *de novo* synthesis, the importance of each pathway is not defined. The parasite might be able to increase flux through the salvage pathway when *de novo* synthesis is hindered (Wang et al., 2004). The predominant folate derivative circulating, of around 80% of total folate pool, in the human host is 5-methyltetrahydrofolate (Belz and Nau, 1998). However, this form of folate was found to have less antagonistic effect on pyrimethamine and chlorcycloguanil compared to folic acid or tetrahydrofolate (Nduati et al., 2008). Curiously, the antagonistic effect was not pronounced for methotrexate and aminopterin (Nduati et al., 2008). Accordingly, 5-methyltetrahydrofolate was found to be a poor substract of PfFT1 and PfFT2 transporters that are able to transport folic acid, pABA and the human folate catabolite *p*ABAG (Salcedo-Sora et al., 2011). Of these pABA was found to be the only salvageable at physiological concentrations (Salcedo-Sora et al., 2011). However, some studies point to salvage of folate or related metabolites can reduce sensitivity to certain antifolate drugs, potentially by bypassing *de novo* synthesis pathway, namely sulfadoxine (Tan-Ariya and Brockelman, 1983; Wang et al., 1999). However, these are strain and type of supplemented folate dependent effects. The contribution of exogenous and *de novo* synthesis is complex, with many contributing factors including parasite genetic background and concentration of folate (or antifolates) in the surrounding environment. However, both pathways seem to be important for the parasite to thrive.

As resistance to chloroquine became widespread, SP was used as a safe and inexpensive alternative for the malaria treatment. However, resistance emerged quickly for this treatment (Heinberg and

Kirkman, 2015). Currently, SP is not recommended treatment and is only being used in certain cases for intermittent preventive treatment in children and pregnant women, but resistance also threatens the effectiveness of these uses (Heinberg and Kirkman, 2015). Other drugs and combinations, such as trimethoprim-sulfamethoxazole, chlorproguanil-dapsone, have also been used but have not been successful long term. Major hinderances for these therapies include quickly emergence of resistance and low half lives, compared to SP (Reeves and Wilkinson, 1979; Winstanley et al., 1997, 1992). Faster clearance of these drugs did not demonstrate to be particularly advantageous for curbing resistance. Moreover, these treaments provided lower profilaxis and treatment compliance (Amukoye et al., 1997; Mutabingwa et al., 2001; Sulo et al., 2002). New more potent antifolates resulting from modeling drug design such as WR99210 faced problems as poor bioavalitity, pharmokinetic profile and toxicity (Ferlan et al., 2001; McKie et al., 1998; Rastelli et al., 2000; Toyoda et al., 1997). Atovaquone-proguanil combination has seen some limited use in profilaxys and in areas of artemisinin combination therapy failure (Blanshard and Hine, 2021). At the moment, the use and interest in these therapies has waned, mostly due to prevalent resistance and the advent of artemisinin derived therapies.

1.5.3 Artemisinin and derivatives

Extracts from plants have been used in traditional Chinese medicine since ancient times to treat sickness, with the plant *Artemisia annua*, commonly named sweet wormwood, used to treat fever (Cui and Su, 2009). Discovery and isolation of artemisinin to treat malaria in the 1970s was a hallmark of medicine of the 20th century. Since the early 2000s, artemisinin is a fundamental part of the first line treatment for uncomplicated malaria, designated by the WHO (Tu, 2017, 2016). The discovery was propelled by efforts of multiple scientist teams all over China, as a request of North Vietnamese leaders due to experiencing heavy losses of soldiers to malaria, during the Vietnam War, in a secret project named Project 523 (Hsu, 2006; Li and Wu, 2003, 1998; Thomas and Ying, 2008). Screening of the traditional Chinese pharmacopoeia led to the identification of multiple plants with good antimalarial properties, including *Artemisia annua*. This plant was extensively used in rural areas of China to treat fevers and malaria (Cui and Su, 2009). Multiple teams explored methods to extract the active ingredient, including Youyou Tu and her team that are credited with the discovery of artemisinin and granted Youyou Tu the 2015 Nobel Prize in Physiology and Medicine (Nobel Media AB 2021, 2021). Project 523 was key to decipher the structure of artemisinin through x-ray crystallography analysis. This structure provided foundation for improvement of the compound leading to several derivatives, including artemether, artesunate and dihydroartemisinin (Figure 6) (Jianfang, 2013). Clinical trials in the 1980s confirmed that

artemisinins were more effective and potent than traditional antimalarial drugs (Cui and Su, 2009; Jianfang, 2013). These antimalarial discovery efforts also propelled the development of several synthetic antimalarial drugs, including pyronaridine, lumefantrine and naphthoquine (Cui and Su, 2009).

Artemisinin has poor solubility in water or oil and so a poor bioavailability. To tackle this problem, semisynthetic derivatives of artemisinin were synthesized such as artesunate, artemether and dihydroartemisinin, which have improved bioavailability and efficacy (Figure 6) (O'Neill and Posner, 2004). All artemisinin derived drugs are converted *in vivo* to DHA, which is the main active metabolite (Tilley et al., 2016).

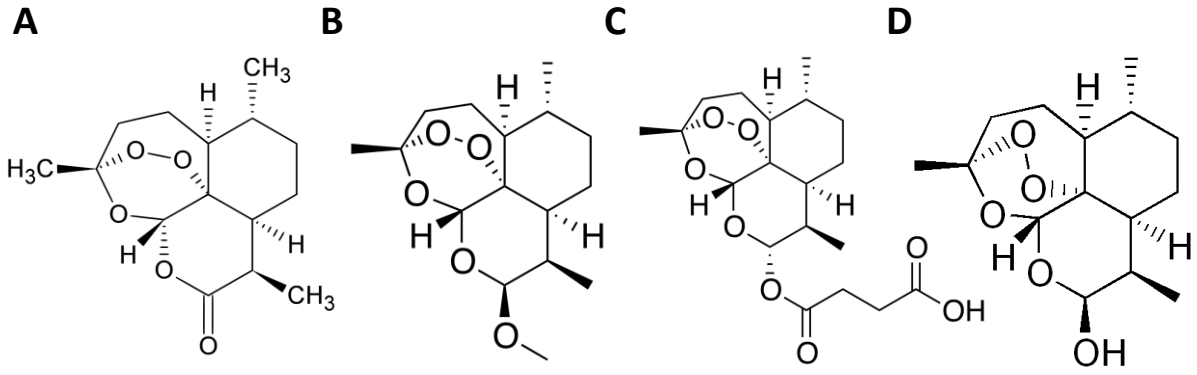


Figure 6. Structure of artemesinin family of antimalarials. A – Artemisinin; **B –** Arthemeter; **C –** Artesunate; **D –** Dihydroartemisinin.

Artemisinin can be produced chemically and through bioengineered microbes, but a major commercial source of artemisinin is still from *Artemisia* plant (Thomas and Ying, 2008). While the plant derived supply is increasing, there are still variations such as weather and agriculture conditions that affect the production output. The introduction of artemisinin compounds in the first-line antimalarial therapy caused a decrease in the plant availability and led to a price rise. This propelled endeavors to develop semisynthetic non plant derived sources (Hale et al., 2007). A method has been devised to produce semisynthetic artemisinin from yeast (Paddon and Keasling, 2014) and commercial production began in 2013. Improvements have been accomplished in recent years and have been reviewed recently, along with the description of the modified strains and chemical reactions that lead to the final artemisinin product (Kung et al., 2018).

Artemisinin is a sesquiterpene lactone containing an endoperoxide bridge that is key for the drug action (Figure 6) (Meunier and Robert, 2010). Artemisinin compounds are active against the intraerythrocytic stages of the parasite and gametocytes but not liver stage, which led to hypothesizing that hemoglobin degradation is key for artemisinin activity (Meshnick et al., 1993). The parasite uptakes hemoglobin using cytostomes, which are endocytic structures resultant from invagination and ingestion of the erythrocyte cytoplasm (Abu Bakar et al., 2010; Aikawa et al., 1966; Spielmann et al., 2020). The

cleavage of the endoperoxide bridge is essential for artemisinin activation, which is mediated by the hemoglobin-derived heme leading to the generation of a radical active species (Denisov, 2011; Xie et al., 2016). Activated artemisinin has been suggested to cause damage to proteins, lipids, and more cellular structures leading to widespread cellular damage (Wang et al., 2015). More recently, the radical activated artemisinin was demonstrated to be responsible for protein damage and function impairment of protein proteasome leading to proteotoxic stress and induction of unfolded protein response that culminates in cell death (Bridgford et al., 2018; Chen et al., 2017; Dogovski et al., 2015; Ismail et al., 2016; Wang et al., 2015; Zhang et al., 2017).

Artemisinins are potent antimalarial drugs that act rapidly, at an early stage of the parasite development and have an unprecedented potency in the elimination of the parasite burden (Antoine et al., 2014). These compounds have unparalleled effect against both asexual and sexual parasite stages (Chen et al., 1994; Kumar and Zheng, 1990; Skinner et al., 1996) and are the faster acting antimalarials, in the present, resulting in rapid clinical responses (White, 2008). Despite the artemisinin extremely fast-acting potency against the intraerythrocytic stage of malaria, as a downside, these compounds have very short *in vivo* half-lives of approximately 1 hour, which limits the use as monotherapy and prophylaxis. Moreover, artemisinins autoinduce cytochrome P450 metabolizing enzymes, which further lowers serum concentrations in subsequent administrations (Ashton et al., 1998; Asimus et al., 2007; Burk et al., 2005; Gordi et al., 2002; Simonsson et al., 2003). The fast elimination in principle prevents the selection of resistant parasites by exposure to residual concentration of the drug. However, the short half-life excludes the use of monotherapy due to poor cure rates and high rates of recrudescence (Menard et al., 2005; Meshnick et al., 1996; Nosten and White, 2007; Veiga et al., 2009; Woodrow et al., 2005). Due to the potent action, variable bioavailability and fast metabolism artemisinins are used in combination therapies combining artemisinin with a long-lasting drug, which is the recommended first-line therapy for treating *P. falciparum* infections (Ashley and White, 2005; Nosten and White, 2007). The advent of artemisinin as a potent antimalarial allowed the treatment of malaria resistant parasites to chloroquine, the previous key malaria therapy, and other therapies as mefloquine and sulfadoxine-pyrimethamine, and provided hope for a malaria-free world. Indeed, efforts have been successful with deaths decreasing over 30%, in recent years.

Early in the 1980s, scientists from the Chinese Academy of Military Medical Sciences explored ways to reduce recrudescence associated with artemisinin monotherapy and prevent the development of resistance, exploring combinations that showed *in vitro* synergistic effects with artemisinin and derivatives. Lumefantrine was chosen as partner drug to artemether, which was shown to be highly

effective even in areas with multi-drug resistance (Makanga and Krudsood, 2009). The combination was registered in China in 1992 and a collaboration granted the pharmaceutical Novartis, in 1999, the international licensing rights, registered as Coartem®. Mefloquine was combined with artesunate and implemented in Thailand in the early 1990s (Carrara et al., 2009; Nosten et al., 2000; Price et al., 1997). Later this combination has been used in several countries in Southeast Asia and South America. Around the same time, artemisinin-piperaquine combination was explored in southern China. Piperaquine was initially made independently by the French company Rhone Poulenc and Shanghai Pharmaceutical Industry Research in the 1960s. Initially widely employed in China the usage declined as monotherapy resistance emerged in the 1980s. Early in the development, the combination was reported that a 7-day regimen was superior to achieve higher cure rates. To shorten the regiment and reduce costs more combinations were pursued such as artesunate-piperaquine phosphate, and ultimately these efforts led to the optimization of dihydroartemisinin-piperaquine phosphate (DHA-PPQ) combination. This combination was a part of the CV8 firstly registered in Vietnam in 1997, which also included trimethoprim and primaquine (Giao et al., 2004). Primaquine and trimethoprim were abandoned and after further adjusting the ratio of drug components DHA-PPQ was registered as Artekin and recommended by the WHO (Bonn, 2004; Cui and Su, 2009; Tangpukdee et al., 2005). Recently, more therapies have been in development that take advantage of artemisinin fast acting mechanisms.

1.5.4 Artemisinin-based combination therapy

Artemisinins are administrated in combination with longer half-life, generally of weeks, partner drugs that have a proposed distinct mechanism of action, in order to avoid treatment failure associated with artemisinin monotherapy and reduce the likelihood of resistance. Resistance emergence to the therapy is reduced due to the lesser chance of parasites to simultaneously develop mutations for two drugs with distinct modes of action and by the elimination of parasites that would survive the initial short burst of artemisinin (Kremsner and Krishna, 2004; Nosten and White, 2007). The immense advantage of reducing the risk and pace of resistance development tackles a critical problem in malaria chemotherapy (White, 2013). The most used ACTs are: artemether-lumefantrine (AM-LF), artesunate-amodiaquine (AS-AQ), artesunate-mefloquine (AS-MQ), artesunate-sulfadoxine-pyrimethamine (AS-SP) and DHA-PPQ.

ACTs are nowadays the recommended therapies for the first line treatment of malaria, due to their high efficacy, resistance is not widespread and are also potent against other human malaria (World Health Organization, 2020). At the present, these therapies are the gold standard for treatment. ACTs have as base the ability of the artemisinin derivative to quickly reduce the parasite burden and thus leaving the

partner drug with fewer parasites to eliminate. Therefore, these regimens reduce the selective pressure on the long-acting partner drug. The artemisinin component is preferably given combined with the slowly

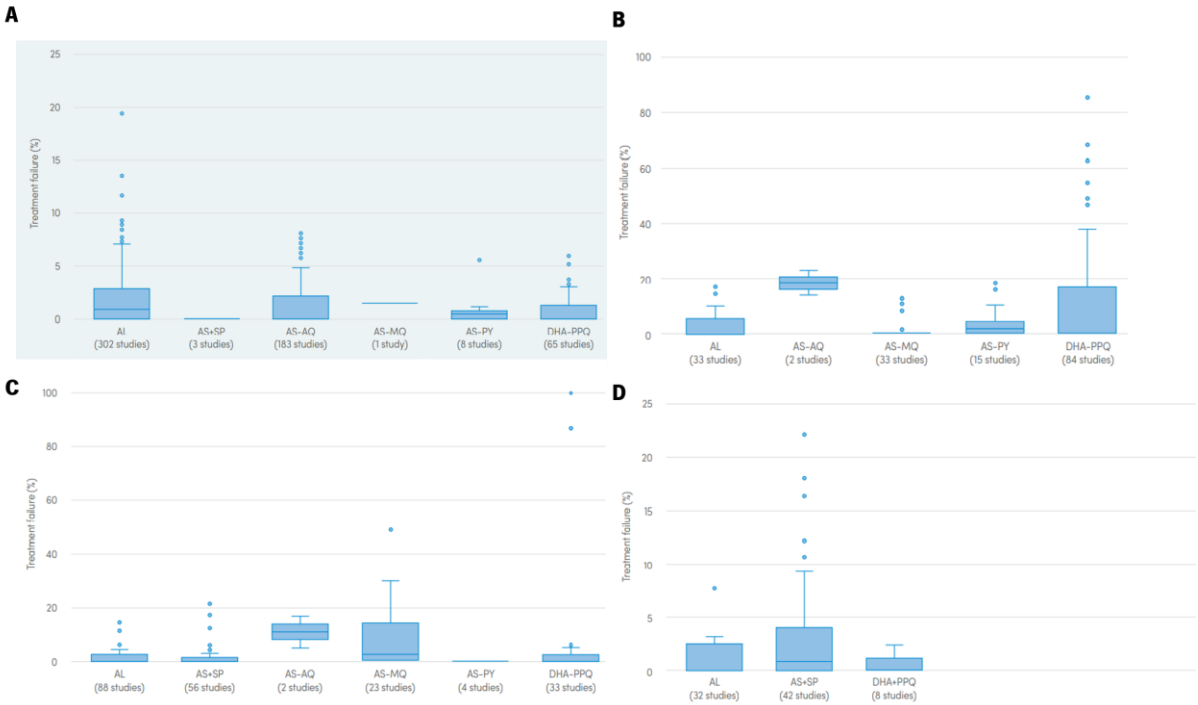


Figure 7. Treatment failture rates of ACTs WHO regions. Depicts the treatment failure rates of arthemeter-lumefantrine (AM-LF), artesunate-sulfadoxine (AS-SP), artesunate-amodiaquine (AS-MQ), artesunate-pyrimetamine (AS-PY), artesunate-mefloquine (AS-MQ) and dihydroartemisinin-piperaquine (DHA-PPQ) in African region (A), Western Pacific region (B), South-East Asian region (C) and Eastern Mediterranean region (D). The Western Pacific region appears to be a focus of resistance to DHA-PPQ. Reproduced from WHO, World Malaria Report 2020 under license CC BY-NC-SA 3.0 IGO.

eliminated antimalarial in a 3-day fixed dose. These 3-day regimens are well tolerated, with little side effects, with most side effects associated with the mefloquine partner drug, and have a good patient compliance (White et al., 2014). Furthermore, the combination tackles the mentioned problems associated with the short half-life of artemisinin (White, 1999). ACTs are still highly efficient and rapidly resolve clinical cases with few associated adverse effects, and except for of foci of DHA-PPQ resistance, in the Western Pacific WHO region, the curative rate remains very high (Figure 7) (Adam et al., 2018; Adjuik et al., 2004; Naing et al., 2019; Saito et al., 2020; Shibeshi et al., 2021; Visser et al., 2014; World Health Organization, 2020).

AM-LF is a highly effective ACT that combines artemether with the quinoline arylaminoalcohol lumefantrine. Artemether reaches peak plasma concentrations within 2 hours of administration and has an elimination half-life of 1 to 3 hours (White et al., 1999). Lumefantrine is a slowly and intermittently absorbed drug, requiring several hours to complete maximal absorption and reaches peak concentrations approximately 6 to 8 hours after administration (Ezzet et al., 2000). The variable bioavailability contributes to inconsistency in pharmacokinetics of populations, thus the lumefantrine drug half-life may vary from 3

to 6 days (White et al., 1999). Nevertheless, this partner drug has the fastest half-life from the approved ACTs. To obtain maximum efficacy, the AM-LF treatment should be given with food or drink, as fat promotes the absorption of lumefantrine (White et al., 1999). Through collaboration with the WHO, Novartis provides AM-LF without profit for distribution in malaria-endemic developing countries (Hamed and Grueninger, 2012). This initiative provides broad access to effective treatment for malaria. Since the distribution program implementation in 2001, 500 million AM-LF treatments have been delivered to more than 60 endemic countries by 2012 (Hamed and Grueninger, 2012) and over 1 billion treatments distributed by 2021 (Novartis, 2021). AM-LF is currently a fundamental therapy in malaria fighting, particularly in sub-Saharan Africa (World Health Organization, 2020). As a result, currently AM-LF is the most widely used antimalarial, the first-line treatment of most sub-Saharan African countries and the second-line in almost all the remaining countries (World Health Organization, 2020). Moreover, adoption of AM-LF is increasing in Southeast Asia due to the development of resistance to other combinations (World Health Organization, 2020). Adoption of AM-LF has been quite successful, for example in KwaZulu-Natal, South Africa malaria related deaths fell by 97% since introduction of AM-LF and an intensified program of indoor residual spraying (Zurovac et al., 2007). Likewise, in Zambia, deaths fell by 66% following similar efforts (Steketee and Campbell, 2010). More locations have reported similar success. So far there are no significant reports of clinical failure of this therapy, despite some early indications of increasing lack of efficacy (Dimbu et al., 2021; Gansané et al., 2021).

Artesunate–mefloquine (AS-MQ) has been widely used in Southeast Asia, this formulation showed great efficacy and tolerability with reduced mefloquine side effects (Taylor and White, 2004). Resistance to the partner drug mefloquine is more prevalent compared to lumefantrine, which led to treatment failures and even replacement of this combination in the Western Pacific WHO region (Leang et al., 2013b; Wongsrichanalai and Meshnick, 2008).

DHA-PPQ combination is commercially available and has been launched extensively in several Asian countries and in Africa. Developed as a replacement of chloroquine, piperaquine was later combined with dihydroartemisinin (Davis et al., 2005). Several clinical trials have demonstrated that this ACT was highly effective and well tolerated (Nosten and White, 2007). DHA-PPQ was introduced to replace failing combinations, particularly AS-MQ in Cambodia, the hotspot of malaria drug resistance (Phyo et al., 2016). However, resistance also emerged in the region and spread in Southeast Asia to Thailand and Vietnam (Amaratunga et al., 2016; Hamilton et al., 2019; Imwong et al., 2017a; Leang et al., 2013a; Saunders et al., 2014; Thanh et al., 2017). Recently, a dominant parasite lineage from Cambodia that harbors both artemisinin and piperaquine partner drug resistance was shown to be widespread in the region,

intensifying concern about highly resistant parasites and threatening DHA–PPQ-based therapy(Figure 7) (Hamilton et al., 2019).

Despite AM-LF being the most effective treatment due to resistance to other therapies, slowly eliminated partner drugs, such as mefloquine and piperaquine, provide longer prophylaxis, whereas reinfections after treatment with AM-LF often emerge within a month. This contributed to AM-LF not being readily adopted in Southeast Asia, compared to Africa. Furthermore, AM-LF was reported to have lower efficacy in Southeast Asia attributed to lower lumefantrine plasma levels, likely due to variances within the host population (Denis et al., 2006).

Several other ACTs, such as artesunate–pyronaridine, AS-AQ, artesunate–sulfadoxine–pyrimethamine (AS-SP), and artesunate–chlorproguanil–dapsone, have been developed, are under clinical trials, undergone clinical trials or have been deployed. ACTs are highly effective against *P. falciparum* malaria, with cure rates exceeding 90%, except for the Western Pacific WHO region where combinations have been replaced due to arising resistance (Figure 7). Nowadays, due to the threat of resistance, there have been efforts in developing triple ACTs. Although, there is debate that managing malaria should be done by making a better usage of existing treatments and developing unrelated antimalarials (Krishna, 2019; van der Pluijm et al., 2021). As downsides, triple combinations expand the risk of adverse events, have lower tolerability and increased price. Additionally, the main failure point of ACTs is the partner drug, so introduction of new partner drugs, in development, could be a more suitable alternative rather than repurposing already failing drugs (Krishna, 2019). Other strategies to prolong current therapies are the extension of therapy duration or alternating ACT combinations. Arguments in favor of triple combinations are the usage of these therapies, as standard, in the treatment of tuberculosis and HIV, and further reduction in the likelihood of resistance emergence. However, implementation comes with hurdles in the balance of pharmacokinetics, pharmacodynamics, dosing, production, deployment and acceptance (van der Pluijm et al., 2021). A recent trial assessed safety, tolerability and efficacy of two triple combinations, DHA-PPQ-MQ and AM-LM-AMQ. These formulations were based in the pharmacokinetic profiles of the compounds and the counterbalance of resistance mechanisms. These therapies showed high efficacy, even in regions of high DHA-PPQ failure, and were shown as safe and well tolerated, despite a slightly higher rate of side effects (van der Pluijm et al., 2021, 2020). Despite that, caution should be used as double combination therapy DHA-PPQ, also showed early signs of effectiveness only to quickly display high failure rates. Other triple ACTs trials are ongoing (van der Pluijm et al., 2021).

Developing an ACT is laborious as one needs to consider the artemisinin component and the partner drugs, which ideally should be structurally unrelated, have longer half-life, but also be effective against

previously generated resistance. Artemisinin has been used as a venue to revive drugs for which resistance has previously arisen, however reintroduction of these partner drugs is somewhat questionable (Nguyen et al., 2003). In Cambodia, resistance exists to mefloquine, but the combination AS-MQ has been deployed with some success reviving mefloquine, for malaria therapy in the area. However, treatment failures led to the replacement of the combination in certain provinces to DHA-PPQ. Due to prevalent piperaquine resistance and associated treatment failures, DHA-PPQ was later replaced back to AS-MQ (US AID, 2018). At first, deployment of ACTs comprising a failing partner drug may seem to curve resistance to the partner drug and be effective, as in the case of AS-MQ and DHA-PPQ, in Thailand and Cambodia. However, these therapies seem to compromise the long term effectiveness of ACTs due to the use of an inappropriate partner drug and can pressure to full widespread artemisinin resistance (Brockman et al., 2000; Nosten et al., 2000; Olliaro and Taylor, 2004). In the most drastic scenario, artemisinin paired with a failing drug could act and have similar problems as artemisinin monotherapy. This is of concern since failing partner drugs put additional pressure on artemisinin, leading to development of parasites less susceptible to artemisinin.

1.6 Antimalarial drug resistance

Chemotherapy has been fundamental in the combat against malaria, unfortunately, as is the case for other diseases, after modern campaigns of drug administration resistance emerged. Nowadays, *P. falciparum* resistance is widespread to nearly all antimalarials. Chloroquine was the first antimalarial to be rendered inefficient in the field due to large scale emergence of resistance in Southeast Asia and subsequent propagation to all hotspots of malaria in the world during the late 20th century (Packard, 2014). New drugs to tackle malaria were deployed soon after such as SP and mefloquine. Resistance to these replacement therapies emerged relatively quickly, although later some of these drugs were repurposed for combination therapies with artemisinin. This sub-chapter will focus on resistance factors particularly on antifolate and artemisinin derived antimalarials. DHA-PPQ and transporter proteins are further explored in sequent chapters, in a drug resistance perspective, due to the emphasis of the thesis in these topics.

1.6.1 Development and mechanisms of drug resistance

The emergence of resistance depends on a multiplicity of factors from the complex interaction between the parasite, human, mosquito and therapy. The parasite main factors that drive resistance are the

mutation rate of the parasite, the fitness costs associated with the resistance mutations and the overall parasite load (Petersen et al., 2011). In the host human population, factors such as the treatment compliance, immune response and pharmacogenetics are important to generate resistance (Petersen et al., 2011).

Mutations associated with drug resistance often impart a fitness cost for the parasite (Fröberg et al., 2013; Hayward et al., 2005; Petersen et al., 2011). Therefore, the altered biological function of the mutated protein is compensated by the selective advantage acquired by becoming drug resistant in the context of drug pressure. Such fitness cost can often be mitigated by the acquisition of compensatory mutations during prolonged drug pressure (Levin et al., 2000). Moreover, fully resistant phenotypes are frequently multifactorial, with early mutations leading to increased tolerance to drugs and subsequent mutations giving rise to full resistance. These mutations are frequently the result of suboptimal drug pressure, which decreases the prevalence of sensitive parasites whereas allowing the survival of more tolerant parasites (Hastings and Ward, 2005). Factors that lead to inadequate drug exposure are improper dosing, counterfeit drugs and infections acquired during the drug elimination phase of a prior antimalarial treatment (Petersen et al., 2011). Therefore, development of drug resistance occurs in several discrete steps, where parasite populations tend to evolve to adapt to the stress situation when exposed to suboptimal levels of drug.

There are various molecular mechanisms described that are associated with drug resistance phenotypes. The main mechanism of resistance explored in *P. falciparum* is the elimination of the drug from the place of action by preventing drug accumulation or promoting drug elimination, through decreased import or increased export, by membrane transporter proteins. Additionally, the parasite can acquire resistance by metabolization of the drug, molecular changes in the drug target and activation of compensatory cellular mechanisms to cope with drug action.

Studies based on *in vitro* culture of the parasite provided a significant development in exploring drug resistance phenotypes and pinpointing specific genome mutations that gave rise to resistance. These *in vitro* studies allow studying the intrinsic parasite susceptibility to a drug without host confounding effects such as immunity and the pharmacokinetics properties of the drug. However, caution must be taken when extrapolating *in vitro* results with *in vivo* phenotypes because there is a plentitude of factors involved in the *in vivo* resistance phenotype which are often not replicable on only *in vitro* assays.

1.6.2 Antifolate resistance

As an alternative to chloroquine, SP was introduced as a safe and inexpensive treatment for malaria. Increased use led to emergence of resistance, and nowadays SP has been replaced by ACTs in the first-line recommended treatment regiments (Müller and Hyde, 2013). Similarly, other antifolate compounds or combinations have had limited use due to emergence of resistance or deployment in areas of previous antifolate failures. The mainly associated antifolate resistance proteins are DHFR and DHPS. These have been identified in *P. falciparum,* with single nucleotide polymorphisms (SNPs) being the key cause of development of resistance (Brooks et al., 1994; Bzik et al., 1987; Peterson et al., 1990, 1988; Triglia and Cowman, 1994).

In *P. falciparum,* the DHFR forms a byfunctional protein with TS, encoded by a single gene on chromosome four. The first 231 aminoacids are from the DHFR domain, follow by 89 residues as junction and 288 aminoacids correspondent to the TS domain (Yuvaniyama et al., 2003). DHFR is the target of antifolates like proguanil, chlorproguanil, trimethoprim, WR99210 and pyrimethamine. *In vivo* and *in vitro* studies demonstrated a rapid development of resistance, compared to other antimalarials, that suggested a simple mechanism of resistance, such as point mutations in a single gene (Dieckmann and Jung, 1986; Nguyen-Dinh et al., 1982; Walliker et al., 1975). Sequencing advancements provided evidence of SNPs being responsible for the resistance phenotype. The SNPs N51I, C59R, 164L, A16V and I164L have been identified as factors of antifolate resistance (Cowman et al., 1988; Foote et al., 1990; Peterson et al., 1988). These mutations impact differentially the various antifolates, but in general these seem to have emerged sequentially to provide higher degrees of resistance at a reduced fitness cost (Foote et al., 1990; Peterson et al., 1990). In general, higher number of mutations correlate with increased resistance, and the mutation S108N is reported as a major mutation for resistance to emerge, and the triple mutant S108N, N51I, C59R, relevant for most resistance phenotypes, with other mutations playing supporting roles (Basco et al., 1995; Crabb and Cowman, 1996; Iyer et al., 2001; Jelinek et al., 1999; Nzila-Mounda et al., 1998; van Dijk et al., 1995; Wu et al., 1995). The SNP S108N was shown to affect drug accommodation rather than access to the active site (Delfino et al., 2002; Yuvaniyama et al., 2003). Although early studies reported gene amplification (Inselburg et al., 1987) evidence in field isolates did not support this as a contributor to resistance.

Similarly to DHFR, the DHPS is a bifunctional polypeptide encoded by a single gene (Cowman et al., 1988; Foote et al., 1990; Hyde and Sims, 2001; Peterson et al., 1988; Sirawaraporn and Yuthavong, 1984). As mentioned previously, DHPS plays a role in *de novo* synthesis of folate and pABA scavenging. There is no known counterpart in the human host. Sulfonamides and sulfones, like sulfoxide and dapsone

act by inhibition of DHPS. These compounds are particularly affected by folate. Although, exogenous folate is thought to be less important under regular conditions and certain parasite strains can grow without folic acid or pABA (Milhous et al., 1985). In some parasite strains folate can still enter the pathway downstream of this enzyme and bypassing the *de novo* pathway (Ferone, 1977; Krungkrai et al., 1989; Trager, 1958; Ping Wang et al., 1997; Wang et al., 1999). Accordingly, addition of folate to culture medium can decrease sulfoxide activity (Chulay et al., 1984; Milhous et al., 1985; P. Wang et al., 1997). In a similar way to DHFR, SNPs on DHPS are sufficient to generate resistance to sulfonamides (Brooks et al., 1994; Dallas et al., 1992; Triglia and Cowman, 1994). The SNPs S436A/F, A437G, K540E, A581G and A613T/S are mediators of resistance (Brooks et al., 1994; Diourté et al., 1999; Plowe et al., 1997; Triglia et al., 1997). SNPs on positions 436, 437 and 540 line the channel to the active site and are likely the main mutations leading to resistance with the SNPs at positions 581 and 613 having a likely compensatory function (Baca et al., 2000; Plowe et al., 1997; Triglia et al., 1998, 1997). The mechanism of resistance is similar to DHFR inhibitors resistance, in which the greater the number of SNPs the higher degree of resistance.

1.6.3 Artemisinin-based combination therapy resistance

For ACTs, as mentioned before, resistance is emerging with increasing treatment failures that already led to treatment replacements in Southeast Asia. Tolerance to artemisinin was first noticed in Southeast Asia, mainly in Cambodia, with detection of a decrease in parasite clearance after treatment (Dondorp et al., 2009; Noedl et al., 2008). Since then, tolerance to artemisinin spread to other areas of Southeast Asia and at the present has been found in multiple countries (Amaratunga et al., 2012; Hien et al., 2012; Kyaw et al., 2013; Phyo et al., 2012). Although still clinical effective, in these regions, failure rates for ACTs are rising (World Health Organization, 2020). Nevertheless, the AM-LF therapy remains highly effective in most parts of the world showing cure rates higher than 95% in the majority of sub-Saharan Africa (Djimde et al., 2015). However, in the Cambodia-Thailand area reports show a consistent delayed parasite clearance and treatment cure rates of less than 90%, lower than the WHO recommendations (Song et al., 2011). This lower efficiency, as mentioned, could be population related and not specifically resistance. Outside the Cambodia-Thailand area, treatment failure of AM-LF is uncommon and not necessarily due to drug resistance, but to several other factors such as non-compliance and counterfeit drugs (Djimde et al., 2015). Despite that, some recent reports show lower efficacy of AL in certain clinical trials, but usually not associated with artemisinin identified markers of resistance (Beshir et al., 2021; Dimbu et al., 2021; Gansané et al., 2021; Plucinski et al., 2017, 2015). Oppositely, DHA-PPQ is the

most concerning ACT having high rate of treatment failure, widespread in Southeast Asia. Resistance to the previously main used antimalarial drugs such as quinolines and SP, emerged in the same region, near Cambodia, and spread across Asia and Africa. In order to prevent the development and propagation of full ACT resistance phenotypes, continuous monitoring of treatment efficacy is necessary and there is a concerted effort to understand the genetic factors that underline the emergence and spread of resistance (Derbie et al., 2020). Even a modest increase of 30% in the failure rates of ACTs could lead to an increase of over 100000 deaths in Africa (Lubell et al., 2014). As such, there is a need to fully understand the mechanisms of action and resistance to ACTs.

Resistance to partner drugs is considered to be the major factor creating selective pressure on the artemisinin component of the ACT, which resulted in tolerant parasites (Dondorp et al., 2011). Artemisinin resistance manifests as increased survival and delayed clearance of young stage ring-stage parasites. Resistance to artemisinin has been defined clinically as increased parasite clearance half-life greater than 5 hours and recrudescence (Ashley et al., 2014; Dondorp et al., 2009) and *in vitro* by parasites having longer ring stage survival after a 6 hour artemisinin exposure (Klonis et al., 2013; Witkowski et al., 2013). At the present only artemisinin tolerance exists, but this phenomenon is usually referred to as artemisinin resistance and the terms have been used interchangeably in the literature. Although there is not yet full failure of the artemisinin component of the therapy, parasites with increased clearance times are expanding all over Southeast Asia, providing evidence of spread of artemisinin tolerance, which in the worst-case scenario could lead to widespread ACT treatment failure (Ashley et al., 2014; Imwong et al., 2017b; Tun et al., 2015). Concerningly, similarly parasites with increased clearance times have recently being reported in Africa (Balikagala et al., 2021).

Surprisingly, recrudescence of ART monotherapy failure does not seem to be associated with parasite clearance times *in vivo*, genetic markers and *in vitro* ring-stage susceptibility (Batty et al., 1998; Breglio et al., 2018; Sá et al., 2018; Wellems et al., 2020). Some reports point to a low fraction of persister parasites by metabolic quiescence. Or that parasites can survive in the spleen or extravascular niches in the bone marrow hiding from the effects of chemotherapy, which can be related with host factors (Henry et al., 2020; Kho et al., 2021; Lee et al., 2018). As of note, the novel partner drug KAE609 and lumefantrine may have the potential to hinder parasites to enter a dormancy state or even to kill dormant parasites, which could be a factor of why AM-LF resistance is rare even considering that this therapy has been deployed for over a decade in Africa (Chavchich et al., 2016).

1.7 Dihydroartemisinin-piperaquine

As mentioned, several combinations of ACT have been implemented so far, one of those is piperaquine used in combination with dihydroartemisinin, the active metabolite of artemisinin type of drugs. As one of the ACT with the highest failure rates and resistance, and one of the thesis focuses, this combination will be further explored in this section. Partner drug resistance is a serious problem, the few drugs available for malarial treatment were repurposed for combination treatment, but previously failing monotherapy drugs pose an additional risk for artemisinin resistance. The combination DHA-PPQ was introduced to save failing combinations in Cambodia, the hotspot of malaria drug resistance, unfortunately resistance emerged and spread in Southeast Asia to Thailand and Vietnam (Amaratunga et al., 2016; Hamilton et al., 2019; Imwong et al., 2017a; Leang et al., 2013a; Saunders et al., 2014; Thanh et al., 2017). Recently, a dominant parasite lineage from Cambodia that harbors both artemisinin and piperaquine partner drug resistance was shown to be widespread in the region, intensifying concern about highly resistant parasites threatening DHA–PPQ based therapy (Hamilton et al., 2019).

1.7.1 Piperaquine resistance

Resistance to piperaquine is thought to be the main factor of DHA-PPQ combination failure. However, detection of piperaquine resistance has been challenging and time-consuming, with conventional *in vitro* dose–response assays frequently yielding non-interpretable data. The first evidence suggested that a *plasmepsin* locus duplication, on chromosome 14, is one of the factors associated with resistance to the drug (Witkowski et al., 2017). Subsequently, the first genetic markers associated with PPQ resistance were identified as decreased copy number variations (CNV) in the *P. falciparum multidrug resistance 1* (*pfmdr1*) gene and increased CNV in *plasmepsin 2* (*pfpm2*) and hybrid *plasmepsin 3-1* (*pfpm3-1*) genes (Amato et al., 2017; Veiga et al., 2012; Witkowski et al., 2017). A bimodal response of resistance to high piperaquine concentration was correlated with increased CNV of *pfpm2* and *pfpm3-1* and a single *pfmdr1* copy (Bopp et al., 2018). However, there was discordance between the degree of resistance suggesting that additional genetic variants or expression profiles are involved in piperaquine resistance (Bopp et al., 2018). More recently, mutations on *P. falciparum* chloroquine resistance transporter (PfCRT) have been shown to confer bimodal resistance to high piperaquine concentrations (Ross et al., 2018). Interestingly, these mutations seem to have appeared later (2016) in genetic backgrounds of multiple *plasmepsins* copies (2 to 10 copies) and correlate with temporal de-amplification of *pfmdr1* (from 2010 to 2016)

(Dhingra et al., 2019; Ross et al., 2018). Subsequently, PfCRT mutations were confirmed to modulate transport of piperaquine and promote resistance *in vitro* (J. Kim et al., 2019).

Conflicting evidence emerged while trying to verify, *in vitro,* the role of the plasmepsins in modulating piperaquine resistance phenotype. Inactivation of *pfpm2* and *pfpm3* led to slightly increased piperaquine susceptibility in the African derived strain 3D7 (Mukherjee et al., 2018). Inconsistently, in the same strain *pfpm2* and *pfpm3* extra gene copy did not lead to susceptibility changes (Loesbanluechai et al., 2019).

As mentioned, the parasite obtains nutrients, namely amino acids, from the degradation of host hemoglobin, in the food vacuole, generating toxic heme byproduct that is detoxified to hemozoin (Coronado et al., 2014). Piperaquine as an aminoquinoline likely has the mode of action related to hemoglobin metabolism and particularly the inhibition of heme detoxification. Plasmepsins are aspartic proteases, which are present in parasite food vacuole and are one of the first enzymes to cleave hemoglobin in the digestion process (Wunderlich et al., 2012). The above-mentioned proteins, PfMDR1 and PfCRT are importer and exporter vacuolar transporter proteins, respectively, that have been implicated in multiple drug resistance phenotypes (Wunderlich et al., 2012). These transporters will be further explored in the context of resistance to other antimalarials in the next sections. There is still no clear mechanism for which piperaquine resistance occurs, but the main factors might be alterations in vacuolar dynamics related with hemoglobin metabolization by action of plasmepsins and transport by PfCRT and PfMDR1.

1.7.2 Artemisinin resistance

Artemisinin resistance, so far, is described as molecular resistance, and is an indicator that susceptibility to artemisinin is decreasing. Clinically, artemisinin tolerance manifests as clearance half-life greater than 5 hours (Ashley et al., 2014). The term artemisinin resistance, currently, does not translate into clinical resistance and there is no strong evidence linking with clinical failure. As referred, resistance to ACT nowadays occurs mainly due to failing partner drugs, nevertheless the molecular evidence of less susceptible parasites prompt preemptive studies in order to monitor emergence of artemisinin resistance. These intermediate less susceptible artemisinin phenotypes can propel the parasite to develop other mechanisms, which can lead to clinical failure. Furthermore, artemisinin reduced sensitivity poses an additional pressure and risk to accelerate the development of full-scale resistance to partner drugs due to a less protective action of artemisinin.

Artemisinin resistance was first suggested in 2005, and subsequently identified in western Cambodia (Dondorp et al., 2009; Noedl, 2005; Noedl et al., 2008). Later in 2014, the molecular basis of the

phenomenon was identified with an observed association of Kelch 13 propeller protein (PfK13) mutations with increased parasite clearance times (Ariey et al., 2014; Ashley et al., 2014; Straimer et al., 2015). Nowadays, artemisinin resistance is primarily associated with point mutations in the PfK13. Following the detection of PfK13 mutants the distribution of these parasites has been monitored, with over 150 mutations detected (Ménard et al., 2016; Ocan et al., 2019). Several of these mutations are now described to reduce artemisinin efficacy, such as N458Y, Y493H, R539T, I534T, R561H, C580Y (Ariey et al., 2014; Ghorbal et al., 2014; Ocan et al., 2019; Straimer et al., 2015). Several other candidate mutations have also been reported to associate with slow clearance or been reported to reduce parasite *in vitro* susceptibility, including both on the propeller domains or in other regions of the protein (Ocan et al., 2019; WWARN K13 Genotype-Phenotype Study Group, 2019). Other loci likely contribute to tolerance phenotype by enhancing stress or compensation of fitness cost (Cerqueira et al., 2017; Mukherjee et al., 2017). Evidence suggest that initially multiple PfK13 mutations appeared independently followed by hard selective sweep events with fitter parasites (Amato et al., 2018; Anderson et al., 2017; Imwong et al., 2017b, 2017a; Miotto et al., 2013; Takala-Harrison et al., 2015). PfK13 mutations are predominant in Southeast Asia, while in Africa, such mutants have remained rare with only 5% of African samples presenting mutations with most of those only detected once (Kamau et al., 2015; Ménard et al., 2016; Ocan et al., 2019; Taylor et al., 2015). The C580Y is the most widespread mutation and a C580Y lineage dominates in eastern Greater Mekong subregion, this lineage also includes *pfpm2* amplification, as mentioned, a marker of piperaquine resistance. (Amato et al., 2018; Imwong et al., 2020, 2017b, 2017a). This lineage seemingly arose in Cambodia and has propagated on the area to Thailand, Laos and Vietnam (Amato et al., 2018; Imwong et al., 2017a, 2017b; Witkowski et al., 2017). As of note, two epidemiological origins of the gene amplifications at the *plasmepsin 2-3* locus were detected. However, parasites resistant to DHA-PPQ belong to a single *plasmepsin 2-3* lineage, which likely emerged independently of PfK13 lineages, although the co-lineage is presently prevalent (Amato et al., 2018). The fast spread of these lineages after the first detection, which occurred in a timeframe of around 10 years, might suggest that artemisinin-resistant parasites can acquire increased biological fitness relatively fast and poses a further risk to failures. As such, surveillance and therapy adaptation to local settings is fundamental. Another less frequent lineage includes the H493Y PfK13 mutation (Amato et al., 2018). In Myanmar, the P446T is a prevalent mutation (Huang et al., 2015; Imwong et al., 2020). The mutations C580Y, through CRISPR-Cas9 and F446I, M476I, Y493H, R539T, I543T and C580Y, through zinc finger nucleases, were validated *in vitro* as able to generate resistance of early rings to short pulses of

artemisinin, in the ring susceptibility assay (Ghorbal et al., 2014; Straimer et al., 2015; Wang et al., 2018).

Some recent evidences point to an development of new PfK13 mutations in Africa, which are emerging independently of Asian mutations (Ocan et al., 2019). Detection of PfK13 C580Y has been reported in Guyana, Papua New Guinea, Equatorial Guinea, Angola, Gana (Huang et al., 2015; Mathieu et al., 2020; Miotto et al., 2020; Yang et al., 2017). In Rwanda, the PfK13 R561H was detected in around 7% of the samples, and through gene editing shown to confer artemisinin resistance *in vitro*. However, therapy cure rates remain high (Uwimana et al., 2020). In contrast, introduction of a frequent African PfK13 mutation A578S, *in vitro*, did not lead to altered artemisinin sensitivity (Ménard et al., 2016). In Uganda, the PfK13 allele A675V, which has been associated with delayed clearance in southeast Asia was detected but in low prevalence and without clinical relevance at the present (Ikeda et al., 2018; Ménard et al., 2016; WWARN K13 Genotype-Phenotype Study Group, 2019). However, recently a longitudinal study, of artesunate monotherapy, in Uganda from 2017 to 2019 suggests increased parasite clearance times for parasites with A675V and C469Y PfK13 mutations (Balikagala et al., 2021). Moreover, the proportion of these mutations is shown to be increasing throughout time concomitantly with an ring-stage increase survivability (Balikagala et al., 2021). However, so far, none of these reports have produced a clear link between PfK13 mutations with combination therapy failure in Africa. Although, a recent study suggests that likely independent emergence of PfK13 mutations might be related with increased clearance, which might propel clinical failures in the future (Balikagala et al., 2021).

PfK13 is a 726 amino acid protein with still unknown function in *P. falciparum.* The protein has motifs that belong to a class of ubiquitination adaptors (Glickman and Ciechanover, 2002). Multiple hypotheses implicated the PfK13 as a responder to downstream effects of ART activation, especially in up-regulation of pathways involved in the cellular stress response and reduced protein translation in the presence of ART-induced stress (Dogovski et al., 2015; Mok et al., 2015). As mentioned, the mechanism of action of ART involves the activation of the endoperoxide bond by hemoglobin-derived heme. The ART radical causes cellular damage and interference on the parasite proteasome (Xie et al., 2016). Several hypotheses emerged as how the parasite can avoid artemisinin damage (Mok et al., 2015), such as enhanced ability to deal with protein damage, enter a quiescent state or a post stress escape mechanism. Indirect analysis showed an increase in expression in genes related to proteostasis in PfK13 mutants and alterations in translation that could make the parasite able to withstand ART (Mok et al., 2015; Zhang et al., 2017). However, observations showed that several PfK13 mutations confer a similar resistance phenotype, which suggests, at least, partial loss of function (Amaratunga et al., 2014b; Ariey et al., 2014).

Accordingly, PfK13 may act as substrate adapter for ubiquitination and a SNP was shown to decrease affinity for *P. falciparum* phosphoinositide 3-kinase, thereby increasing its steady-state levels by reducing ubiquitination and proteasomal degradation and elevating phosphatidylinositol 3-phosphate that associates with artemisinin resistance (Mbengue et al., 2015). Moreover, PfK13 mutant parasites resume protein turnover faster and have enhanced recovery after 6 hours of DHA exposure (Dogovski et al., 2015; Yang et al., 2019). Despite that, proteomic analysis with WT and mutant PfK13 parasites demonstrated that basal level and turnover rate of stress response proteins is similar, disagreeing with the theory that PfK13 mutations directly mediate the parasite ability to deal with stress (Siddiqui et al., 2017; Yang et al., 2019). Instead, PfK13 could be involved in lowering the damage caused by ART (Siddiqui et al., 2017; Yang et al., 2019).

Recently, a novel mechanism of PfK13 mediated resistance was proposed by demonstrating that PfK13 acts upstream as a conduit for ART activation (Birnbaum et al., 2020; Yang et al., 2019). PfK13 localizes in vesicles close to cytostomes, which are erythrocyte-cytosol containing structures used by the parasite in the uptake of hemoglobin (Birnbaum et al., 2020; Yang et al., 2019). There, PfK13 interacts closely with endocytosis related proteins independent of clathrin, and more proteins with still unknown function (Birnbaum et al., 2020). PfK13 and interacting proteins in this compartment next to cytostomes are required for uptake of hemoglobin, with PfK13 being essential only in young ring-stage parasites (Bhattacharjee et al., 2018; Birnbaum et al., 2020, 2017). Mutations in PfK13, such as R539T and C580Y, were shown to reduce this protein concentration, which led to reduced hemoglobin uptake and enhanced ring-stage survival (Birnbaum et al., 2020; Siddiqui et al., 2017; Yang et al., 2019). Moreover, PfK13 knockdown, through mislocalization, was sufficient to reduce sensitivity to ART and reduce uptake of host cytosol and trafficking to digestive vacuole (Birnbaum et al., 2020; Yang et al., 2019). Additionally, expression of episomal PfK13 C580Y allele demonstrated that raising the abundance of protein, albeit carrying only the resistant allele, eliminated the ART tolerance phenotype (Birnbaum et al., 2020). These evidences show that reduced PfK13 protein levels alone can explain the resistance phenotype (Birnbaum et al., 2020; Yang et al., 2019). Recollecting, ART activation occurs via reaction with heme group, resultant from hemoglobin digestion, and therefore reduced hemoglobin uptake results in less ART activation (Birnbaum et al., 2020). As of now, reports have mentioned the puzzling observation that while mutations in PfK13 reduce protein levels, reduced transcript level has not been linked with artemisinin resistance (Mok et al., 2015; Xie et al., 2020). Undeniably, more studies to understand transcript levels of PfK13 are necessary. All these discoveries relate the PfK13 to hemoglobin endocytosis and trafficking. Accordingly, partial loss of PfK13 function reduces hemoglobin digestion, more pronounced at the early

ring stage, leading to less ART activation, which enhances cell survival. Nevertheless, the catabolism of hemoglobin provides the parasite with a fundamental source of amino acids and consequently PfK13 mutations carry a biological tradeoff for the parasite (Birnbaum et al., 2020). Therefore, mutant PfK13 impaired endocytosis occurs mainly in rings and not in trophozoites, which explains why these mutations have been selected rather than other genes involved in hemoglobin endocytosis, as the fitness cost is likely too high (Birnbaum et al., 2020). In the same way, PfK13 complete knock out is not viable *in vitro*, and only partial mislocalization, which mimics reduced expression of mutants is achievable (Birnbaum et al., 2020). On that note, there is also a possibility that the parasite upregulates pathways to deal with lack of hemoglobin. Accordingly, PfK13 mutants appear to alter multiple systems in parasite biology, such as unfolded protein response, protein degradation, vesicular trafficking and even mitochondrial metabolism (Mok et al., 2021).

Alterations in other proteins involved in hemoglobin uptake or trafficking to the digestive vacuole also impact artemisinin susceptibility, similarly to PfK13. Likewise, the knockout of *falcipain 2a*, a hemoglobinase, also delays the onset of artemisinin sensitivity in early ring-stage parasites (Xie et al., 2016). Accordingly, *falcipain 2a* has been implicated in artemisinin resistance, after artificial selection *in vitro*, and recently mutations have been detected in field parasites (Ariey et al., 2014; Klonis et al., 2011; Rocamora et al., 2018; Siddiqui et al., 2018). Moreover, mutations in coronin, AP2 adaptor, and F-acting-binding protein, which are proteins involved in endocytosis processes, also reduce sensitivity to artemisinin (Demas et al., 2018; Henriques et al., 2015, 2014, 2013; Milani et al., 2015; Yuan et al., 2014). Mutations in the deubiquitinase, UBP1, which reduced the rate of deubiquitination of trafficking regulators, are predicted to have an impact in hemoglobin uptake process and associated with failure to combination therapy and decreased artemisinin susceptibility (Henriques et al., 2014). Altogether, these evidence demonstrate the importance of hemoglobin endocytosis and metabolism in the activation of artemisinin. The disruption of the process leads to reduced artemisinin sensitivity, particularly in early-stage parasites.

Remarkably, since the first reports of extended clearance time of parasites after ACTs, there has not been a significant increase in clearance half-life. This fact might be related with the essentiality of the hemoglobin uptake and metabolism for the parasite. Indeed, *in vivo,* only PfK13 mutations that give a slight reduced hemoglobin uptake only at early ring stage have been selected and full PfK13 disruption is not tolerated. Conceivably, it might be unlikely that parasites can further evolve mechanisms to impair hemoglobin catabolism to drive artemisinin resistance, particularly in the trophozoite stage where there is an intense need for the products of hemoglobin degradation. Accordingly, the R539T and I543T PfK13

mutations have been shown to give higher artemisinin resistance, however the C580Y mutation that confers a lower fitness cost is more widespread (Hamilton et al., 2019; Straimer et al., 2017). In Africa, the rate of infections that remain untreated are higher, which selects against parasites with impaired growth and might be a factor for PfK13 mutations to not be, for now, as prevalent. Nonetheless, even mild losses of efficacy increase the pressure on partner drugs, which are especially limited, and can give rise to ACTs failure (Xie et al., 2020). Moreover, alternative mechanisms for artemisinin resistance can arise employing an entirely different pathway and these are more likely to arise in already tolerant parasites.

1.8 Antimalarial resistance associated transporter proteins

Beyond the resistance proteins mentioned above such as PfPMs, PfMDR1 and PfCRT as modulators of piperaquine resistance, and PfK13 as a major ART resistance factor, other proteins have been implicated in diverse antimalarial resistance. In this section, transporter proteins will be explored as mediators of resistance, as PfMDR1 and PfCRT are implicated in resistance to virtually all antimalarials these will be further explored in the next sections in context of antimalarial drugs, apart from DHA-PPQ (Figure 8).

1.8.1 ATP-binding cassette transporters (ABC)

The ABC transporter super-family is known to transport a wide variety of substrates across cellular membranes against a concentration gradient, an action driven by ATP hydrolysis. These transporters are present in multiple life forms, from prokaryotes to the most complex eukaryotes (Rees et al., 2009). The overall architecture of ABC transporters is conserved, with two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) that provide energy through ATP hydrolysis. Some ABC transporters have an additional membrane spanning domain, which appears to mediate trafficking of the protein or retention at the target location, while others are half-transporters containing only one of each domain (Dean and Allikmets, 2001; Deeley and Cole, 2006). The NBDs are highly conserved and considered the hallmark of ABC transporter family, while most variation occurs on TMDs (Ter Beek et al., 2014). ABC transporters can mediate active influx and efflux of structurally and functionally diverse compounds across membranes, and are classified as importers or exporters, respectively (Ter Beek et al., 2014). Importers are present mainly in prokaryotes and plants and frequently rely on additional substrate binding protein (SBP) for recognition and transport of substrate (Arnold et al., 2020; Lee et al., 2008; Lewinson and

Livnat-Levanon, 2017; Rempel et al., 2020; Shitan et al., 2003; Terasaka et al., 2005; Wang et al., 2020; Wanke and Üner Kolukisaoglu, 2010). SBPs can be present in periplasm or membrane-bound and interact with TMDs (Lewinson and Livnat-Levanon, 2017). Exporters are widespread across kingdoms, in prokaryotes and eukaryotes, and do not require extra SBP for transport. Various classifications have been employed, such as importers divided into three classes and exporters into two based on their 3D conformation and mechanism of transport (Lewinson and Livnat-Levanon, 2017; Rees et al., 2009). Recently ABC transporters have been reclassified based on TMD folds obtained from high-resolution X-ray crystallography and single-particle cryo-electron microscopy (Thomas et al., 2020). This new grouping has revealed that transporters of the same type with a similar scaffold can be functionally diverse, acting as importers, exporters, ion channels, or lipid floppases (Thomas et al., 2020).

Transporter proteins are key in *Plasmodium* biology, mediating the uptake of nutrients and extruding waste products and toxic compounds. Due to their capacity to direct substrates between cellular compartments, transporters are frequently associated with drug resistance. The *P. falciparum* encodes in the genome at least 16 ABC proteins, from which 11 are putative transporters (Kavishe et al., 2009; Martin, 2020).

1.8.1.1. *P. falciparum* multidrug resistance protein 1 (PfMRP1)

The Multidrug Resistance Protein (MRP) family, which belongs to the ABC transporters super-family, is frequently associated to phenomena of multidrug resistance in multiple organisms. The physiological characteristic substrates of MRPs are amphipathic organic anions, like glutathione, glucuronide or sulphate-conjugated compounds and have been linked as a homeostatic modulator of oxidative status and a player on oxidative stress response. The human MRPs are the best characterized transporters and are associated with drug resistance due to the ability to transport a wide variety of anticancer drugs, alone or conjugated with glutathione, glucuronate and sulphate. In *P. falciparum*, this family comprises two transporters, the PfMRP1 and PfMRP2.

P. falciparum Multidrug Resistance-associated Protein 1 (PfMRP1) and 2 (PfMRP2) have been linked to resistance but so far no clear evidence emerged about their role (Dahlström et al., 2009a, 2009b; Nogueira et al., 2008; Raj et al., 2009; Veiga et al., 2014, 2011). The biological role and substrates of these transporters also remain largely unexplored, and identification of such could pave a way to development of new antimalarial therapies. Between these proteins, the PfMRP1 role in antimalarial drug resistance has been more explored, however the complete importance of this protein in the overall drug response remains to be elucidated and is one of the main focuses of this dissertation.

The PfMRP1 is composed of 1823 amino acids and has been localized at the parasite plasma membrane in the asexual erythrocytic stages (Raj et al., 2009). In *P. falciparum*, PfMRP1 was suggested as a potential glutathione transporter and as a folate transporter (Atamna and Ginsburg, 1997; Bozdech and Ginsburg, 2004; Raj et al., 2009; Rijpma et al., 2016b). Similarly to hMRPs, the PfMRP1 also appears to transport glutathione, probably to maintain redox homeostasis and act as a mechanism to cope with oxidative stress. Thus antimalarial resistance could occur by transport of PfMRPs, in the same fashion anticancer drug resistance is mediated by hMRPs (Raj et al., 2009).

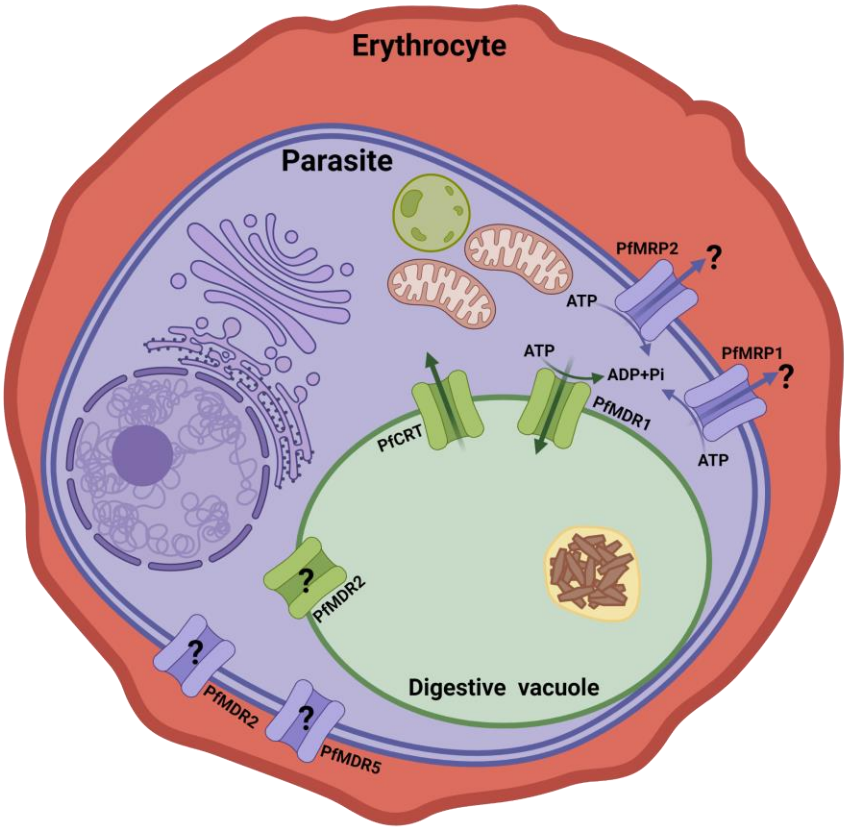


Figure 8. Schematic representation of the most explored transporters in *P. falciparum*. PfCRT and PfMDR1 are known to be key in antimalarial resistance being the most well comprehended transporters in modulating resistance. PfCRT transporter uses proton-motive force to efflux small peptides derived from hemoglobin degradation and has been implicated in resistance to several antimalarials. PfCRT is the key factor for chloroquine resistance. PfMDR1 is an active transporter using the energy provided by ATP to transport compounds into the vacuole. The physiological function is still unknown, but it has been implicated in transport of antimalarials into vacuole and polymorphisms lead to antimalarial resistance. PfMRP1 and PfMR2 are also active transporters belonging to the same family of PfMDR1, the ATP-binding cassette transporters, and are proposed to localize on plasma membrane. PfMRP1 has been implicated in some drug resistance phenotypes, folate metabolism and GSH redox. The PfMDR2, PfMDR5 and PfMRP2 are transporters that have some minor evidence linking them to resistance phenotypes, but their role remains largely unknown. Image created with BioRender.com

The PfMRP1 was hypothesized to be associated with the antimalarial drug response, since chloroquine was reported to be transported by the hMRPs (Vezmar and Georges, 1998). Furthermore, the antimalarials chloroquine, quinine, mefloquine and primaquine can inhibit hMRP mediated transport and reverse certain anticancer drug resistance. Therefore, these drugs are potential substrates of hMRP and

potentially PfMRP1 (Vezmar and Georges, 2000). PfMRP1 has been implicated in drug resistance with early reports associating SNPs with different sensitivities to the antimalarials chloroquine and quinine (Mu et al., 2003). Further studies on *in vitro* drug response of clinical isolates and *in vivo* malaria chemotherapy data appeared to support these associations (Dahlström et al., 2009b, 2009a; Phompradit et al., 2014b, 2014a; Raj et al., 2009; Veiga et al., 2011). PfMRP1 appears to be significantly polymorphic with distinct geographic patterns, with generally fewer polymorphisms observed in Africa. The most prevalent and associated to resistance SNPs include the: H191Y, S437A, I876V, F1390I and K1466R. The H191Y was associated with chloroquine and quinine resistance (Mu et al., 2003; Phompradit et al., 2014b) and decreased susceptibility to mefloquine in recrudescences (Phompradit et al., 2014a). The polymorphism S437A was associated with chloroquine, quinine (Mu et al., 2003) and mefloquine (Phompradit et al., 2014a) susceptibility, moreover an association was described of recrudescence carrying the 437A amino acid and artesunate sensitivity (Phompradit et al., 2014a). The I876V polymorphism stands out as having an apparent global spread. This polymorphism was found to be under selective pressure after AM-LF treatment, which led to near disappearance of the valine allele. This was attributed to reinfections and exposure to sub therapeutic levels of lumefantrine (Dahlström et al., 2009a). Moreover, the I876V polymorphism was associated in recrudescent cases with mefloquine and artesunate susceptibility (Phompradit et al., 2014a). The F1390I polymorphism was associated with an increase in susceptibility to chloroquine and quinine (Phompradit et al., 2014b). In addition, carriers of F1390 were observed to be less susceptible to artemisinin, mefloquine and lumefantrine (Veiga et al., 2011). At last, the 1466K polymorphism was selected in recrudescent cases from SP treatment (Dahlström et al., 2009b). Since this protein is proposed as a folate transporter (Rijpma et al., 2016b), the impact of the K1466R polymorphism could be related to alterations in folate transport and homeostasis, since increased folate concentrations have been associated with increased resistance to antifolates (Nduati et al., 2008; Wang et al., 1999).

Despite the field evidence pointing to involvement of PfMPR1 in antimalarial drug resistance, while trying to validate PfMRP1 role in drug resistance, *in vitro,* conflicting reports emerged. One study provided evidence that asexual blood stage parasites with disrupted *pfmrp1* accumulated more intracellular glutathione, quinine and chloroquine than WT parasites. These disrupted parasites also displayed increased susceptibility to several antimalarials, such as quinine, chloroquine, artemisinin, piperaquine and primaquine (Raj et al., 2009). This suggested that PfMRP1 is involved in the efflux of these drugs and absence of the transporter could lead to increased intracellular accumulation and, therefore, more drug can reach the site of action. However, these *pfmrp1* disrupted lines showed impaired growth rates,

particularly at higher parasitemia, thus confounding any evidence of a direct role of the protein in the drug responses, since the increase in drug susceptibility could be due to the less fit state of the parasite. Moreover, the possible hindering of response mechanisms to oxidative stress could render the parasite less able to cope with the drug action instead of the action of direct transport. Contradictory reports demonstrated no impact of a *pfmrp1* disrupted line on parasite susceptibility to most of the antimalarials tested and no apparent fitness cost (Rijpma et al., 2016b, 2016c). These studies differences in the parasite physiological responses could be due to the genetic background of parasite strains, the genetic manipulation technique and culture conditions. One study used chloroquine resistant *P. falciparum* W2 strain, using single recombination approach and standard static culture conditions (Raj et al., 2009). The other used chloroquine sensitive *P. falciparum* NF54 strain, using a double crossover with removal of the selectable marker through FLPe mediated recombination and semi-automated culture system (Rijpma et al., 2016b, 2016c). These study dependent differences highlight the importance of careful reporting of all strains, techniques and even culture conditions. Nevertheless, the PfMRP1 seems dispensable for asexual blood stage development, at least in certain *in vitro* conditions. Additionally, parasites with disrupted *pfmrp1* presented a decrease in folate concentration in the parasitized erythrocytes, suggesting a folate exporter function (Rijpma et al., 2016b). However, there was no distinction in this study of folate from the parasite versus the erythrocyte (Rijpma et al., 2016b). These parasites also had decreased sensitivity towards methotrexate, an antifolate compound and folate analog, but not for other antifolate compounds such as pyrimethamine, WR99210 and trimethoprim, which are structurally different from folate or other antimalarial drugs (Rijpma et al., 2016c, 2016b).

1.8.1.2. *P. falciparum* multidrug resistance protein 1 (PfMDR1)

PfMDR1 is a well-known modulator of antimalarial resistance (Calçada et al., 2020; Duraisingh and Cowman, 2005; Ferreira et al., 2011; Veiga et al., 2016; Wurtz et al., 2014). This transporter is composed of 1419 amino acids and is present on the membrane of the parasite DV. The ATP binding loops are orientated facing the cytoplasmic side, and therefore this transporter is considered a vacuolar membrane importer but cytoplasm exporter, which can sequester various compounds including toxic metabolites and antimalarials on the DV (Duraisingh and Cowman, 2005; Ferreira et al., 2011). The PfMDR1 was identified in the search for a homolog of the mammalian P-glycoprotein (P-gp), which is a key player in drug resistance in mammalian tumor cells due to the capability to transport a wide range of chemotherapeutic agents (Higgins, 2007). PfMDR1 was thought to be the major player in chloroquine resistance due to homology with the mammalian P-gp. While this initial hypothesis was proved not to be

accurate, the PfMDR1 seems able to modulate susceptibility to chloroquine, dependent on the parasite genetic background, mainly the PfCRT protein (Calçada et al., 2020; Reed et al., 2000; Sá et al., 2009; Veiga et al., 2016). The PfMDR1 biological functions are still unknown, but the transporter seems essential to the asexual stages of the parasite, as no knockout has been accomplished and the gene appears refractive to disruption (Rijpma et al., 2016a; Zhang et al., 2018).

Several polymorphisms, particularly, at the codons 86, 184, 1034, 1042 and 1246 of PfMDR1 have been linked with altered susceptibility to several antimalarial drugs (Duraisingh et al., 2000; Ferreira et al., 2011; Pickard et al., 2003; Reed et al., 2000; Sidhu et al., 2005). The SNPs at positions 86 and 184 are more prevalent in Africa, while at positions 1034, 1042 and 1246 are more frequent in South America. Transport studies have shown that PfMDR1 can transport antimalarials and that solute transport is modulated by the C-terminal polymorphisms at codons 1034, 1042 and 1246 (Reiling and Rohrbach, 2015; Rohrbach et al., 2006; Sanchez et al., 2008). The S1034C, N1042D and D1246Y polymorphisms seem to be involved in resistance to mefloquine, quinine and chloroquine (Reed et al., 2000), in a manner dependent on the genetic background (Sidhu et al., 2005).

Earlier studies, implicated the N86Y SNP in resistance to antimalarials, where the 86N was selected by AM-LF (Sisowath et al., 2007, 2005) and mefloquine (Price et al., 1999), while the 86Y by chloroquine (Khalil et al., 2005), amodiaquine (Holmgren et al., 2006) and piperaquine (Mungthin et al., 2017). Moreover, the position 86 was found to modulate transport to different drugs, as the N86Y polymorphism changes the specificity of transport from quinine and chloroquine to halofantrine transport (Sanchez et al., 2008). Additionally, parasites harboring the N86, 184F and D1246 SNPs have been linked with selection after AM-LF treatment (Conrad et al., 2014; Malmberg et al., 2013; Venkatesan et al., 2014). More recently, through *in vitro* allelic exchange, lines harboring N86Y polymorphism demonstrated that the 86Y increased resistance to amodiaquine and chloroquine and the N86 increased resistance to lumefantrine, mefloquine and dihydroartemisinin, corroborating the *in vivo* associations (Calçada et al., 2020; Veiga et al., 2016). The 184F mutation was found to have a limited impact, showing a slight decrease in piperaquine sensitivity when paired to N86 (Veiga et al., 2016) and instead seems to potentiate the N86 resistance phenotype by further increase of substrate transport (Calçada et al., 2020).

Furthermore, the *pfmdr1* gene is also found amplified with consequently higher protein expression that associates with *in vitro* resistance to multiple antimalarials such as quinine, mefloquine, lumefantrine, dihydroartemisinin and halofantrine (Calçada et al., 2020; Cowman et al., 1994; Gil and Krishna, 2017; Price et al., 2004; Sidhu et al., 2006; Wilson et al., 1989). This amplification was found to be the most predictive of treatment failure to mefloquine and AS-MQ (Price et al., 2004), as well as increased risk of

failure to AM-LF (Price et al., 2006), likely due to these drugs having targets of outside the DV. *Pfmdr1* amplifications are mainly distributed in South America (Pacheco et al., 2019) and Southeast Asia (Calçada et al., 2020). Recently, *pfmdr1* amplifications were demonstrated to be expanding in Southeast Asia carrying along the N86 and 184F polymorphisms. This *pfmdr1* genotype was shown *in vitro* through allelic exchange, to contribute to resistance to mefloquine, lumefantrine and dihydroartemisinin (Calçada et al., 2020).

Adapting therapies to the prevalent polymorphism in different world regions is important. Especially for PfMDR1 certain polymorphisms frequently have different impact depending on the drug, which highlights the importance of fully characterizing a transporter in the problematic of drug resistance.

1.8.1.3. Other ABC transporters

Besides the ABC transporters described above, the PfMDR2, PfMDR5 and PfMDR6 have been somewhat studied. However, the role of these proteins in antimalarial resistance largely remains to elucidate. PfMDR2 is proposed to be localized both on the DV and plasma membrane and appears to transport and generate resistance to heavy metals (Rosenberg et al., 2006). PfMDR2 had some early evidences linking to chloroquine and mefloquine response (Cowman et al., 1994; Wilson et al., 1989) and more recently to pyrimethamine (Briolant et al., 2012), however the transporter was not further explored concerning drug resistance. Recently, a genome-wide association study suggested PfMDR2 polymorphisms to associate with artemisinin resistance, as a contributor for a genetic background that creates a favorable background for PfK13 mutations to arise (Miotto et al., 2015). PfMDR5 was demonstrated to be a half transporter localized primarily on the plasma membrane, and with regards to drug resistance an association was reported between microsatellite repeats with lumefantrine (Okombo et al., 2013), piperaquine, chloroquine, amodiaquine and pyronaridine susceptibilities (Gendrot et al., 2019). PfMDR2 and PfMDR5 were shown to be dispensable to the parasite asexual stages. Moreover, the deletion of these proteins resulted in minor, although significant, decreases in sensitivity to mefloquine and quinine, for PfMDR2, and an increase in sensitivity to artemisinin, for PfMDR5 (van der Velden et al., 2015). The PfMDR6 was found to be under positive selection from a genome-wide association study (Mu et al., 2010). Additionally, microsatellite polymorphisms in PfMDR6 were linked with sensitivity to dihydroartemisinin (Wang et al., 2012), piperaquine, lumefantrine (Okombo et al., 2013) and quinine (Gendrot et al., 2017), however these associations were region-specific. Recently, the ABCI3 transporter was identified as possible modulator of drug resistance and drug target (Murithi et al., 2021).

1.8.2 *P. falciparum* chloroquine resistance transporter (PfCRT)

The PfCRT was identified in the search for the molecular mechanism of resistance to chloroquine (Fidock et al., 2000). This transporter is a transmembrane protein containing 424 amino acids with 10 transmembrane helices, and is localized on the DV of the parasite (Cooper et al., 2002; Fidock et al., 2000; J. Kim et al., 2019). Recalling, this organelle is where digestion of the erythrocyte cytosol occurs with the breakup of hemoglobin to provide essential amino acids for parasite growth and multiplication (Krugliak et al., 2002). Moreover, in the DV, the resultant byproducts of metabolism are detoxified. Consistent with the crucial role of DV, this cellular compartment is the local of action of multiple antimalarial drugs, with the most explored being chloroquine (Martin et al., 2018). The PfCRT is essential to the parasite asexual blood stage survival and has been identified as a drug target (Bushell et al., 2017; Waller et al., 2003; Zhang et al., 2018). Inhibition of PfCRT causes parasite death and downregulation leads to impaired growth and DV swelling (Pulcini et al., 2015). Although, PfCRT is essential for the parasite and has been demonstrated to be crucial for multiple drug resistance phenotypes, the native function and substrate specificity remained unresolved for a long time as many attempts to characterize the transporter produced widely disparate results (Bakouh et al., 2017; Juge et al., 2015; Nessler et al., 2004; Patzewitz et al., 2013; Warhurst et al., 2002; Wieczorek et al., 1991; Zhang et al., 2002). Recently, PfCRT was proposed to be a proton mediated exporter of host-derived peptides, of 4 to 11 residues in length, from the DV to the cytoplasm. Moreover, the transporter does not appear to transport non-specifically other metabolites or ions (Shafik et al., 2020). Furthermore, the net charge of PfCRT was demonstrated to be negative, suggesting substrates are likely to be positively charged (Shafik et al., 2020). Piperaquine and chloroquine are thought to accumulate in the acidic vacuole as protonated species (CQ^{2+} and PPQ^{4+}), where they bind to toxic Fe^{3+}-heme resultant from hemoglobin metabolism and inhibit the conversion into the chemically inert hemozoin. These drugs as positively charged can bind to PfCRT (Sullivan, 2017). Drug resistant variants of PfCRT were shown to reduce the capacity of PfCRT to transport peptides, which explains the fitness cost, and also increase the capacity of PfCRT to extrude drugs from their local of action on the DV (J. Kim et al., 2019; Shafik et al., 2020).

As mentioned, polymorphisms have been identified in the PfCRT that associate with resistance to chloroquine in field isolates (Ecker et al., 2012). Of particular note, the 76 amino acid position is fundamental in the chloroquine drug resistance phenotype, as chloroquine resistant field isolates carry a threonine at position 76 (76T) instead of the WT lysine (K76) (Ecker et al., 2012). Furthermore, allelic exchange experiments with resistant field isolate parasites demonstrated that the K76T mutation is fundamental for *in vitro* resistance to chloroquine (Lakshmanan et al., 2005). The mechanism of

resistance is a gain of function that makes PfCRT more capable to export chloroquine from the DV (J. Kim et al., 2019; Lehane et al., 2008; Lehane and Kirk, 2008; Martin et al., 2009; Shafik et al., 2020; Sidhu et al., 2002). While the K76T polymorphism seems to be sufficient to decrease the susceptibility to chloroquine (Martin et al., 2009), the degree of the resistance is imparted by the parasite genetic background and likely additional factors are necessary for a full resistant phenotype, including other PfCRT polymorphisms (Ecker et al., 2012; Gabryszewski et al., 2016b; Valderramos et al., 2010). As mentioned, the K76T polymorphism confers the parasite a fitness cost, which accounts for an increase on the population predominant allele back to WT after drug discontinuation (Kublin et al., 2003; Laufer et al., 2010). Hence, removal of chloroquine drug pressure can lead to virtual disappearance of mutant PfCRT in favor of fast-growing WT parasites (Chan et al., 2006; Frosch et al., 2014; Laufer et al., 2006). Regardless, polymorphisms in this gene have been demonstrated to impart a fitness cost to the parasite and therefore are predominantly selected in the context of drug pressure, although variants without fitness cost have recently evolved (Frosch et al., 2014, 2014; Lewis et al., 2014; Ord et al., 2007; Petersen et al., 2015). In fact, genetic and mathematical modeling show evidence that mutations within *pfcrt* occurred in bursts, in a balance to gain resistance and minimize the reduced fitness (Chan et al., 2012; Gabryszewski et al., 2016b; Mehlotra et al., 2005). Accordingly, other mutant forms of PfCRT harboring more mutations through the positions 72-76 (haplotype SVMNT) remain highly prevalent in South America even in the absence of drug pressure, highlighting the importance of compensatory mutations (Sa and Twu, 2010). These mutations spanning the codons 72-76 (Ibraheem et al., 2014) are more variable than the K76T, which might reflect the different histories of drug usage in the different geographic regions. Also, a highly mutant strain, named Cam734, with 5 different mutations, is reported to have the ability to confer chloroquine resistance without an associated fitness cost (Gabryszewski et al., 2016a). In sum, the 76T is the most predictive polymorphism of chloroquine treatment failure (Ecker et al., 2012), however compensatory mutations eliminate the fitness cost and propel a higher resistance phenotype.

The role of PfCRT is not that well-defined for other antimalarials. Studies with genetically altered lines demonstrated that mutant PfCRT can increase susceptibility to artemisinins (Sidhu et al., 2002; Valderramos et al., 2010). Specifically, AM-LF seems to select for the WT K76 allele (Sisowath et al., 2009; Venkatesan et al., 2014), whereas AS-AQ appears to select for the 76T mutant form in field isolates (Folarin et al., 2011; Venkatesan et al., 2014). This differential selection by distinct alleles is helpful to adapt therapy in the field. Consequently, there is value to use both therapies simultaneously as first-line treatments, or to adapt therapies based on the population predominant allele. As such, owing to the demonstrated importance of this protein is important to monitor PfCRT, in particular for ACT therapies.

Moreover, PfCRT mutations have been shown to at least modulate susceptibility to several drugs including lumefantrine, mefloquine, amodiaquine and piperaquine (Blasco et al., 2017; Dhingra et al., 2017; Fidock et al., 2000; Martin et al., 2018; Ross et al., 2018; Sá et al., 2009; Sidhu et al., 2002; Sisowath et al., 2009). Regarding piperaquine, recent evidence shows an increasingly important role of PfCRT in generating resistance to this drug. As one of the main focuses of this thesis, and because PPQ resistance is multifactorial this topic was covered in chapter 1.7.1. As of note, PfCRT has been demonstrated to transport chloroquine and piperaquine (Martin et al., 2009; Summers et al., 2014). More recently new PfCRT polymorphisms, F145I and C350R, were shown to mediate piperaquine efflux concomitant with reduced chloroquine transport (J. Kim et al., 2019). This new data suggests that PfCRT has drug specific transport, and the ability to transport chloroquine and piperaquine depends on mutations in distinct sites in the central cavity (J. Kim et al., 2019). These mutations can differentially impact electrostatic and other properties of the cavity, and importantly show that antimalarials can exert opposing selective pressures on this transporter (J. Kim et al., 2019).

1.9 Genetic engineering in *P. falciparum*

The capability to genetically manipulate *P. falciparum* is required to functionally understand the genome. Knockout, knockdown and overexpression of genes allow evaluating the overall function of a gene in a phenotype. Moreover, allelic exchange can provide crucial information about the role of SNPs in the drug resistance phenotype. *P. falciparum* has a haploid genome, at the human host, and therefore genetic manipulation involves only targeting of a single allele and the ability to propagate this specie *in vitro* allows the use of blood stages of the parasite to perform transfection-based experiments. There has been a considerable development in the technologies used for transfection and manipulation of *P. falciparum* genome (De Koning-Ward et al., 2015).

All these methods require *in vitro* transfection of DNA into the parasite being in use since 1995. However, transfection efficiency on this organism is very low and laborious. Therefore, only recently methods with higher success rate have been adopted in a wider scale. The main technologies used for parasite genetic manipulation are the serine integrase mycobacteriophage Bxb1 system, selection linked integrase (SLI), zinc finger nucleases (ZFNs) and CRISPR-Cas9 (Table 1). Although other methods of genetic manipulation exist such as homing endonucleases and transcription activator-like effector nucleases (TALENs) these are not routinely used in *P. falciparum*. A summary of current genome editing is present in Table 1 and described in the next chapters.

1.9.1 Site-specific nucleases

Site-specific nucleases, such as ZFNs and CRISPR/Cas9, share the ability to selectively trigger a double-strand break at a defined site in the genome. Genome engineering exploits the cellular response to the induced breaks (Lee et al., 2014). In many organisms, repair occurs by the error-prone pathway of non-homologous end joining, which frequently leads to indels generating gene disruptions. However, *Plasmodium* lacks the canonical pathway, a double-edged sword that on one side eliminates one facile method for generating gene disruptions but decreases the concern about potential off-target effects from cleavage at unintended loci (Lee et al., 2014). An alternative end joining pathway has been proposed (Kirkman et al., 2014). However, this mechanism appears much less efficient and *P. falciparum* primarily uses homologous recombination when provided a repair template (Kirkman et al., 2014). As a response to induced genome breaks the parasite and by giving an exogenous homologous sequence the endonuclease system can be exploited to introduce genome mutations (Lee et al., 2014). The specifics of donor design are as diverse as the potential uses of nuclease gene disruption, such as single nucleotide modification, tagging or marker-free insertion of fluorescent reporters, conditional control elements or knockout. Some aspects of donor design must be taken into consideration such as the length of template, distance from the nuclease cut site and presence of silent mutations to prevent nuclease cut of recombinants. Although a possibility, until now, off target alterations have not been reported for *Plasmodium* after ZFN or CRISPR editing experiments (Lee et al., 2019).

The *Plasmodium* genome comprises multiple multigene families (Gardner et al., 2002). High degree of homology between family members is a potential challenge in targeting one specific gene and unintended repair from the paralogous gene sequences can occur rather than the provided donor. Careful design of homology sequence, as well as guide RNA (gRNA) (for CRISPR-Cas9 system, explained below) is essential, as well as careful genotyping (Lee et al., 2019). A general caution for any genome-editing strategy should be the consideration of unexpected deletions and rearrangements that are difficult to identify using the standard PCR-based genotyping.

1.9.1.1. Zinc finger nucleases (ZFN)

ZFNs are DNA-binding proteins engineered to couple an endonuclease enzyme designed to target specific locations in the genome inducing double-strand breaks (Straimer et al., 2012). Developing of ZFNs is usually outsourced as it is a costly and laborious process. However, nowadays, specialized companies provide highly optimized zinc fingers with high rates of success (Moraes Barros et al., 2015; Ross et al., 2018; Straimer et al., 2012). The zinc fingers are usually encompassed in a donor plasmid

encoding a ZFN pair (ZFN Left (ZFNL) and ZFN Right (ZFLR)) that can be co-expressed from a single promoter using the viral 2A ribosomal skipping peptide (Straimer et al., 2012). Following expression, the ZFNL and ZFNR dimerize and assemble an artificial enzyme, which includes FokI enzyme domain capable to induce double strand breaks, guided by the ZFN pair to the desired genome location (Straimer et al., 2012). The DNA double strand break triggers the genome repair mechanisms. The homologous recombination pathway, as mentioned before, can then be exploited to make genome modifications, such as gene knockouts and allelic exchange. Gene editing with ZFN is expensive and requires a new protein design for each target locus. Due to the high cost and time to generate custom ZFNs although demonstrating high efficacy to generate allelic exchange early on, the technology has been used mainly for editing of difficult and critical targets related with drug resistance and is being largely replaced by CRISPR approaches. The ZFN genome editing tool has been employed in *P. falciparum*, particularly for the study of drug resistance to perform allelic exchanges in PfCRT, PfMDR1 and PfK13 (Moraes Barros et al., 2015; Ross et al., 2018; Straimer et al., 2015; Veiga et al., 2016).

1.9.1.2. CRISPR

The initial CRISPR-Cas9 systems for *P. falciparum* were developed using a two-plasmid system to deliver the endonuclease Cas9, gRNA and donor template (Ghorbal et al., 2014; Wagner et al., 2014). A targeting gRNA guides the Cas9 to a genome location, where the endonuclease mediates the double strand genome break. More recently, Lee *et al.* exploited the use of shorter promoters to create an all-in-one delivery plasmid (Lee et al., 2019). Additionally, CRISPR systems were also developed recently for *in vitro P. knowlesi* manipulation, which coupled with higher transfection efficiency of this species can facilitate large scaling screening of genetic modifications (Mohring et al., 2019).

The main feature of the Cas9 system is that, unlike ZFNs and TALENs, the nuclease does not require modification to alter target specificity, greatly simplifying the design phase and cost of the technology. Specificity is instead conferred by the gRNA, particularly the first 20 nucleotides (Ribeiro et al., 2018). The gRNA consists of a sequence that can be divided into crRNA and trRNA. The trRNA is a non-variable part optimized for the system that mediates interaction of the mature RNA with the endonuclease (Ribeiro et al., 2018). The crRNA is the specificity conferring region and can be made to target specific DNA locations by designing a complementary sequence to the genome target (Ribeiro et al., 2018). Approaches using a single-guided RNA (sgRNA) have been optimized to facilitate the design process by requiring only target zone design. Careful designing of gRNA is fundamental, with multiple parameters affecting the efficacy. The gRNA needs to be designed complementary to the target on the genome

followed by a PAM sequence (-NGG for *Streptococcus pyogenes* derived Cas9), taking into consideration off targets, and be as close as possible to the required site of modification (Ribeiro et al., 2018). Several algorithms have been developed that facilitate the gRNA construction process (Lee et al., 2019). Furthermore, there is usually a need to do parallel experiments with multiple sets of gRNAs to expedite the process, since even gRNA with high scores can end up failing. Despite that, Cas9 is considerably more cost-effective than ZFN and TALENs, as the later require engineering of specific enzymes, which is not viable for most laboratory groups, and even designing several gRNAs is several times more cost-efficient. An additional challenge to the current CRISPR-Cas9 system is the AT-rich genome of the parasite, comprising 80% of the genome (Gardner et al., 2002), which limits the availability of -NGG PAM regions for gRNA construction and reduces the quantity of available spots for genome editing. Novel variants of CRIPSR nucleases in development, particularly Cas12a (originally named Cpf1) are better suited for AT-rich genomes with a -TTTN PAM region (Safari et al., 2019; Zetsche et al., 2015).

The CRIPSR-Cas9 system was demonstrated to be efficient in *P. falciparum* with gene knockouts and single nucleotide substitutions achieved in short times (Bansal et al., 2017; Crawford et al., 2017; Ghorbal et al., 2014; Nacer et al., 2015; Ng et al., 2016; Wagner et al., 2014). One of the main impacts of this technology so far was to validate the PfK13 C580Y SNP in ART resistance (Ghorbal et al., 2014).

As with many other organisms, CRISPR-Cas9 is quickly becoming the preferred strategy because of simplicity and cost, and currently is the technology that is evolving faster for genome editing and in *P. falciparum*.

1.9.2 Bxb1 integrase system

Nuclease-based strategies, due to the nature of genome break are more appropriate to gene modifications such as knockouts and allelic exchange. However, the study of *P. falciparum* genes that are important during erythrocytic growth has been hampered because of the haploid parasite genome, and thus conventional knockout approaches can lead to parasite death or severe growth defects if the targeted gene has essential functions to the parasite. The mycobacteriophage Bxb1 integrase approach facilitates the integration of transgenes and reporters (Nkrumah et al., 2006). This serine integrase on contrary to other integrases does not require DNA super coiling, divalent cations or bacterial host factors for activity, making the use in heterologous systems more appealing (Balabaskaran-Nina and Desai, 2018). The Bxb1 integrase can efficiently catalyze recombination between an *attP*-containing plasmid and an *attB* site integrated into the parasite genome (Ghosh et al., 2008; Gupta et al., 2018; Kim et al., 2003; Nkrumah et al., 2006). This system requires the use of genetic modified lines containing the attB site,

such as the 3D7^{attB} and Dd2^{attB}, integrated at the erythrocyte stage non-essential cg6 locus (Nkrumah et al., 2006). The transient expression of the integrase in the nucleus of the parasite allows genome modifications. An *attP*-containing plasmid encompassing a gene of interest (GOI) can be used to insert a copy of this gene in the parasite genome (Nkrumah et al., 2006). This system has been employed in *P. falciparum*, such as for the localization of H-protein by introducing a tagged protein into the genome (Spalding et al., 2010). Another approach using this system was the introduction of the *attB* site into an intron of a desired target, which allowed modifications in the downstream sequence (Balabaskaran-Nina and Desai, 2018). However, intronic sequences are not prevalent in the parasite genome limiting this approach to selected genes. To circumvent that, an intronic sequence with *attB* can be placed in the middle of the gene. However, this process is more laborious and involves multiple transfections. This technique can be also used to carry out site-directed mutations, internal insertions or deletions (indels), domain swaps, addition of C-terminal epitope tags on the encoded protein, modify codons, or alter transcriptional regulatory elements. Moreover, after successful *attB* introduction the system in several studies reported rapid appearance of recombinants with a near-homogeneous population of integrant parasites within 2–4 weeks of the second transfection (Balabaskaran-Nina and Desai, 2018; Nkrumah et al., 2006; Spalding et al., 2010). However, there is a limitation in cases where the desired transgene is a modified version of a parasite gene since the endogenous copy is not affected by the transfection and remains functional, then a nuclease-based engineering is preferred.

1.9.3 Selection linked integrase (SLI)

Localization and functional analysis of *P. falciparum* proteins is essential to understand the biology of this parasite and to uncover new drug targets, but progress in this respect has been slow. Selection linked integrase (SLI) is based on homologous recombination by chance but was improved to overcome the inefficiency of the randomness by allowing selection of parasites with genomic integrations (Birnbaum et al., 2017). In this approach, a promoterless targeting region on the plasmid to be integrated is linked with an additional selectable marker that is separated by a 2A skip peptide (Birnbaum et al., 2017). The selection marker will only be expressed after successful single crossover integration into a locus with a promoter and the use of the skip peptide makes the marker not attached to the target. Parasites carrying a modification of interest can be selected faster using the corresponding drug pressure for the selected resistance marker. This method has been exploited to generate green fluorescent protein (GFP) knockins, in which full endogenous genes are fused to a GFP sequence for localization studies, knockouts by disrupting the target gene and knock sideways, in which a target is tagged with a mislocalizer protein to

move out of the site of function to bypass problems of disrupting essential genes (Birnbaum et al., 2020, 2017). This method has also been employed to modify alleles. While this method leaves a larger footprint compared to CRISPR-Cas9 or ZFNs, has the advantage to dispense parasite cloning, which allows the rapid assessment of many mutations. Moreover, SLI is more cost-effective compared to ZFNs and improves on the transfection efficiency in CRISPR methods that use two plasmids. Nevertheless, the system requires the use of two selection markers (Birnbaum et al., 2017), one to select for episomal plasmid to increase the likelihood of integration event and another to select for the integration event, as such this technique is less suitable for multiple sequential genome manipulations.

Table 1. Current used technologies for *P. falciparum* genome modification, simple description, main uses, advantages and disadvantages.

Method	Mechanism	Essential elements	Main uses	Advantages	Disadvantages
Zinc finger nuclease (ZFN)	Homologous repair after induced genome break by engineered specific ZFN.	Custom engineered zinc finger nuclease. Homologous template to induce repair and desired modification.	Allelic exchange Knock-outs	Highest efficiency with high precision Allows sensitive changes with low genomic impact such as one nucleotide change.	High cost Requires engineering of ZFN for each new target
CRISPR-Cas9	Homologous repair after induced genome break by endonuclease Cas9 guided to target genome location by specific gRNA.	Cas9 endonuclease. Specific gRNA designed to target genome location near PAM sequence. Homologous template to induce repair and desired modification.	Allelic exchange Knock-outs	Lower cost of implementation than ZFNs, allows design of changes in multiple targets. Allows sensitive changes with low genomic impact such as one nucleotide change.	Not fully optimized for P. falciparum Modifications restricted to near PAM sites Careful gRNA design, often needing parallel experiments with different gRNAs.
Bxb1 integrase	Bxb1 integrase mediates recombination between contents of exogenous DNA with attP site with parasite lines modified with attB site.	Bxb1 integrase. Parasite line engineered to have attB site. Plasmid with attP site with exogenous content to introduce on genome.	Introduction of transgene, overexpression of genes Tagging, modification of regulatory elements	Rapid gene editing in attB lines Allows higher range of gene modifications, such as tagging and modification of regulatory elements Reduces risk of off target modifications	Laborious introduction of attB site into parasite genome Leaves higher genomic fingerprint, due to whole plasmid introduction and selection marker.
Selection linked integration (SLI)	Homologous recombination selection driven.	Plasmid with homologous region and a linked selection marker.	Tagging, modification of regulatory elements Knock sideways and Knock-outs	Does not require enzyme expression. Possible to do knock sideways on essential genes to circumvent limitations of knockout strategies. Good for tagging and location studies. Simple design, usually fast and efficient.	Depends on random recombination, with some targets that are less susceptible to recombination. Leaves higher genomic fingerprint

2. AIMS

This dissertation has a multifactorial approach in major topics concerning parasite molecular pathways that are involved in the problematic of antimalarial resistance both *in vivo* and *in vitro*. The dissertation covers resistance to ACTs, particularly the assessment *in vivo* in Africa of resistance molecular markers (Study 1 and 2). Moreover, the dissertation covers *in vitro* validation of piperaquine mediators of resistance (Study 3). Finally, the dissertation explores the PfMRP1 in antimalarial resistance and further elucidation of the transport properties (Study 4).

The specific aims of the studies presented in this dissertation are described here.

Study 1 - *Plasmodium falciparum plasmepsin 2* duplications, West Africa: Identification of molecular markers related with ACT resistance, in Africa, that can be used to evaluate and predict emergence of resistance. This study explores the possible presence in Africa of a molecular marker of piperaquine resistance, the increased copy number of *plasmepsin* locus.

Study 2 - *Plasmodium falciparum* K13 expression associated with parasite clearance during artemisinin-based combination therapy: The role of *pfk13* transcriptional response has not been explored, despite PfK13 loss-of-function being key for artemisinin reduced susceptibility. This study explores the *in vivo pfk13* transcriptional response and parasite clearance outcomes after artemether-lumefantrine treatment.

Study 3 - Multigenic architecture of piperaquine resistance trait in *Plasmodium falciparum*: Resistance to ACTs, in the present, is mostly due to failing partner drugs. As such, identification of mediators of resistance is fundamental to tackle this problem. This study aims to clarify the role of piperaquine mediators of resistance. Therefore, through parasite genetic manipulation, *in vitro*, parasites were developed containing *plasmepsin* gene duplications and analyzed in a piperaquine drug resistance perspective.

Study 4 - The *Plasmodium falciparum* protein PfMRP1 functions as an influx ABC transporter: Transporter proteins are essential for antimalarial resistance phenotypes. This study sought to clarify the role of PfMRP1 in antimalarial resistance. Through parasite genetic manipulation, *in vitro*, parasites were developed with disrupted *pfmrp1* gene. Due to a key finding that this transporter appears to be an importer protein, this study aims to explore the PfMRP1 transport functionality.

3. MATERIAL AND METHODS

3.1 Materials

3.1.1 Reagents, solutions and mediums

- Super optimal broth (SOB): 0.5% (w/v) yeast extract (Millipore, 70161), 2% (w/v) tryptone (Millipore, T7293), 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$ prepared in milli-Q H_2O. pH was adjusted to 7 with 1M NaOH. Sterilized by autoclaving.
- Dulbecco's Phosphate-Buffered Saline (Corning, 20-030-CV)
- Lysogeny broth (LB): 10 g/L tryptone (Millipore, T7293), 5 g/L yeast extract (Millipore, 70161) and 10 g/L NaCl, prepared in milli-Q H_2O. For plates 15 g/L of agar was used. Sterilized by autoclaving.
- 1X Tris-acetate-EDTA (TAE) buffer: 40 mM Tris (pH 7.6) (Sigma, TRIS-RO), 20 mM acetic acid (Sigma, 695092), 1 mM EDTA (Supelco, 1.08418), prepared by diluting 50X in distilled H_2O.
- Malaria culture medium (MCM): RPMI 1640 (Gibco, 23400062) supplemented with 2 mM L-glutamine (present on RPMI), 200 µM hypoxanthine (Merck, H9377), 0.25 µg/mL gentamycin (Gibco, 345815), 25 mM HEPES (present on RPMI), 0.2% $NaHCO_3$, and 0.25% AlbuMAX™ II Lipid-Rich BSA (Gibco, 11021037)]. Filter sterilized.
- RPMI without folate (Gibco, 27016021): Supplemented with 2 mM L-glutamine (present on RPMI), 200 µM hypoxanthine (Merck, H9377), 0.25 µg/mL gentamycin (Gibco, 345815), 25 mM HEPES (Gibco, 15630080), and 0.25% AlbuMAX™ II Lipid-Rich BSA (Gibco, 11021037)]. Filter sterilized.
- Parasite freezing solution: 28% glycerol (Sigma, G5516-1L), 3% sorbitol (Sigma, S3889-1KG) and 0.65% NaCl, prepared in milli-Q H_2O. Filter sterilized.
- Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, prepared in distilled H_2O, and pH adjusted to pH 7.4 with HCl. Sterilized by autoclaving.
- Cytomix: 10 mM K_2HPO_4/KH_2PO_4, 120 mM KCl, 0.15 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES (Gibco, 15630080), 2 mM EDTA (Supelco, 1.08418), prepared in milli-Q H_2O and pH adjusted to 7.6 with 10 M KOH. Filter sterilized.

- Lysis solution with 4X SYBR Green: 20 mM Tris (pH 7.5), 5 mM EDTA, 0.008 % (W/V) saponin (Merck, 47036), 0.08 % (V/V) Triton X-100 (Sigma, X100) and 4X SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO (Invitrogen, S7564)
- Cytometry drug assay staining solution: 2X SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO (Invitrogen, S7564) and 1 µM of MitoTracker™ Deep Red FM (Invitrogen, M22426) in PBS

3.1.2 Chemicals

All compounds otherwise stated were filter sterilized and stored in aliquots at -80 °C before use. Working solutions for intermediary dilutions were otherwise stated performed in MCM.

- Blasticidin S HCL (Sigma, 1520S-25MG): Stock prepared at 10mg/mL in milli-Q H_2O.
- G418 (Merck, 108321-42-2): Stock prepared at 400 mg/mL in milli-Q H_2O.
- WR99210 (Gift from Jacobus Pharmaceuticals): Stock prepared at 1mg/mL in DMSO. Working solution prepared by 1/100 dilution in culture medium and stored at 4 °C for 6 weeks.
- Chloroquine (Merck, C6628): Stock prepared at 1.94 mM in milli-Q H_2O.
- Mefloquine (Sigma, M2319-100MG): Stock prepared at 24.1 mM in DMSO.
- Lumefantrine (Sigma, L5420-5MG): Stock prepared at 2.36 mM in DMSO.
- Dihydroartemisinin (Sigma, D7439-50MG): Stock prepared at 17.59 mM in acetone.
- Pyrimethamine (Sigma, 46706-250MG): Stock prepared at 40.2 mM (10 mg/mL) in DMSO.
- Trimethoprim (Sigma, 91131-1G): Stock prepared at 68.8 mM in DMSO.
- Amodiaquine (Sigma, 1031004): Stock prepared at 10 mM in milli-Q H_2O.
- Methotrexate (Merck, 454-126-100MG): Stock prepared at 200mM in milli-Q H_2O.
- Aminopterin (Sigma, A1784): Stock prepared at 23 mM (10 mg/mL) in 1M NaOH.
- Piperaquine (Sigma, C7874-5mg): Stock prepared at 5 mM in H_2O.
- Verapamil (Sigma, V4629-1G): Stock prepared at 101.82 mM (50 mg/mL) in methanol.
- Elacridar (Sigma, SML0486-10MG): Stock prepared at 3.8 mg/mL in DMSO.
- MK571: Stock prepared at 9.31 mM in H_2O.
- MitoTracker™ Deep Red FM (Invitrogen, M22426): Stock solution prepared at 500 µM in DMSO (184 µL of DMSO into one stick of 50 µg of lyophilized compound).
- Methotrexate, Fluorescein, triammonium Salt (F-MTX) (Invitrogen, M1198MP): Stock solution prepared at 1 mM in DMSO.
- SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO (Invitrogen, S7564)

- DAPI Solution (Thermo Scientific, 62248, 1 mg/mL): Working solution was prepared in PBS (see below) at 1 µg/mL and filter sterilized before use.
- Folic acid (Sigma, F8758): Working solution prepared at 5 mg/mL in 1M of NaOH. Solution prepared fresh for every experiment.
- DTT (Fermentas, R0862): Working solution was prepared at 1 M in milli-Q H_2O. Prepared fresh every experiment.
- GSH (Cayman Chemical Company, 1.800.364.9897): Working solution prepared at 32.3 mM in milli-Q H_2O. Filter sterilized. Prepared fresh every experiment.
- GSSG (Cayman Chemical Company, 15491): Working solution prepared at 10 mM in milli-Q H_2O. Filter sterilized. Prepared fresh every experiment.
- H_2O_2 (Sigma, H1009): Working solution prepared at 9.8 mM in milli-Q H_2O. Prepared fresh every experiment.
- Carbenicillin (NZYTech, MB16501): Stock prepared at 100 mg/mL in milli-Q H_2O. Stored at 4 °C for 4 weeks.
- Fluo-4, AM, cell permeant (F14201)

3.1.3 Cells and plasmids

- Super *E. coli* XL10 Gold® ultracompetent cells (Agilent, 200315)
- *Plasmodium falciparum* 3D7 strain (MR4-Malaria Resources, MRA-120)
- *Plasmodium falciparum* Dd2 strain (MR4-Malaria Resources, MRA-156)
- *Plasmodium falciparum* $Dd2^{attB}$ strain (MR4-Malaria Resources, MRA-843)
- pDC2 plasmid (kindly provided by David Fidock, (Straimer et al., 2012))
- pINT-based vector (kindly provided by David Fidock, Adjalley et al., 2010)
- p415TEF cyto roGFP2-Grx1, (a gift from Tobias Dick; Addgene plasmid # 65004; http://n2t.net/addgene:65004; RRID:Addgene_65004)

3.1.4 Enzymes, mixes and kits

- NZYSpeedy qPCR Green Master Mix (NZYTech, MB22401)
- SYBR™ Green PCR Master Mix (Applied Biosystems, 4309155)
- Supreme NZYTaq II 2× Green Master Mix (NZYTech, MB36001)
- NZYSpeedy qPCR Green Master Mix (2x) (NZYTech, MB224)
- NZYMiniprep kit (NZYTech, MB010)

- NZYMidiprep endotoxin free kit (NZYTech, MB27901, discontinued)
- NZYMaxiprep endotoxin free (NZYTech, M39901)
- CloneJET PCR Cloning Kit (Thermo Scientific, K1231)
- NZYGelpure (NZYTech, M011)
- QuikChange Multi SDM Kit (Agilent, 200514)
- T4 DNA Ligase (Thermo Scientific, EL0011)
- Phusion Blood Direct PCR Master Mix (Thermo Scientific, F175S)
- NZY Blood gDNA Isolation kit (NZYTech, MB13602, discontinued)
- NZY Tissue gDNA Isolation kit (NZYTech, MB135, replacement for MB13602)
- NZY Total RNA Isolation kit (NZYTech, M13402)
- First-Strand cDNA Synthesis Kit (NZYTech, MB125)
- Restriction enzymes: BglII (Thermo Scientific, FD0083); XhoI (Thermo Scientific, FD0694); NheI (Invitrogen, IVGN0066); ApaI (Invitrogen, IVGN0326); SacII (Thermo Scientific, ER201); XamJI (AvrII) (Thermo Scientific, FD1564)

3.2 Study 1 - *Plasmodium falciparum* Plasmepsin 2 Duplications, West Africa

3.2.1 Clinical material – Study site, sample collection and ethics

A subset of archived *P. falciparum* DNA samples were analyzed from clinical infections derived from a set of large, multicenter comparative ACT efficacy trials conducted in West Africa by the West African Network for Antimalarial Drugs (Pan African Clinical Trials Registry (PACTR201105000286876) (West African Network for Clinical Trials of Antimalarial Drugs (WANECAM), 2018). These trials, performed from October 2011 to February 2016 in Mali, Burkina Faso, and Guinea, had a randomized double-blind design with a 2-year follow-up for monitoring repeated treatment. The study focused on the DHA-PPQ trial conducted at the village of Bougoula-Hameau in Mali, located ≈350 km south of the capital city of Bamako, near the border with Burkina Faso. The weekly control follow-up for each episode at Bougoula-Hameau was 63 days, and the DHA-PPQ arm involved a total of 224 patients, who were over 6 months of age.

We conducted a pilot study analyzing the 96 recurrent infections associated with the shortest inter-episode periods, assuming that this subgroup, among whom initiation of recurrent infection ranged from 23 to 65 days posttreatment (Figure 11), would be the most likely to include *pfpm2* duplications. The

study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology, University of Sciences, Techniques and Technology of Bamako.

3.2.2 Molecular analysis

The copy number of *pfpm2* (PF3D7_1408000) was determined by using a SYBR-green–based quantitative PCR (ThermoFisher Scientific, Waltham, MA, USA) in a protocol modified from the one previously described (Witkowski et al., 2017). DNA was purified using NZY Blood gDNA Isolation kit. *P. falciparum* β-tubulin (*pfβtub,* PF3D7_1008700) gene was used as the internal nonduplicated standard and the *P. falciparum* 3D7 strain DNA as a parallel single *pfpm2* copy control. Quantitative PCR was performed with SYBR™ Green PCR Master Mix in a 10 µL total volume and 400 nM of each primer. Thermal cycle was performed at 98 °C for 3 minutes, followed by 45 cycles at 98 °C for 15 seconds, 63 °C for 20 seconds, and 72 °C for 20 seconds on a C1000 Thermal Cycler (Bio-Rad, Marnes-la-Coquette, France) with the CFX96 Real-Time System (Bio-Rad) detection system.

Copy number was calculated using the *pfβtub* as the endogenous control gene by the $2^{-\Delta\Delta_{Ct}}$ method and using 3D7 DNA as a single copy calibrator (Livak and Schmittgen, 2001). Primers used are in Table 2. Primers and experimental setup were validated using serial dilutions of *P. falciparum* DNA to verify linearity over the ranged of concentrations used (Figure 9). All procedures were performed in triplicate.

Table 2. Primers used for quantitative PCR of *plasmepsin 2 (pfpm2)* copy number

Gene	Primer	Sequence (5'-3')
pfpm2	*pfpm2*_fw	TGGTGATGCAGAAGTTGGAG
PF3D7_1408000	*pfpm2*_rv	TGGGACCCATAAATTAGCAGA
pfβtub	*pfβtub*_fw	TGATGTGCGCAAGTGATCC
PF3D7_1008700	*pfβtub*_rv	TCCTTTGTGGACATTCTTCCTC

3.3 Study 2 - *Plasmodium falciparum* K13 expression associated with parasite clearance during artemisinin-based combination therapy

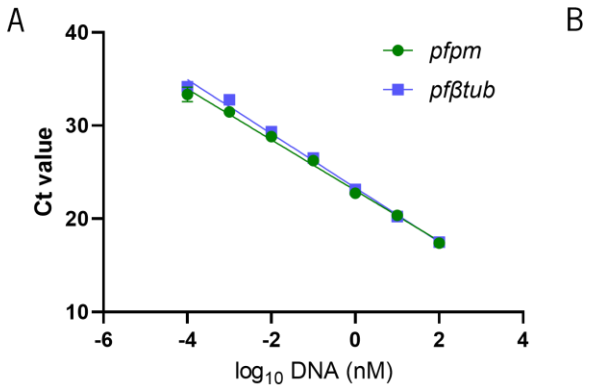
3.3.1 Clinical material - Study site, sample collection and ethics

The trial under study was conducted at the Fukayosi Primary Health Care Centre, Bagamoyo District, Tanzania in accordance with the Declaration of Helsinki and Good Clinical Practice (Carlsson et al., 2011). Ethics clearance was granted by the National Institute for Medical Research, Dares Salaam, Tanzania,

and the Regional Ethics Committee, Stockholm, Sweden. Informed consent was obtained from parents or guardians of all children enrolled (Clinical Trials (US), identifier NCT00336375). Briefly, 50 children with microscopically confirmed uncomplicated *Plasmodium falciparum* malaria were hospitalized and received AM-LF treatment in six doses over 3 days. Venous blood samples were collected before treatment initiation (0 hours) and at nine more time points (2, 4, 8, 16, 24, 36, 48, 60 and 72 hours) after initiation of treatment. Parasite densities were determined by Giemsa staining, and parasite clearance was measured and documented as the proportion of patients with positive microscopy.

3.3.2 RNA extraction

RNA was extracted from venous blood of the 50 enrolled patients using an ABIPRISM H6100 Nucleic Acid PrepStation (Applied Biosystems, Fresno, CA, USA). Not expecting to find parasitic RNA 24 hours



B

	pfpm	*pfβtub*
Slope	-1,737	-2,902
Y-intercept	22,57	23,33
R^2	0,9943	0,9931

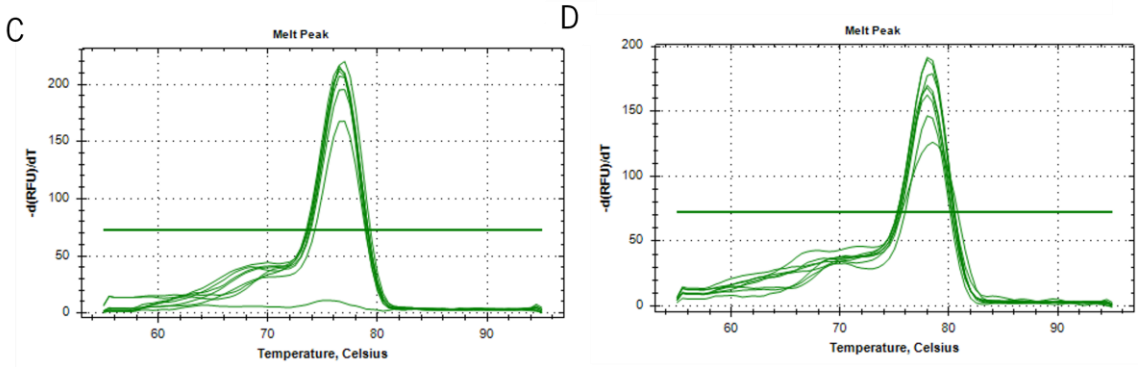


Figure 9. Validation of q-PCR setup with primers of Table 2. A – Serial diluted samples of *P. falciparum* DNA and the corresponding Ct values for respective primers. B – Slope, Y-intercept, R2, and equation corresponding to A. C – Melting peak of *pfpm2* amplification. D – Melting peak of *pfβtub* amplification. C and D were obtained from BioRad CFX Manager 3.1 software.

post-treatment, total RNA (human and parasite) quality and quantity were measured using the Agilent RNA6000 Pico total RNA assay in an Agilent 2100 Bioanalyser™ (Agilent, Santa Clara, CA, USA) and standardized prior to cDNA synthesis for the first six timepoints [0 hours (before treatment) and 2, 4, 8,

16 and 24 hours after treatment initiation]. Three out of 50 patients sampled had low total RNA quality and therefore were excluded from further transcript analysis.

3.3.3 Quantitative PCR

Quantitative PCR was performed in triplicate with custom MGB TaqMan® probes for the *pfk13* (PF3D7_1343700) gene, in an ABIPRISM® 7900HT Sequence Detection System (Applied Biosystems). The housekeeping gene serine-tRNA ligase (PF3D7_0717700), shown to be transcribed stably throughout different intraerythrocytic stages (Bozdech et al., 2003; Magallón-Tejada et al., 2016; Ngwa et al., 2017; Veiga et al., 2010), was used as an endogenous control (normalizer). Oligonucleotides used are presented in Table 3.

Table 3. Primers used for quantitative PCR of *pfk13* expression

Gene	Probe/Primer	Sequence (5'-3')
pfk13	6-FAM, NFQ probe	ACGCCAGCATTGTTG
PF3D7_1343700	Primer fw	GTGGATTTGATGGTGTAGAATATTTAAATTCGA
	Primer rev	GCTTTTTTGGTAGACATAGGTGTACACA
serine–tRNA ligase	VIC, TAMRA *probe*	ACGCCAGCATTGTTG
PF3D7_0717700	*Primer fw*	TGAAACTATAGAATCAAAAAGGTTACCACTCAAATACGCT
	Primer rev	TGTGCCCCTGCTTCTTTTCTA

Amplification reactions were done in quadruplicate in 384 well plates with 10 µL containing TaqMan® Gene Expression Mastermix (Applied Biosystems™, Fresno, CA, USA), 300 nM of each forward and reverse primer, 100 nM of TaqMan® probe and 2 µL of amount-normalized cDNA. Amplification conditions were 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. For each patient, *pfk13* transcript fold change in expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) in which the threshold cycle number (C_t) was normalized to the C_t of the housekeeping gene serine-tRNA ligase (PF3D7_0717700) for all time points (ΔC_t) and $\Delta\Delta C_t$ calculated using the ΔC_t from time point 0 h (before treatment) as a calibrator. Spearman correlation was applied to assess linear relationships between *pfk13* expression variation throughout time and between parasite clearance (PC) parameters. Patient data were stratified in two groups defined as *pfk13* transcript expression of more or less than 1-fold change (1-fold change represents no *pfk13* transcript difference after treatment; $\Delta\Delta C_t$=0; 2°=1). The Mann–Whitney test was used on the patient and PC data grouped with more or less than 1-fold change of *pfk13* transcripts. The Cox proportional hazards analysis (Kaplan–Meier curve) was used to assess the effect of *pfk13* transcripts on the $PC_{50\%}$ outcome. The analyses were done with GraphPad Prism version 7.00

software. Sequencing of the *pfk13* resistant loci, encompassing the SNP PfK13 C580Y, was performed in all infections by PCR amplification using the primers 5'-CAAATATTGCTACTGAAACTATG-3' and 5'-TGTGCATGAAAATAAATATTAAAGAAG-3', and sequencing primer 5'-AGGTGGATTTGATGGTGTAGAA-3'.

3.4 Studies 3 and 4 - Common methods

3.4.1 DNA cloning in *Escherichia coli*

Plasmid transformation was performed into *E. coli* XL10 Gold® ultracompetent cells using the heat shock transformation method. Briefly, *E. coli* competent cells were thawed on ice and up to 2 µL (10 to 50 ng) of DNA was added to 20 µL of competent cells. The mixture was incubated on ice for 20 minutes. After incubation, the mixture was heat shocked for 45 seconds at 42 °C, on a thermoblock, and then placed on ice for 2 minutes. Then, 300 µL of SOB medium was added and the cells were incubated at 37 °C with shaking, at 200 rotations per minute (rpm), for 1 hour. The cells were plated into LB agar plates with the appropriate antibiotic. The plates were incubated overnight at 37 °C.

3.4.2 Plasmid DNA isolation

Miniprep was performed to obtain plasmid DNA quantities of up to 30 µg, at concentrations ranging from 100 ng/µL to 500 ng/µL, mainly for plasmid manipulation. On miniprep, plasmid DNA was isolated using NZYMiniprep kit following manufacturer recommendations.

Maxiprep was performed to obtain plasmid DNA quantities of up to 500 µg, at concentrations of 1 µg/µL, mainly for transfection into *P. falciparum*. On maxiprep plasmid DNA was isolated using NZYMidiprep endotoxin free kit and NZYMaxiprep endotoxin free following manufacturer recommendations.

After the isolation of plasmid DNA, concentration and purity were measured using NanoDrop™ 1000 spectrophotometer (Thermo Scientific).

3.4.3 Agarose gel electrophoresis of DNA

Agarose gels were prepared by mixing agarose in 1X TAE buffer, at a final agarose concentration of 1% to 3%, depending on the expected fragment sizes, heated until boiling and then cooled until approximately 50 °C. GreenSafe Premium was added into the mixture, in order to visualize the DNA bands under UV light. The dissolved agarose was poured into a horizontal gel cast and left at room temperature (RT) in order to solidify. The GeneRuler™ 1 kb plus, 1 kb and 100 bp DNA Ladder were used as molecular weight

markers. The bands to be analyzed were stained with 6X DNA Loading Dye or, when available, with respective colored kit buffers. All electrophoresis were run at 6 V/cm in 1X TAE buffer. Image of the gels were recorded using Gel Doc™ EZ System (Bio-Rad).

3.4.4 *P. falciparum in vitro* culture

Plasmodium falciparum strains used in this thesis were 3D7, Dd2, Dd2^{attB} and generated mutants. Asexual stage parasites were propagated in human erythrocytes with MCM under a controlled atmosphere of 3% O_2/5% CO_2/92% N_2 maintained in 25 cm^2 flasks or on culture plates in a hypoxia chamber (Billups-Rothenberg, Inc., CA, USA). Cultures on 25 cm^2 flasks were maintained at 4% hematocrit in 5 mL of MCM, cultures on 24 well plates were maintained at 2% hematocrit in 2 mL of MCM and cultures on 96 well plates were maintained at 1% hematocrit in 200 µL of MCM. Medium changes were performed daily, unless otherwise stated. Cultures healthiness and parasitemia were regularly monitored by microscopy.

Parasites were stored by freezing predominantly ring stage cultures (>2% parasitaemia, over 70% of rings) by careful dropping of an equal volume of a freezing solution [28% glycerol (Sigma, G5516), 3% sorbitol (Millipore, 1.07758) and 0.65% NaCl)] and flash freezing on liquid N_2. Parasites were stored for short term at -80 °C and long term in liquid N_2.

Parasites were thawed by careful addition of 0.2X of original pellet volume of a 12% NaCl solution, incubated for 5 minutes, followed by careful addition of 10X of original pellet volume of a 1.6% NaCl solution and centrifugation at 1500 rpm for 5 minutes without brake. Following, the pellet was careful resuspended in 10X of original pellet volume of 0.9% NaCl and 0.2% dextrose solution and centrifuged at 1500 rpm for 5 minutes. The final pellet was resuspended in MCM at 4% hematocrit and cultured as referred.

Synchronization of parasites was performed using 5% sorbitol (Millipore, 1.07758) solution, which selectively leads to burst of erythrocytes infected with trophozoites and schizonts parasites, synchronizing the culture at ring stage.

3.4.5 *P. falciparum* growth analysis

P. falciparum culture growth was analyzed under microscope or by flow cytometry.

For the microscopic analysis a blood smear was prepared, fixed with 100% methanol and stained with 10% Giemsa's azur eosin methylene blue solution for 15 minutes. Parasitemia was calculated by counting the number of parasitized erythrocytes per total of erythrocytes counted. For precise calculations at least 10000 erythrocytes were counted and compared with cytometry.

For the flow cytometry analysis, 40 µL of 1% hematocrit *P. falciparum* culture was incubated with 40 µL of staining solution [2X SYBR™ Green I Nucleic Acid Gel Stain (Invitrogen, S7564) and 1.6 µM MitoTracker™ Deep Red FM (Invitrogen, M22426)] for 30 minutes at 37 °C. After the incubation, 300 µL of PBS was added and the cells were analyzed on LSRII flow cytometer (BD Biosciences). SYBR Green strains DNA (parasitized cells) and MitoTracker strains based on mitochondrial membrane potential (alive parasites). Data was analyzed using FlowJo™ Software for Windows Version 10 (Ashland, OR: Becton, Dickinson and Company; 2019). Gating was done on the SYBR Green, on Alexa Fluor 488 channel (excitation: 490 nm / emission: 525 nm), and MitoTracker, on APC-Cy7 channel (excitation: 633 nm / emission: 780 nm), positive cells, giving the parasitemia present against total number of cells. Data was analyzed using FlowJo™ V10 software (Becton, Dickinson and Company).

3.4.6 *P. falciparum* transfection

Ring-stage sorbitol synchronized cultures at ≈5% parasitemia were washed, at 1500rpm for 5 minutes, in 1X cytomix and electroporated with 50 µg of purified plasmid DNA eluted in 1X cytomix, using Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.2 cm gap (BioRad). The electroporation was performed at 0.31 kV with capacitance of 950 µfD on the Gene Pulser Xcell™ (BioRad) electroporator. After the electroporation, the parasites were washed twice in 5 mL of MCM and cultured at 4% hematocrit as referred.

3.5 Study 3 - Multigenic architecture of piperaquine resistance trait in *Plasmodium falciparum*

3.5.1 Generation of *plasmepsin* 2 and *plasmepsin* 3-1 transgenic parasite lines

Plasmepsin 2 or hybrid *plasmepsin* 3-1 genes were introduced into the Dd2^{attB} *P. falciparum* line using the *attB-attP* recombinase system. This was performed by co-transfecting the mycobacteriophage Bxb1 integrase coding plasmid (pINT) with an *attP* containing plasmid with *pfpm2* or *pfpm3-1* (Figure 13) (Nkrumah et al., 2006). The hybrid *pfpm3-1* gene was PCR amplified from a Thai isolate from previous work (Carlsson et al., 2011; Veiga et al., 2012) and *pfpm2* from Dd2, using primers on Table 4, which contain introduced AvrII and XhoI restriction enzyme recognition sites. PCR reaction was performed with Phusion Green High-Fidelity DNA using recommended conditions, with an annealing temperature of 55 °C and 30 seconds of extension time.

Table 4. Primers used for genomic amplification of *plasmepsin* genes

Gene	Primer	Sequence (5'-3')
pfpm2	*pfpm2_gen*_fw	ATCACCTAGGATGGATATTACAGTAAGAGAACATGATTTT
	*pfpm2_gen*_rv	ATAACTCGAGTTATAAATTCTTTTTAGCAAGAGCAATACC
pfpm3-1	*pfpm3.1*_fw	ATCACCTAGGATGGATATTACAGTAAGAGAACATGATTTT
	*pfpm3.1*_rv	ATAACTCGAGTTATAAATTCTTTTTAGCAAGAGCAATACC

PCR reaction was verified by agarose gel electrophoresis and fragments corresponding to *pfpm2* and *pfpm3-1* were introduced into pJET1.2/blunt plasmid following manufacturer recommendations using the CloneJET PCR Cloning Kit. *pfpm2* and *pfpm3-1* were verified by sequencing using the pJET1.2 forward and reverse sequencing primers (Thermo Scientific). *pfpm2* and *pfpm3-1* were transferred using the restriction enzymes AvrII and XhoI into pDC2 plasmid (kindly provided by David Fidock) under the *P. falciparum* calmodulin (*pfcam;* PF3D7_1434200) promoter and the *hsp86* terminator (PF3D7_0708500) for expression in *Plasmodium falciparum.* Briefly, cut *pfpm2* and *pfpm3-1* fragments (from pJET) and open pDC2 were run in agarose gel and purified using NZYGelpure. The purified fragments were ligated with 1 U T4 DNA Ligase using 50 ng of vector and 15 ng of insert, according to a plasmid to insert ratio of 1 to 3. Plasmid was extracted from 10 obtained colonies. To confirm the correct gene insertion, the plasmids were cut with AvrII and XhoI and further sequenced using primers 5'-TGTATTATTCCAATGTGCATG-3' and 5'-TCATGTCTCAGTAAAGTCTTTC-3'. A correct plasmid for each gene was stored and these were denominated pDC2-*pfpm2* and pDC2-*pfpm3-1*.

Plasmids pDC2-*pfpm2* or pDC2-*pfpm3-1* were co-transfected with pINT in the Dd2^{attB} strain and selected with 2.5 μg/mL of BSD and 400 μg/mL of G418 for 5 days. These compounds select for blasticidin S deaminase gene present on pDC2 and the neomycin resistance gene present in pINT. Live parasites were observed by microscopy after 18-20 days, post-selection, and were further selected with 2.5 μg/mL of BSD for pDC2 integration into the genome. After culture reemergence, correct genomic integration was confirmed by PCR using primers previously described (Table 5) (Spalding et al., 2010) and individual clones were generated by limiting dilution. Reaction was performed with Supreme NZYTaq II 2x Green

Table 5. Primers sequences used for *plasmepsins* edited cultures genotyping

Primer sequence (5' → 3´)
P11 - GATGCGCAATTAACCCTCACTAAAGGG
P12 - GCACAGATGCGTAAGGAGAAAATACC
P13 - GATAGCGATTTTTTTTACTGTCTG
P14 - AAATGTATAAAAGATGAACATGGTGAA

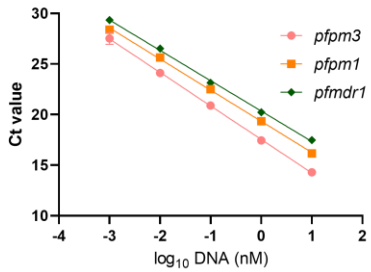
Master mix with 0.5 μM of each primer, 10 ng of template DNA (extracted from 100 μL of blood from cultures with NZY Tissue gDNA Isolation kit) in a 25 μL reaction. Amplification conditions were performed

following enzyme recommendations with an annealing temperature of 60 °C and 30 seconds of extension using Eppendorf Mastercycler EP Gradient S.

3.5.2 Quantitative PCR of *plasmepsin* transgenic parasite lines

Parasite DNA was extracted from blood of parasite culture using the NZY Blood gDNA Isolation kit. This kit has been discontinued and replaced with NZY Tissue gDNA Isolation kit. RNA was purified after a tight synchronization of parasites with sorbitol. Sorbitol was added to the cultures to synchronize for rings and added again after 24 hours (to synchronize for late rigs) and 40 hours (to synchronize for early rings). RNA was extracted using the NZY Total RNA Isolation kit and cDNA was synthesized using the First-Strand cDNA Synthesis Kit. *pfpm2* (PF3D7_1408000), *pfpm3* (PF3D7_1408100), *pfpm1* (PF3D7_1407900) and *pfmdr1* (PF3D7_0523000) copy numbers and expression were measured by qPCR using a CFX96 real-time PCR machine (Bio-Rad). The endogenous control *β-tubulin* (PF3D7_1008700) gene was used as a single copy gene. Amplifications were performed in 20 µL reaction mixtures for *pfpm2*, *pfpm3*, *pfpm1*, *pfmdr1* and *pfβ-tubulin* genes, primers used are described in Table 6. Amplification reaction was performed with NZYSpeedy qPCR Green Master Mix, with 0.4 µM of each primer, 50 ng of template cDNA in a 20 µL reaction. Amplifications were performed under the following conditions: 95 °C for 3 minutes, followed by 45 cycles of 95 °C for 15 seconds, 63 °C for 20 seconds, 72 °C for 20 seconds. Fluorescence data were collected after the 72 °C extension step. Copy number was calculated using the *pfβtub* as the endogenous control gene by the $2^{-\Delta\Delta Ct}$ method and using Dd2 DNA as a single copy

A



B

	pfpm1	*pfpm3*	*pfmdr1*
Slope	-3,079	-3,312	-3,001
Y-intercept	19,33	17,54	20,34
R^2	0,9972	0,9989	0,9985

C

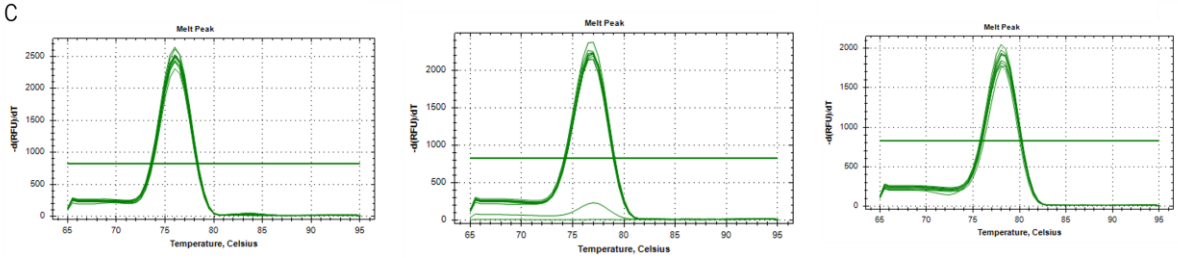


Figure 10. Validation of q-PCR setup with primers of Table 6. A – Serial diluted samples of *P. falciparum* DNA and the corresponding Ct values for respective primers. B – Slope, Y-intercept, R2, and equation corresponding to A. C – Melting peak of *pfpm1, pfpm2 and pfmdr1* (left to right) amplification. *Pfpm2 and pfβtub* Melting peaks were obtained from BioRad CFX Manager 3.1 software.

calibrator. Validation and standard curve analysis of linearity for *pfpm1, pfpm3* and *pfmdr1* reactions were performed using *P. falciparum* cDNA in a 10-fold serial dilution (Figure 10). Amplifications were performed in 3 technical replicates in 3 independent reactions.

Table 6. Primers used for quantitative PCR of *plasmepsins* copy number and expression

Gene	Primer	Sequence (5'-3')
pfpm2	*pfpm2*_fw	TGGTGATGCAGAAGTTGGAG
	*pfpm2*_rv	TGGGACCCATAAATTAGCAGA
pfpm3	*pfpm3*_fw	CACCTTCATGAAAAATGAAGAATC
	*pfpm3*_rv	AAGAAAAACCTCCTGCCAAAA
pfpm1	*pfpm1*_fw	CAATGGTTTCGAACCAGCTT
	*pfpm1*_rv	GGTAAAAACGGCTTGTTCGAT
pfmdr1	*pfmdr1*_fw	TGCATCTATAAAACGATCAGACAAA
	*pfmdr1*_rv	TCGTGTGTTCCATGTGACTGT
pfβtub	*pfβtub*_fw	TGATGTGCGCAAGTGATCC
	*pfβtub*_rv	TCCTTTGTGGACATTCTTCCTC

3.5.3 Drug susceptibility assays and PfCRT/PfMDR1 inhibition assays

Plasmodium falciparum drug susceptibility assays were performed, using tightly synchronized with sorbitol ring cultures, in flat-bottom 96-well plates at 1% hematocrit and 0.3% starting parasitemia. The plates were pre-dosed with titrated concentrations of piperaquine (24-wells of sequential 1:2 dilutions) and mefloquine (12-wells of sequential 1:2 dilutions). Verapamil and elacridar, chemical inhibitors of PfCRT and PfMDR1 were used at a constant concentration of 0.8 μM and 0.1 μM, respectively (Calçada et al., 2020; Martin et al., 1987; Martiney et al., 1995).

Assays were incubated for 72 hours at 37 °C with a gas mixture of 92% N_2, 3% O_2 and 5% CO_2. Growth was measured based on an optimized assay by staining, after freeze-thaw of assay plates, with 4X SYBR Green I on a lysis solution for 3 hours and read on a fluorimeter configured at 485 nm of excitation wavelength and the fluorescence detected at 525 nm (Dery et al., 2015). IC_{50} values were calculated by using non-linear regression curve-fitting algorithm log(inhibitor) vs. response–variable on GraphPad Prism 7.01 software.

Piperaquine bimodal dose–response does not follow a IC_{50} curve-fitting response. Instead, the area under the second response curve (AUC) was measured. AUC was calculated on GraphPad Prism 7.01 software using the range of concentrations 0.25 μM to 800 μM.

All assays were performed in independent triplicates. Statistical significance was determined via parametric two-tailed, Student's *t*-tests using GraphPad Prism 7.01 software.

3.5.4 Fluo-4 accumulation assays

Plasmodium falciparum-infected erythrocytes were washed with D-PBS and were incubated with 5 μM Fluo-4 AM for 4 hours at 37 °C with a gas mixture of 92% N_2, 3% O_2 and 5% CO_2. In the last 30 minutes, 1.6 μM MitoTracker Deep Red FM was added. Chemical inhibitors of PfCRT and PfMDR1 were used at a constant concentration of 0.8 μM verapamil, 0.1 μM elacridar, and both inhibitors combined. After incubation, the parasites were washed twice with D-PBS and prepared for cytometry or transferred into a slide for microscopy. Fluo-4 fluorescence was measured on LSRII BD cytometer by gating live parasites with MitoTracker Deep Red on APC-Cy7 channel (excitation: 633 nm / emission: 780 nm). Data was analyzed using FlowJo™ V10 software (Becton, Dickinson and Company).

The area of DV on the cell lines analyzed was assessed through fluorescence microscopy using an Olympus BX61 microscope equipped with a visible light laser, and the images were recorded with a digital camera (DP70). The parasites incubated with Fluo-4 were excited at 488 nm with the emission in the 505-nm filter, and an exposure time of 400 ms was applied to obtain the images. Single images were obtained using a 100X objective lens. Regions of interest within the infected RBC, including the parasite cytosol and the parasite DV, were recorded with Cell^P software (Electro optics, UK) and the image overlays were obtained using Image J software version 1.52a. A mean of 10 parasites per condition using the area around Fluo-4 and outside MitoTracker staining. ImageJ version 1.52a software was used to calculate digestive vacuole areas.

3.6 Study 4 - The *Plasmodium falciparum* protein PfMRP1 functions as an influx ABC transporter

3.6.1 Plasmid construction

A plasmid for *pfmrp1* disruption was constructed using a starting pDC2-based vector. CompoZr® Custom ZFNs were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) to induce a double-stranded break in the *pfmrp1* coding sequence. Two ZFNs (left and right), binding to adjacent sequences on opposite strands of the DNA helix, are required to induce the double-strand break. These were supplied on separate plasmids. The ZFN pair was designed to bind to the sequence TTATGCATCCTCATTtatatgCAAGTGGAATAA (the DNA cut site is shown in lower case letters and is situated 1208 bp upstream of the *pfmrp1* stop codon). The plasmid encoding the right ZFN also encoded the 2A 'ribosome skip' peptide, which enables polycistronic expression of 2A-linked genes. The two plasmids encoding the ZFNs were digested with BglII and XhoI and combined to yield an intermediate plasmid with 2A-linked ZFN sequences. The ZFN fusion was then digested with NheI and XhoI and subcloned downstream of the *pfcam* (PF3D7_1434200) promoter and upstream of the *hsp86* (PF3D7_0708500) 3' UTR in a pDC2-based vector with the human dihydrofolate reductase (h*dhfr*) selectable marker, yielding the plasmid pDC2-ZFN*pfmrp1-hdhfr*. Due to the nature of PfMRP1 being associated with folate transport the h*dhfr* selectable cassette, which codes for the human enzyme that bypasses the action of *P. falciparum* antifolate drugs was changed from h*dhfr* to blasticidin S deaminase gene (*bsd*). *Bsd* was introduced using ApaI and SacII restriction to yield the plasmid pDC2-ZFN*pfmrp1_pfmrp1-bsd*. A 2459 bp donor sequence, encompassing from the *pfmrp1* coding sequence from nucleotides 2999 to 5455, was amplified from Dd2 genomic DNA with the primers p6 and p7 (Table 7) and inserted into the BstAPI and AatII sites of pDC2-ZFN*pfmrp1-bsd* to yield the editing plasmid pDC2-ZFN*pfmrp1_pfmrp1-bsd*. To prevent edited genomes from being further cleaved, three silent mutations were engineered into the donor sequence at the ZFN-binding site via site-directed mutagenesis with QuikChange Multi SDM Kit using the

Table 7. Primers sequences used for construction of pDC2-ZFN*pfmrp1_pfmrp1-bsd*

Primer sequence (5' → 3´)
p6 - GTAGCATATGGTGCTTATTTCGTAAAAGGGAATACGGAGAG
p7 - GTTGACGTCTAACAAATGTGATAATTCAGATTGCG
p9 - ACCCACACTTATATGCTTCTGGTATAATCAAATTATATAAAGAAAAAAATTATG
p10 - TACCAGAAGCATATAAGTGTGGGTGCATAATAATGTAAGTAGTTAATAAC

primers p9 and p10 (Table 7). Figure 16 depicts the ZFN-mediated *pfmrp1* double-stranded break (thunderbolt) that was repaired by recombination with the *pfmrp1* homologous region on the plasmid, leading to single-crossover plasmid introduction and *pfmrp1* disruption.

A plasmid to detect the fluctuation of glutathione reduction-oxidation status in the parasite was constructed on a pINT-based vector (Adjalley et al., 2010). The plasmid was constructed to episomal express the genetically encoded redox roGFP1-Grx1 sensor, under the *pfcam* (PF3D7_1434200) promoter and with the neomycin phosphotransferase II selectable marker, enabling G418 selection. RoGFP1-Grx1 was obtained from p415TEF cyto roGFP2-Grx1, (a gift from Tobias Dick; Addgene plasmid # 65004; http://n2t.net/addgene:65004; RRID:Addgene_65004) (Morgan et al., 2013) and subcloned downstream of *pfcam* promoter and upstream of the *hsp86* (PF3D7_0708500) terminator in a pDC2-based vector using AvrII and XhoI restriction enzymes. RoGFP1-Grx1 was cut with the *pfcam* promoter using ApaI and XhoI restriction and ligated onto pINT, replacing the integrase originally on that plasmid (Nkrumah et al., 2006).

Restriction reactions were performed with Anza restriction enzymes (Thermo Scientific) using recommended guidelines. The purified fragments were ligated with 1 U T4 DNA Ligase using 50 ng of vector and 15 ng of insert, accordingly to plasmid to insert ratio of 1 to 3. From 10 obtained colonies, plasmid was extracted. All created plasmids were verified by restriction and sequencing.

3.6.2 Parasite culture and transfection

Transfection was performed in $3D7^{attB}$ and $Dd2^{attB}$ strain. To enable transient expression of ZFN, homology repair and disruption of *pfmrp1* 2.5 µg/mL of BSD selection was applied 24 hours post electroporation for 5 days. After selection, parasites were cultured without drug. After microscopic growth was observed 19-22 days post-transfection, the cultures were genotyped by PCR and tested for gene disruption. Amplification was performed with Phusion Blood Direct PCR Master Mix using 0.5 µM of each primer and 1 µL of blood from transfected cultures, in a 10 µL reaction using primers on Table 8. PCR reaction was performed according to manufacturer guidelines, primer annealing was performed at 55 °C and extension was performed at 30 seconds per 1 kb. The cultures were subjected to another similar round of selection if the PCR was negative, or the mutant population was present in minimal quantities. Positive bulk cultures were cloned by limiting dilution and positive wells were detected and genotyped by PCR. One clone from each strain was selected to proceed further experiments.

To enable continuous episomal expression of roGFP1-Grx1 sensor selection with G418 was applied continuously at concentration of 250 µg/mL, a day after transfection. After microscopic growth was observed the cultures were run on LSRII flow cytometer (BD Biosciences) and observed under microscope for sensor derived fluorescence on the Alexa Fluor 488 channel (excitation: 490 nm / emission: 525 nm).

Table 8. Primers sequences used for *pfmrp1* edited cultures genotyping

Primer sequence (5' → 3´)
p1 - AAGGACATATTTATTAAACCGCAGAG
p2 - AAACTTACACACACCCATGC
p3 - CCTTCAAAAATGTTTAATGGATGAT
p4 - CTCATGGTTATGGCAGC
p5 - TGTGTTAATTTGTGATTCCA

3.6.3 *In vitro* antimalarial drug assays

Drug susceptibility assays were performed using a flow cytometry-based approach described previously, with minor modifications (Amaratunga et al., 2014a). Assays were performed in flat-bottom 96 well plates in 200 µL total volume with MCM, 1% hematocrit and 0.3% starting parasitemia. Assays using reduced folate were performed using RPMI without folate and supplemented with 3 ng/mL and 100 ng/mL of folic acid. The plates were pre-dosed with descending concentrations of test compound, two-fold dilution each consecutive well. Compounds used were chloroquine, mefloquine, lumefantrine, dihydroartemisinin, pyrimethamine, trimethoprim, WR99210, methotrexate and aminopterin. To perform drug assays with transporter proteins inhibitors these were added at a fixed concentration to all wells, verapamil (Sigma) was used at 800 nM, elacridar at 100 nM and MK-571 at 1600 nM. Two wells were left without drug as positive growth control. After 72 hours, parasitemia was assessed in the flow cytometer. For the flow cytometry analysis, 60 µL of each well from drug assay was incubated with 40 µL of staining solution (2X SYBR™ Green I Nucleic Acid Gel Stain and 1.6 µM MitoTracker™ Deep Red FM) for 30 minutes at 37 °C. After the incubation, the cells were diluted in 300 µL of PBS and passed on LSRII flow cytometer (BD Biosciences). SYBR Green fluorescence was captured on Alexa Fluor 488 channel (excitation: 490 nm / emission: 525 nm) and MitoTracker on APC-Cy7 channel (excitation: 633 nm / emission: 780 nm). SYBR Green stains DNA (parasitized cells) and MitoTracker stains based on mitochondrial membrane potential (alive parasites). Data was analyzed using FlowJo™ V10 software (Becton, Dickinson and Company). Gating was done on the SYBR Green and MitoTracker positive cells giving the parasitemia. Assays were performed with 3 to 10 independent replicates. Data normality was assessed using Shapiro-Wilk test. Statistical evaluations comparing WT and Δ*pfmrp1* strains were performed using two-tailed unpaired *t*-

tests with Welch's correction. Statistical evaluations comparing strains between different folate conditions were performed using one-way ANOVA with Tukey's multiple comparisons test. GraphPad Prism 9 was used for statistical analysis for all assays.

3.6.4 Fluorescein methotrexate accumulation assay

Accumulation of F-MTX was measured using flow cytometry over time and microscopy. Parasites were incubated in 1mL of MCM in a hematocrit of 4% at around 2-4% parasitemia with F-MTX at 1000 nM. Conditions without F-MTX or with uninfected red blood cells were used as controls for baseline and autofluorescence.

For the flow cytometry analysis, 1.6 µM MitoTracker™ Deep Red FM was incubated with F-MTX. After the incubation, at the time points 1 hour, 2 hours and 3 hours, 40 µL of 1% hematocrit of the cells were diluted in 300 µL of D-PBS and run on LSRII flow cytometer (BD Biosciences). Another condition was performed at 3-hours with parasites similarly incubated with 5 µM of Fluo-4, instead of F-MTX. Data were analyzed using FlowJo™ V10 software (Becton, Dickinson and Company). Gating was performed on the APC-Cy7 channel (excitation: 633 nm / emission: 780 nm) of the MitoTracker positive cells to determine parasitemias. Mean Alexa Fluor 488 (excitation: 490/emission: 525) fluorescence of live parasites was calculated and plotted. Total of 100000 events were recorded (erythrocytes) with at least 1000 MitoTracker positive live cells (at least 1% parasitemia). Assays were performed in three independent replicates.

For the microscopy analysis, the parasites were collected after a 3-hour incubation time point and further stained for 5 minutes with 0.1 µg/mL of DAPI, in addition to MitoTracker. Parasites were centrifuged at 1500 rpm for 5 minutes and a microscopic slide of the red blood cells pellet was made for live fluorescent imaging. Fluorescence pictures were captured using an Olympus LPS Confocal FV3000 microscope. For quantification pictures captured using Olympus BX61 microscope equipped with a visible light laser and recorded with a digital camera (DP70). Fluorescence was quantified with ImageJ software version 1.52a (NIH) by circling the parasite area. 11 parasites were counted per condition on the first replicate and 7 on the second independent replicate.

Data normality was accessed using Shapiro-Wilk test. Statistical evaluations comparing Dd2 WT and Dd2$^{\Delta_{pfmrp1}}$ were performed using two-tailed unpaired *t*-tests with Welch's correction. Statistical evaluation of F-MTX accumulation over time between Dd2 WT and Dd2$^{\Delta_{pfmrp1}}$ was performed using two-way ANOVA with Šídák's multiple comparisons test.

3.6.5 Competition assays

F-MTX competition assays were performed using a flow cytometry-based approach. The assay was performed in 96-well plates with pre-dosed descending concentrations of test compound, with a two-fold dilution each consecutive well, with the highest compound concentration of 2000 nM. Two wells were left without drug as negative control. All wells were also pre-dosed with 1000 nM of F-MTX, including controls. In the last 30 minutes of the assay, parasites were incubated with 1.6 µM MitoTracker™ Deep Red FM. After 4 hours, the cells were diluted in 300 µL of D-PBS and processed on a LSRII flow cytometer (BD Biosciences). Gating and data analysis was performed similarly as fluorescein accumulation assay.

3.6.6 Growth assays

Assays were performed in flat-bottom 6-well plates at 4% hematocrit and 0.05% starting parasitemia. Before starting the assay, parasites were cultured in RPMI without folic acid for 14 days to reduce possible folate pools derived from culture medium. Parasites were grown under normal culture conditions or in RPMI without folic acid. Moreover, supplementation of medium without folic acid was done with addition of 100 µg/L of folic acid. Every 24 hours, parasitemia was assessed in the flow cytometer. Flow cytometry was performed incubating 20 µL of each culture with 40 µL of staining solution (2X SYBR™ Green I Nucleic Acid Gel Stain and 1.6 µM MitoTracker™ Deep Red FM) for 30 minutes at 37 °C. After the incubation, the cells were diluted in 300 µL of PBS and examined on a LSRII flow cytometer (BD Biosciences). SYBR Green stains DNA (parasitized cells) and MitoTracker detects the mitochondrial membrane potential (live parasites). Data were analyzed using FlowJo™ V10 software (Becton, Dickinson and Company). Gatings were performed as described above for drug susceptibility assays to calculate parasitemia. Assays were performed in three independent replicates

3.6.7 Redox assays

Ring stage cultures were tightly synchronized with sorbitol. After 24 hours, 300 µL of tightly synchronized trophozoites at 4% hematocrit, were run on LSRII flow cytometer (BD Biosciences). After 60 seconds, for baseline monitoring, and after 120 seconds, for double stimuli assay, parasites were challenged with 20 µL of H_2O_2 at 10 mM, 20 µL DTT at 10 mM, 20 µL of GSH at 30 mM and 20 µL of GSSG at 30 mM. On redox antimalarial assays parasites after 60 seconds of baseline monitoring parasites were challenged with the antimalarials chloroquine, mefloquine, aminopterin, pyrimethamine, dihydroartemisinin, lumefantrine, amodiaquine, and the transporter inhibitor MK571 at 1 mM. Gating was done in Alexa Fluor 488 positive cells to select live and sensor positive parasites. Ratio of fluorescence

between Alexa Fluor 488 (excitation: 488 nm, emission: 525 nm) and AmCyan channels (excitation: 470 nm, emission: 500 nm) was calculated using derived function on FlowJo™ V10 software (Becton, Dickinson and Company). Data were exported and assays were normalized for baseline or for first stimuli in case two stimuli were provided and plotted.

3.6.8 Transcriptome sequencing and analysis

Parasites were tightly synchronized with 5% sorbitol in intervals of 20 hours for 3 consecutive life cycles. RNA was extracted from trophozoite stages, estimated between 28 and 32 hours old, using a RNeasy Mini Kit (Qiagen). Libraries and sequencing were outsourced (Macrogen Inc), using TruSeq RNA Illumina Library v2 construction (nonstranded polyA enrichment) and HiSeq2500 Illumina 2x100bp 2Gb (20M pair-end reads) throughput-based sequencing. Transcriptome datasets for both 3D7 and Dd2 were mapped against the 3D7 v3 reference sequence (Böhme et al., 2019) using HISAT2 2-2.0.0-beta (D. Kim et al., 2019) (–rna-strandness RF –max-intronlen 5000). Read counts were determined using HTSEQ v0.6.0 (-r pos -s no) (Anders et al., 2015). Deconvolution analysis was performed as described previously (Aunin et al., 2020). Differentially expressed genes were determined using edgeR (Robinson et al., 2010), with genes expressed at ≤ 3 counts per million (cpm) excluded. Because there were no biological replicates for any condition, we used the exact test with dispersion set to 0.01, as suggested for data concerning genetically identical model organisms (https://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf). We used a false discovery rate (FDR) cut off of 0.01 and a fold change cut off of 2 to call differentially expressed genes.

3.6.9 Phylogenetic analysis

A dataset of 117 publicly available amino acid sequences from ABC transporters spanning a wide taxonomic range was retrieved with BLASTp by querying the Landmark dataset with the *pfmrp1* amino acid sequence (XP_001351050) (McGinnis and Madden, 2004). These sequences were aligned using MAFFT v. 7.221 (Katoh and Standley, 2013) and used to make phylogenetic reconstructions using IQ-TREE multicore version 1.5.5. The best fitting substitution model was LG+F+R8 as determined by ModelFinder according to AIC (Minh et al., 2013; Nguyen et al., 2015). Node support for the inferred maximum likelihood trees was computed using 5000 ultrafast bootstrap replicates (Minh et al., 2013).

3.6.10 Data availability

The RNA-Seq data from this work have been deposited to: Gene Expression Omnibus GSE195649 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195649)

4. RESULTS AND DISCUSSION

4.1 Contribution to the results

The results section is divided by the main studies outlined on the aims. Study 1, 2 and 3 are based on original published peer-reviewed articles. Study 4 presents the results of a manuscript under submission and published on a pre-print server. The original articles references can be found in each of the sub-chapters. The presentation on the dissertation differs slightly from the articles by making a shorter introductory section to avoid extensive repetition with the main introduction and by exploring some additional results that did not fit the original publications.

The dissertation author contribution to the specific studies were the following:

Study 1: Experimental procedures as DNA extraction, qPCR and copy number analysis. Revising and reviewing the manuscript with co-authors.

Study 2: Experimental procedures as RNA extraction, qPCR and expression number analysis. Revising and reviewing the manuscript with co-authors.

Study 3: Design of the *in vitro* strategy and experiments together with project leader. All experimental procedures from the development of plasmids to the phenotypic assays. Data analysis. Co-writing the manuscript with corresponding author.

Study 4: Design of the *in vitro* strategies and experiments together with project leaders along the project. All experimental procedures. Data analysis except phylogenetic analysis (performed by co-author Nuno Osório) and transcriptomic analysis (performed by co-author Adam Reid). Writing the manuscript.

4.2 Study 1: *Plasmodium falciparum* Plasmepsin 2 Duplications, West Africa

Published: Emerging Infectious Diseases, 24(8): 1591-1593. 17 August 2018.

doi: 10.3201/eid2408.180370

Artemisinin combination therapy is key for the control of malaria in sub-Saharan Africa. While AM-LF and AS-AQ are the primary deployed treatments in the region, due to the prone capacity of *P. falciparum* to develop resistance, DHA-PPQ has been included as a second line antimalarial drug. This decision is prudent and is based on reports of treatment inefficacy following AM-LF therapy that had markers associated with lumefantrine resistance, indicating possible emergence of resistance (Plucinski et al., 2015). DHA-PPQ has shown high efficacy levels in clinical trials conducted in Africa, making the combination a prospect for therapy and intermittent preventive approaches in the region (Gutman et al., 2017). However, treatment failure of DHA-PPQ is prevalent in Southeast Asia, mainly due to piperaquine failure and increased pressure of tolerant parasites to dihydroartemisinin (Amato et al., 2017; Witkowski et al., 2017). Piperaquine resistance is mostly associated with increased CNV in the *plasmepsin* locus, including the *pfpm2* gene (PF3D7_1408000) and PfCRT polymorphisms (Amato et al., 2017; Dhingra et al., 2019, 2017; Ross et al., 2018; Witkowski et al., 2017).

CNV is usually considered to emerge at relatively rapid mutation rates, with several orders of magnitude higher compared to SNPs, generating substantial diversity (Alonso et al., 2013; Cheeseman et al., 2016, 2009; Conrad and Hurles, 2007). However, most CNVs are deleterious and only rare cases become fixated and are often associated with drug pressure. Therefore, preexisting *pfpm2* duplications in Cambodia might have been rapidly selected by DHA-PPQ, aided by the less protective action of DHA due to the presence of tolerant artemisinin lineages. Additionally, these could have conferred a favorable background for SNPs in *pfcrt* to emerge, as will be explored later in this dissertation in the Study 3. There is a likelihood of these amplifications occurring in Africa, in which case selection due to piperaquine pressure could lead to development of resistance. As such, there is an importance to monitor *plasmepsin* duplication status before further deploying piperaquine based treatments in Africa.

This study rational was to investigate the *pfpm2* amplification occurrence in the African setting. For this, a subset of archived *P. falciparum* DNA samples from clinical infections were analyzed. These samples are derived from a set of large, multicenter comparative ACTs efficacy trials conducted in West Africa (West African Network for Clinical Trials of Antimalarial Drugs (WANECAM), 2018). This pilot study analyzed 96 samples from recurrent infections with the shortest interepisode periods. This subgroup had recurrent infection after 23 to 65 days posttreatment and was assumed to be more likely to include markers of piperaquine resistance, such as *pfpm2* duplications.

The analysis was conclusive in 65 of the 96 samples and confirmed the presence of 7 infections carrying 2 copies of *pfpm2*, representing ≈10% of the successfully analyzed infections. We did not identify any trend of earlier recurrence associated with this group of infections (Figure 11), a preliminarily observation that needs to be further explored in a larger sample set.

Our results clearly show that piperaquine resistance–associated *pfpm2* duplications are probably already frequent in Africa, which is of concern given the long half-life of piperaquine (>20 days). In high-transmission areas, this long period of decreasing drug exposure is likely to progressively select more tolerant parasites, potentially carrying of *pfpm2* extra copy. Parallel studies conducted in these areas have not detected substantial altered parasite clearance dynamics or PfK13 mutations associated with artemisinin-derivative therapy (Kamau et al., 2015; Maiga et al., 2012), indicating that these *pfpm2* duplications are emerging despite the overall efficacy of dihydroartemisinin. Further studies are urgently needed to clarify the clinical implications of *plasmepsin* amplification and piperaquine susceptibility in African setting. Moreover, monitoring occurrence of duplication in other areas of high malaria transmission in Africa is important to monitor emergence of these mutations and for decisions of alternative therapies deployment.

More recently, additional studies also demonstrated multiple copies of *plasmepsin* 2 in high proportion in Burkina Faso and Uganda (Leroy et al., 2019). Concomitantly, there was also a recent description of

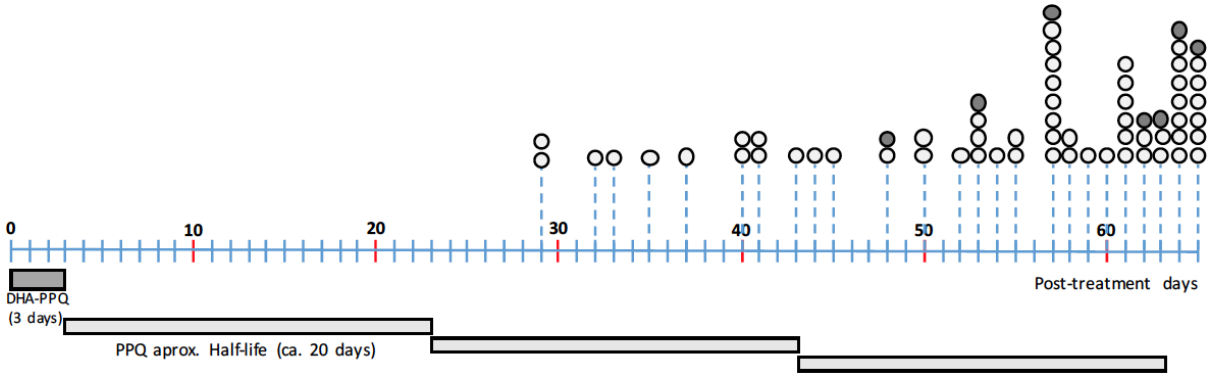


Figure 11. Timeline distribution of the *pfpm2* CNV status. White filled circles: 1 copy, dark gray filled circles: 2 copies.

increased sensitivity to piperaquine in Ugandan parasite isolates (Rasmussen et al., 2017). However, this sensitization can be related with the massive use of AL in the area (Ebong et al., 2021). *In vitro* overexpression of *plasmepsins* was shown to not impact susceptibility to piperaquine in the 3D7 strain, derived from Africa (Boonyalai et al., 2020), but was shown to increase piperaquine resistance, including a bimodal response in a Dd2 strain, derived from Asia (Study 3). This highlights that the genetic background might play an important role in the modulation of piperaquine response by the plasmepsins. The evidence of *pfpm2* duplication presence in Africa by our study and recent studies indicates the need

to further evaluate the *in vitro* susceptibility profiles to piperaquine of duplicated *pfpm2* African isolates. This is important for the introduction of programs using DHA-PPQ, which are under evaluation due to increased post treatment prophylaxis, for mass drug administration campaigns using intermittent preventive treatments. New combinations are being tested for introduction in the field, such as artefenomel-piperaquine. Therefore, this knowledge is essential to not deploy a new combination in areas of piperaquine resistance, which would immediately compromise the combination while increasing selective pressure on artefenomel (Eisele, 2019; Gerardin et al., 2015; Macintyre et al., 2017; WHO, 2018).

4.3 Study 2: *Plasmodium falciparum* K13 expression associated with parasite clearance during artemisinin-based combination therapy

Published: Journal of Antimicrobial Chemotherapy, 74(7): 1890-1893. 1 July 2019.
doi: 10.1093/jac/dkz098

Sustained ACT efficacy is dependent on the actions of both the long half-life component and the artemisinin derivative. In Southeast Asia, a change in the parasite response to artemisinins has been observed, essentially characterized by a significant increase in infection clearance times (Dondorp et al., 2009; Noedl et al., 2008). This phenotype has been associated with clinical failure after ACT treatment (Amato et al., 2017; Noedl et al., 2008; Witkowski et al., 2017). Of particular concern is the possibility that the reduction of artemisinin efficacy may also spread to Africa, where 90% of all malaria deaths occur, jeopardizing the United Nations sustainable developmental goal of ending the malaria epidemic by 2030 (United Nations, 2015).

Increased parasite clearance (PC) times have been firmly associated with sequence variation in the gene coding for the cytoplasmic PfK13 propeller-like protein (PF3D7_1343700) (Ariey et al., 2014). Genetically modified PfK13 loci confirmed the role of PfK13 propeller mutations in artemisinin resistance

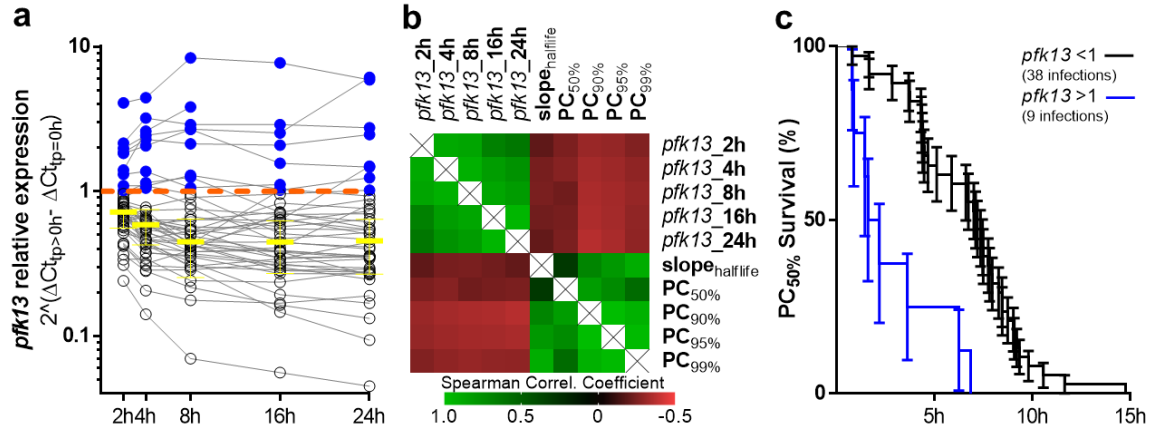


Figure 12. Changes in *pfk13* transcripts and parasite clearance in patients treated with artemether-lumefantrine. (a) *pfk13* relative expression by fold change after treatment compared with before treatment for every analyzed patient sample over time. Connection lines demark individual patient samples over time. The dashed red line delineates the threshold for *pfk13* upregulation (blue circles, >1-fold) and downregulation (open circles, <1-fold). Median *pfk13* expression by time point is represented with a yellow dash and standard error of the 47 samples. *pfk13* expression fold change was calculated by the $2^{\Delta\Delta_{Ct}}$ method normalized with housekeeping gene PF3D7_0717700 and calibrated with pair infection data before treatment initiation (time 0h). **(b)** The heat map shows the Spearman correlation coefficients of pairwise comparisons of the *pfk13* relative expression at different time points and the PC parameters of matching patients. **(c)** Kaplan-Meier survival analysis defining the expected $PC_{50\%}$ duration in time for the two groups defined in (a). χ^2 [Log-rank (Mantel-Cox) test]: 17.7; P <0.0001. $PC_{50\%}$ median survival: *pfk13*<1=7.1; *pfk13*>1=1.9.

(Ghorbal et al., 2014; Straimer et al., 2015). Intriguingly, it was observed that the levels of artemisinin resistance exhibited by strains with genetically modified PfK13 were lower compared with those of clinical isolates carrying the same mutations (Straimer et al., 2015), indicating the existence of other determinants beyond the known mutations. This is supported by field reports of artemisinin resistance independent of PfK13 mutations, emphasizing the need for an understanding of resistance factors beyond PfK13 mutations (Mukherjee et al., 2017). One barely possibility studied is the influence of *in vivo* PfK13 expression levels, prior to treatment and after artemisinin impact. In this work, we have studied the *in vivo pfK13* expression in Tanzanian patients treated with AM-LF and explored association of expression levels with patients' PC parameter outcomes.

Table 9. Characteristics of the studied population and differential *pfk13* expression

	TOTAL (n=47)			***pfk13*<1 * (n=38)**		***pfk13*>1 * (n=9)**		**P-value**
	mean	**SD**	**range**	**mean**	**SD**	**mean**	**SD**	
Patient data at admission								
Age (months)	50.0	30.0	12 - 119	50.7	28.6	46.9	37.1	0.510
Weight (kg)	14.3	5.5	8 - 30	14.3	5.4	14.0	6.4	0.474
Parasite density (Pf/mm³ blood)	60171	51805	2120 - 200400	61744	50076	53529	61420	0.397
Temperature (°C)	37.9	1.0	36.2 - 40.8	38.0	1.1	37.7	0.5	0.995
Parasite clearance data								
Slope half-life	2.5	1.1	0.5 - 5.2	2.6	1.1	2.1	1.1	0.343
$PC_{50\%}$ (h)	6.0	3.2	0.8 - 14.8	6.7	2.9	3.0	2.4	0.0008
$PC_{90\%}$ (h)	11.6	4.8	2.1 - 21.8	12.6	4.3	7.3	4.8	0.007
$PC_{95\%}$ (h)	14.1	5.7	2.6 - 26.2	15.2	5.2	9.4	5.8	0.016
$PC_{99\%}$ (h)	19.9	8.0	3.9 - 37.8	21.2	7.5	14.4	8.3	0.052

* - *pfk13* expression profile after 2 hours of treatment. *pfk13* > 1 represent the infections group with fold change relative expression above one and *pfk13* < 1 represent the infections group with fold change relative expression below one.

We obtained *pfk13* transcript expression data for the 47 infections analyzed at the six timepoints under review, except for the 24 hours point, at which 3 of the 47 infections did not provide reliable *pfk13* transcript results. After AM-LF treatment initiation, a wide range of *pfk13* transcript fold change in expression was observed throughout all timepoints (Figure 12a). Using the *pfk13* expression levels of each infection before treatment initiation (0 hours) to understand the fold change in expression after treatment initiation, the expression ranged from 0.2 to 4.1-fold after 2 hours, 0.1 to 4.4-fold after 4 hours, 0.1 to 8.3-fold after 8 hours, 0.1 to 7.7-fold after 16 hours and 0.04 to 6.1-fold after 24 hours. Strong correlations of *pfk13* expression were observed between all post-treatment timepoints (Figure 12b), with individual infection variation defined at the first time point after treatment (2 hours) and maintained over

time (Figure 12a; gray connection lines). The data were not normally distributed (Shapiro–Wilk, W=0.47–0.66, P<0.0001), showing skewness for decreased expression of *pfk13* response (Figure 12a; yellow bar showing median values). This observed differential expression was not linked to genetic variability at the *pfk13* gene-resistant loci, since all infections were found to be WT from amino acid 464 up to the 3'-terminus of the open reading frame. By taking parasite clearance (PC) data (Table 9) together with the *pfk13* expression profile over time, we noticed a clear clade based on Spearman correlation, inferring that *pfk13* down-regulation is associated with longer PC time (Figure 12b).

To confirm this, infections were stratified into two groups defined by infections with increased or decreased expression of *pfk13* by more than or less than 1-fold, respectively (Figure 12a, red dashed line cut-off of 1 signifying no variation compared with before treatment initiation; $2^{-\Delta\Delta_{Ct}}=2^{0}$). Reinforcing the negative correlation result (Figure 12b), after 2 hours of treatment, a significant difference (P<0.001) in PC times was observed, with higher mean $PC_{50\%}$ values in the infections group with reduced *pfk13* expression. This significant difference was maintained for the $PC_{90\%}$, $PC_{95\%}$ and $PC_{99\%}$, but did not reach significance for the PC slope half-life (Table 9). To better understand the risk factor of the two groups (*pfk13*<1 and *pfk13*>1) to predict the PC time, we performed a Kaplan–Meier survival analysis using the $PC_{50\%}$. Significant difference in survival time for $PC_{50\%}$ is observed between the two groups (median *pfk13*<1=7.1 hours; *pfk13*>1=1.9 hours) (Figure 12c).

SNPs at *pfk13* have been identified as molecular markers of ACT resistance among Southeast Asian patients (Miotto et al., 2015). These have been found in <3% in Africa (Kamau et al., 2015), possibly explaining the lack of extreme cases of long PC time. Variation in this phenotype nevertheless exists and has been well documented (Kone et al., 2020; Ouattara et al., 2015). The question remains of whether there are other factors beyond open reading frame variation that might explain clearance differences in Africa. In this molecular prospective approach, we explored for the first time the transcriptional profile of *pfk13* during AM-LF therapy. Since transcriptional variation appears to be higher than genetic variation in our study, we have hypothesized that it might have an important role in the early stage of development of drug resistance. Previously, a large *ex vivo* transcriptomic study of *P. falciparum* isolates from Southeast Asian and African patients, collected before treatment and encompassing normal and delayed PC time, revealed high variation in *pfk13* expression, although not correlated with artemisinin resistance (Mok et al., 2015). Our prospective study describing *pfk13* expression during treatment also showed a large range of transcription levels, with 20 times the dynamic range observed after 2 hours of treatment. Interestingly, the intrinsic transcription features of each individual infection prevailed during the 24 hours of study (Figure 12a). The PfK13 propeller domain encodes six Kelch motifs theoretically playing a role in protein

degradation via polyubiquitination or involved in cell response to oxidative stress. The C580Y mutation was shown to decrease affinity for a protein substrate, thereby increasing its steady-state levels by reducing ubiquitination and proteasomal degradation (Mbengue et al., 2015). Parasites responding with decreased expression of *pfk13* could therefore act as a similar outcome, translated into a slow phenotype and manifested as longer PC times. This hypothesis could explain the clear negative correlation observed between the clearance parameters of the infections herein analyzed and their *pfk13* transcriptional response (Figure 12b). Moreover, recently PfK13 mutations were shown to decrease hemoglobin endocytosis leading to less artemisinin activation and consequent resistance, which happens due to reduced protein abundance (Birnbaum et al., 2020; Yang et al., 2019). Reduced transcript levels are expected to lead to a similar reduced in protein abundance that can explain the higher PC values. The difference between the *pfk13* gene expression groups could also be explained by the different ring developmental stage at the sample collection time, as recently described (Gibbons et al., 2018). These authors explored the transcriptional profiling of a recombinant PfK13 strain, with increased susceptibility to artemisinins, revealing down-regulation of *pfk13* expression at early rings and up-regulation at the early trophozoite stage. They could distinguish this short period of stage development through a full transcriptomic approach and not by microscopic observation of morphological changes, constraining the validation of this hypothesis in our study. A Kaplan–Meier survival analysis using the $PC_{50\%}$ for the two groups (*pfk13*<1 and *pfk13*>1) revealed a significant difference in survival time ($PC_{50\%}$ medians: *pfk13*<1=7.1 h; *pfk13*>1=1.9 h) (Figure 12c), demonstrating that there is a prognostic value of the *pfk13* transcriptional profile to predict the PC time. A larger study with full transcriptomic approach would be needed to dissect this phenotypic clearance outcome more comprehensively. Our study thus revealed the *pfk13* transcripts as a potential additional factor relevant for *in vivo* PC outcome of ACT, at least in the scenario of AM-LF treatment in PfK13 WT African parasites.

4.4 Study 3: Multigenic architecture of piperaquine resistance trait in *Plasmodium falciparum*

Published: The Lancet Infectious Diseases, 20(1): 26-27. 1 January 2020.
doi: 10.1016/S1473-3099(19)30689-9

As mentioned in Study 1, treatment failure of DHA-PPQ is prevalent in South East Asia, due to piperaquine failure (Amato et al., 2017; Witkowski et al., 2017) and there is concern that further implementation of this therapy or piperaquine derived therapies in Africa could lead to fast emergence of resistance. William L Hamilton and colleagues and Rob W van der Pluijm and colleagues described the genomic evolution of *Plasmodium falciparum* malaria and the spread of DHA-PPQ resistance in this species in Southeast Asia (Hamilton et al., 2019; van der Pluijm et al., 2019). Resistance in the region has been associated with *pfcrt* polymorphisms and CNV in *plasmepsins* and *pfmdr1* genes (Bopp et al., 2018; Dhingra et al., 2019, 2017; Hamilton et al., 2019; Ross et al., 2018; van der Pluijm et al., 2019; Witkowski et al., 2017). Despite compelling evidence regarding the determinant effect of *pfcrt* polymorphisms on piperaquine resistance (Dhingra et al., 2019, 2017; Ross et al., 2018), the roles of *plasmepsins* and *pfmdr1* genes, which were first identified in a 2017 phenotype–genotype association study, remain unclear (Witkowski et al., 2017).

Therefore, in the Study 3, *P. falciparum* parasite lines were generated with an extra copy of the *plasmepsin 2* (*pfpm2*) or the hybrid of the *plasmepsin 1* and *plasmepsin 3* genes (*pfpm3–1*), which is a hybrid *plasmepsin* gene created by a duplication event (Amato et al., 2018; Ansbro et al., 2020). These lines were generated in the southeast-Asian derived Dd2 genetic background to study the contribution of *plasmepsins* to piperaquine resistance (Figure 13). Moreover, this study aimed to validate, *in vitro*, the *plasmepsins* amplification in mediating piperaquine resistance in the field.

4.4.1 *Plasmepsin* disruption

An extra *pfpm2* or *pfpm3-1* copy was introduced using the *attP-attB* system in the *P. falciparum* Dd2 strain, which originates from Southeast Asia (Figure 13) (Antony et al., 2016; Guinet et al., 1996; Walliker et al., 1987). *Pfpm2* and *pfpm3-1* were amplified from Dd2 strain and a field isolate genomic DNA, respectively, and introduced into the *attP* containing plasmid named pDC2. This

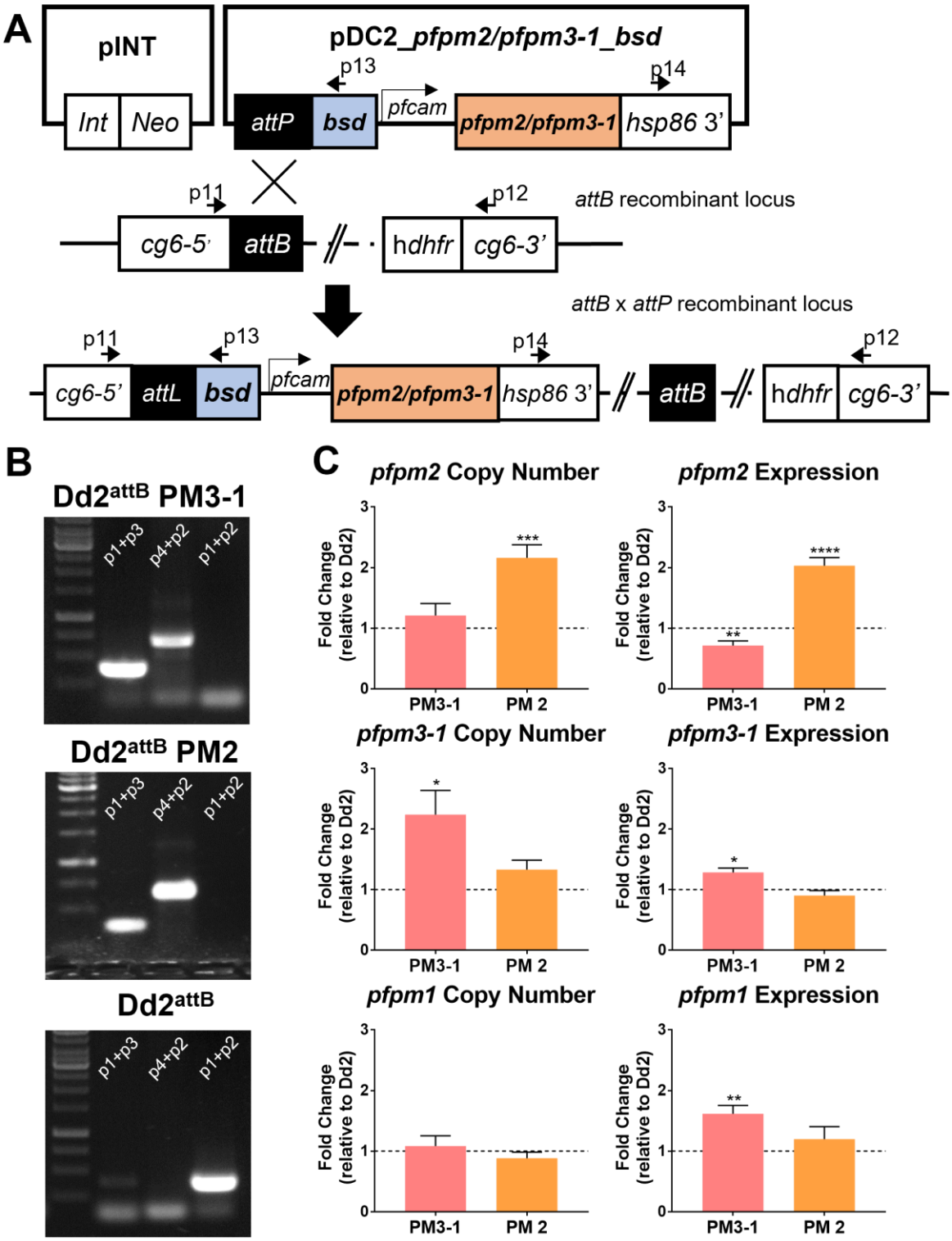


Figure 13. Generation of plasmepsin 2 (PM2) extra copy and hybrid plasmepsin 3-1 (PM3-1) Dd2 parasite lines. (A) – A copy of *plasmepsin 2* (*pfpm2*) and *plasmepsin 3-1* (*pfpm3*-1) was introduced into the Dd2^{attB} line using the *attB*-*attP* recombinase system. pINT contains the Bxb1 integrase (*Int*) and G418 resistance gene (*Neo*). *Pfpm2* and *pfpm3-1* hybrid were amplified from genomic DNA and introduced into the pDC2 plasmid, which has blasticidin selection marker (*bsd*) and *attP* that recombines with *attB* on the genome of the Dd2^{attB} line. (B) – PCR confirmation of *plasmepsins* extra copy integration into the genome of Dd2^{attB}. Primers are represented in the scheme on A. (C) – Quantitative PCR data presenting expression and copy number of *pfpm3-1*, *pfpm2*, *plasmepsin 1* (*pfpm1*) on edited lines relative to Dd2. Data are presented as mean with SEM. Statistical significance was determined via two-tailed, student's *t*-test comparing mutated lines with Dd2 WT. Three independent assays were performed. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001.

plasmid was co-transfected with pINT, an integrase mycobacteriophage Bxb1 coding plasmid, into the Dd2^{attB} strain. This strain is modified to contain an *attB* site into the *cg6* non-essential locus, and the integrase can catalyze the recombination between the *attP* plasmid into the *attB* locus, effectively introducing an extra *plasmepsin* copy into the genome (Figure 13).

After transfection, selection was applied for 5 days with G418, which selects for pINT plasmid, and BSD, which selects for pDC2 that expresses the *bsd* selection marker. Parasite growth was observable by microscopy after 14 days post drug selection, and *bsd* selection was applied continuously to select for integrated parasites, which reappeared after 10 days. The integration event was confirmed by PCR, which identified the integration at both 5' and 3' and the absence of wild type cg6 allele (Figure 11B). The resulting lines are denoted Dd2^{attB} PM2 and Dd2^{attB} PM3-1. Figure 13 illustrates the strategy and shows an electrophoresis gel of editing events.

Plasmepsin copy numbers were confirmed by qPCR for both strains (Figure 13C). *Plasmepsin* expression was also evaluated through qPCR, in which the Dd2^{attB} PM2 displayed 2-fold increased *pfpm2* expression but the Dd2^{attB} PM3-1 only had a 1.2-fold *pfpm3* increase in expression, despite having two *pfpm3* copies in the genome (Figure 13C). This effect might be related to feedback or compensatory mechanisms. Indeed, on the Dd2^{attB} PM3-1 line, we detected increased expression of *plasmepsin 1*. Curiously, this effect only seems to happen for the hybrid *pfpm3-1* gene and not for the extra copy of *pfpm2*. Another hypothesis could be a decreased transcription due to the hybrid DNA or even instability of the hybrid mRNA.

4.4.2 *Plasmepsin* amplification leads to decrease *pfmdr1* copy number

In the course of generating the transgenic lines, we observed a de-amplification of the three *pfmdr1* copy number detected in Dd2 parasites, in both Dd2^{attB} PM2 and Dd2^{attB} PM3-1 mutant strains (Figure 14C), which led to increased susceptibility to mefloquine as expected (Figure 14F). This result corroborates observations of piperaquine-resistance selection in southeast Asia (Bopp et al., 2018) and suggests that the reduction of *pfmdr1* copy number occurs as a result of a cellular physiological digestive-vacuole adaptation to withstand *plasmepsin* gene amplification events rather than a direct process of selection for piperaquine resistance. To explore this, we used the Fluo-4 that has been demonstrated to accumulate on digestive vacuole with kinetics depending on PfMDR1 transport and independent of other loci (Calçada et al., 2020; Friedrich et al., 2014; Reiling and Rohrbach, 2015; Rohrbach et al., 2006). Intriguingly, the Dd2^{attB} PM2 and Dd2^{attB} PM3-1 lines present higher Fluo-4 fluorescent values, even though these lines have fewer copies of *pfmdr1* (Figure 12A and C).

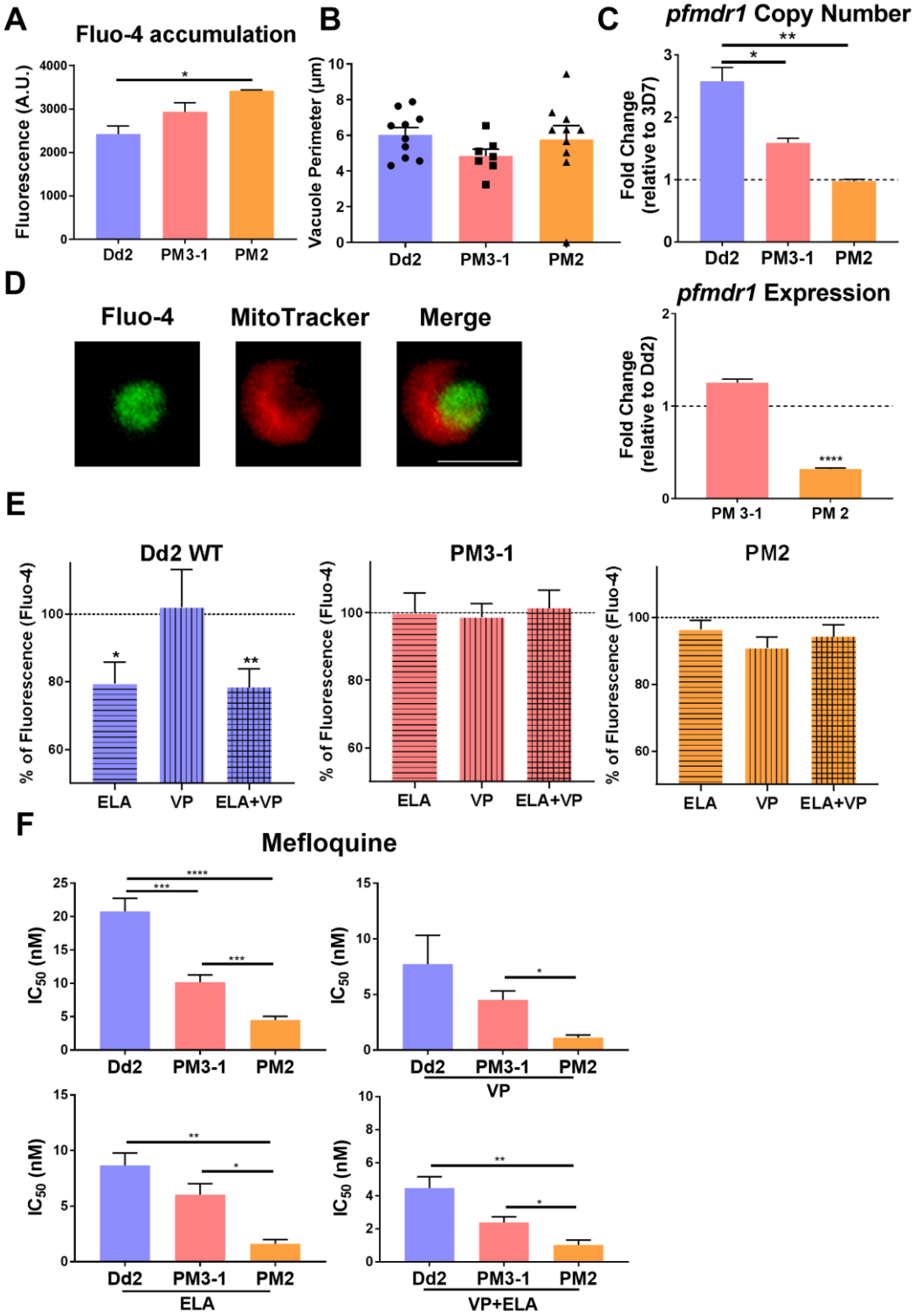


Figure 14. Fluo-4 accumulation assays, *pfmdr1* expression and mefloquine drug susceptibility assays. (A) – Fluo-4 accumulation after 4 hours of incubation measured through flow cytometry. Data is presented in fluorescence arbitrary units (A.U.) (B) – Parasite vacuole area measured on fluorescent microscopy using the area around Fluo-4 and outside MitoTracker staining. (C) – Quantitative PCR data presenting copy number and expression of *pfmdr1*. (D) – Fluorescence microscopy of a parasite incubated with Fluo-4 and MitoTracker. Scale = 5 µm (E) – Fluo-4 accumulation after 4h of incubation with verapamil (VP) and elacridar (ELA), and both combined measured through flow cytometry. Data is presented in percentage of fluorescence related to untreated control. (F) – IC_{50} derived from nonlinear regression from 12-fold dilution assays of mefloquine susceptibility, and in presence of VP, ELA and VP+ELA. Data are presented as mean with SEM. Three independent assays were performed. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001.

Moreover, as expected, using elacridar, a chemical blocker of PfMDR1, there was a decrease in Fluo-4 fluorescence in Dd2 WT strain and no effect was observed using verapamil, a chemical blocker of PfCRT. Intriguingly, on Dd2^{attB} PM2 and Dd2^{attB} PM3-1 strains, there was no impact of elacridar on Fluo-4 fluorescence. The area of the vacuoles remained unchanged between mutants and WT strain (Figure 14B). These results point to an alteration in vacuolar physiology that impacts the probe fluorescence. This probe has been shown to be highly dependent on pH (Rohrbach et al., 2005), so pH change of vacuole due to increased *plasmepsin* copy number is a possible hypothesis. Nevertheless, this assay is not enough to clearly pinpoint which specific changes occurred in the dynamics of the vacuole.

Since piperaquine has been shown to select for *plasmepsin* increased copy numbers, these results suggest that decreased *pfmdr1* copy numbers in the field is an indirect consequence of piperaquine pressure in addition to the discontinuation of mefloquine-based therapies. Likewise, this data supports the use of mefloquine and piperaquine in combination for triple combination therapies, as these compounds exert simultaneously different *pfmdr1* pressures.

4.4.3 *Plasmepsin* amplification leads to high grade resistance to piperaquine

Drug susceptibility assays were performed to explore the impact of *plasmepsin* duplication in piperaquine resistance. As somewhat expected, no significant alteration of these parasite lines was detected at the half maximal inhibitory concentration (IC_{50}) of piperaquine, as usually the phenotype manifests as high-grade resistance (Figure 15A). Moreover, given the association of *pfcrt* mutations and *pfmdr1* with piperaquine resistance, drug assays were performed using verapamil as an inhibitor of PfCRT and elacridar as an inhibitor of PfMDR1. No alteration in IC_{50} was observable for the Dd2^{attB} PM2 and Dd2^{attB} PM3-1 strains. Additionally, in similar assays, the range of piperaquine concentrations was extended to detect possible bimodal resistance phenotype, as previously reported (Bopp et al., 2018). Both *plasmepsin 2* and *plasmepsin 3–1* gene amplifications contributed to bimodal piperaquine resistance. This phenotype was enhanced in the presence of the verapamil and elacridar inhibitors, creating a bimodal effect on piperaquine susceptibility (Figure 15B), similar to that previously described in Cambodian *P. falciparum* isolates and genetically engineered strains with PfCRT mutations (Bopp et al., 2018; Dhingra et al., 2017; Ross et al., 2018). This effect was higher with PfCRT inhibition (area under the curve [AUC] was 74 [95% CI 67–82] for *plasmepsin 2* and 42 for *plasmepsin 3–1*) than with PfMDR1 inhibition (AUC was 47 [95% CI 40–54] for *plasmepsin 2* and 29 for *plasmepsin 3–1*; (Figure 15C).

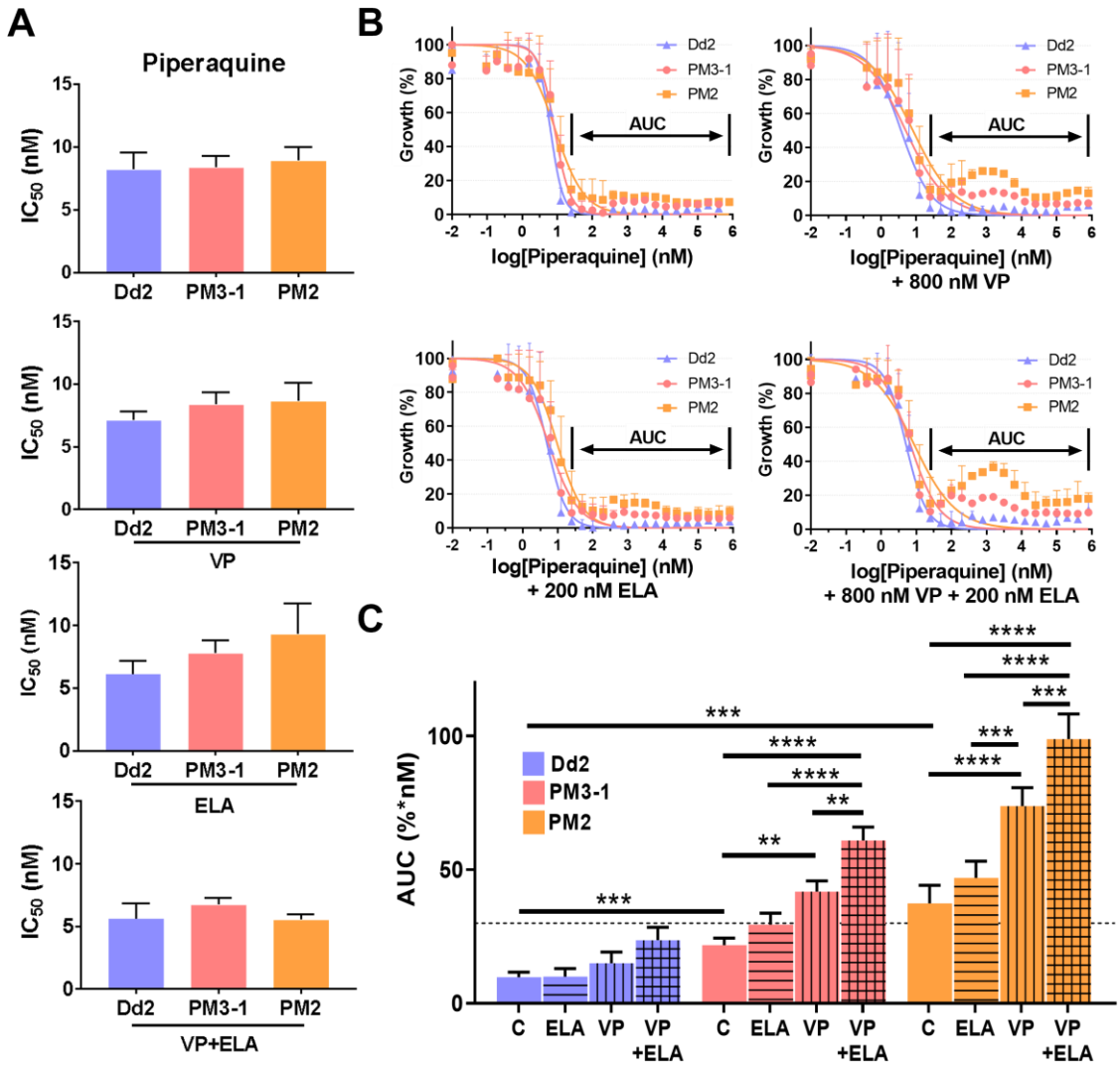


Figure 15. Piperaquine susceptibility assays. (A) – IC_{50} derived from nonlinear regression from 12-point dilution assay of piperaquine susceptibility (B) – Growth curve for the piperaquine extended susceptibility assay of 24-point dilution. (C) – Area under the curve (AUC) calculated from the second curve of response from the bimodal susceptibility assay, as depicted in (B). Threshold of 30 (dashed horizontal line) was defined as threshold of piperaquine resistance, as previously reported (Bopp et al., 2018b). Verapamil (VP) was used as chemical blocker of PfCRT and elacridar (ELA), as chemical blocker of PfMDR1, verapamil plus elacridar were used combined (VP+ELA). Data are presented as mean with SEM. Three independent assays were performed. Statistical significance was determined via ANOVA. **p<0.01 ***p<0.001 ****p<0.0001.

4.4.4 Discussion

The first evidences of piperaquine resistance associated *plasmepsins* duplications with the phenotype (Witkowski et al., 2017). Moreover, later studies identified *pfmdr1* decreased copy number variations and *pfcrt* polymorphisms (Bopp et al., 2018; Ross et al., 2018). Subsequently, the role of PfCRT has been confirmed *in vitro* as a mediator of piperaquine resistance (Dhingra et al., 2019; Ross et al., 2018), while the role of *plasmepsin* duplication in this phenotype remains elusive. Hypothesis for the reason why *pfcrt*

mutations were not identified earlier suggest that a proportion of piperaquine-resistant isolates to not involve mutations in *pfcrt* but rely on alternative pathways such as one involving *plasmepsins* duplications. Alternatively, *pfcrt* mutations recently evolved and therefore were not captured in initial studies. Moreover, another possibility is that these *pfcrt* mutations need specific backgrounds to develop, such as one provided by *plasmepsins* duplication.

Temporal data suggests that the *pfcrt* mutations associated with piperaquine resistance arose on a genetic background of amplified *plasmepsins* and correlate with temporal de-amplification of *pfmdr1* (Dhingra et al., 2019; Ross et al., 2018). Most of the *pfcrt* mutation are observed after 2010 becoming more prevalent between 2011-2013 and became the dominant allele by 2016 (Dhingra et al., 2019; Ross et al., 2018). These mutations seem to have arisen independently in strains with Dd2 PfCRT background and mutant PfK13 (Ross et al., 2018). Curiously, high *plasmepsin* copy number (4 and more copies) were more frequent in 2012 and 2013, and by 2016 *plasmepsin* duplication (2-3 copies) was the predominant amplification status (Dhingra et al., 2019). Additionally, survival rates of piperaquine-treated field isolated adapted parasites increased, *in vitro,* over the years (Dhingra et al., 2019).

Together with our data, these findings suggest an initial selection of high number of *plasmepsin* locus amplifications with a simultaneous decrease in *pfmdr1* copy number variations to accommodate the *plasmepsin* amplification events. These mutations might not cause a full resistant phenotype. Instead, these events likely generated a low grade piperaquine resistance that created a favorable *P. falciparum* genetic background for novel *pfcrt* mutations to arise in the context of piperaquine pressure. These parasites can have increased resistance to high concentrations of piperaquine, likely at a lower fitness cost.

The initial F145I, M343L and H97Y PfCRT mutations were shown to confer a high fitness cost and are being replaced by less resistant but fitter T93S and I218F mutations (Dhingra et al., 2019; Ross et al., 2018). These initial mutations H97Y, F145I and G535V were also shown to occur in parasites with 3 to 7 copies of *pfpm2* (Ross et al., 2018). Moreover, in this study all piperaquine resistant PfCRT mutants had *pfmdr1* single copy, despite the presence of field samples with multiple *pfmdr1* copies, which further implies the *pfmdr1* single copy as important to resistance and not just a consequence of mefloquine removal (Ross et al., 2018). Interestingly, in previous studies using *pfcrt*-edited parasites with a single *plasmepsin* copy, these parasites developed an enlarged and translucent digestive vacuole, while this phenotype was not observed for piperaquine resistant field parasites harboring PfCRT mutations combined with multiple copies of *pfpm2* (Boonyalai et al., 2020; Ross et al., 2018). This suggests that *plasmepsins* can directly counteract vacuole disruption caused by mutant PfCRT, which in the field are

likely to be associated with a fitness cost (Dhingra et al., 2019). *In vitro* removal of PfCRT mutations coincides with improved growth rates and *pfpm2* de-amplification, linking these two factors (Ross et al., 2018). Accordingly, in the field *plasmepsin* duplication still seems necessary as these parasites seem to retain at least *plasmepsin* locus duplication (Ross et al., 2018). Even though, *pfcrt* mutations *in vitro* seem sufficient to generate resistance and might dispense the *plasmepsin* duplications (Ross et al., 2018). As such, likely the initial expansion of mutations in PfCRT could have been propelled by *plasmepsin* duplications which could have a beneficial impact on the fitness cost caused by these mutations.

Recently, the structure of PfCRT was unraveled, and the piperaquine resistance associated mutation C350R that emerged in the 7G8 isoform and the mutations T93S, I218F, F145I, H97Y, G353V that emerged in the Dd2 isoform were located in the helices that line the central negatively charged cavity (J. Kim et al., 2019). The mutation M343L, which confers low grade piperaquine resistance in the Dd2 isoform, was located deeper into the central cavity closer to the parasite cytosol (J. Kim et al., 2019). Contrary to other mutations, the M343L was shown to not confer a fitness cost when introduced into the Dd2 strain. However, it confers a fitness cost in a Cambodian adapted parasite with a Dd2-like *pfcrt* background (Ross et al., 2018). Location of the mutation is important for fitness cost, possibly due to alterations in solute transport, but additional factors including mutations in other genes are important for the overall fitness cost of the strain. PfCRT 7G8 isoform was shown to bind to piperaquine but not to transport, which was consistent with the sensitive role of the strain (J. Kim et al., 2019). The F145I and C350R mutation were shown to transport piperaquine when introduced into the 7G8 strain and associated with piperaquine resistance phenotype (J. Kim et al., 2019). Interestingly, these mutations have reduced chloroquine transport (J. Kim et al., 2019). On the contrary, the introduction of F145I mutation into the Dd2 strain did not increase piperaquine transport. Notably, in another study there was no significant difference in intracellular piperaquine concentration on the edited Dd2 piperaquine sensitive control line versus the Dd2 piperaquine resistant variants expressing the PfCRT mutations F145I, M343L, or G353V, suggesting that these mutations do not confer resistance by accumulating less piperaquine in the Dd2 background (Ross et al., 2018). Moreover, there was an increase in the intracellular accumulation of piperaquine resistant edited field isolates when removing PfCRT resistant conferring mutations (Ross et al., 2018). These reflect the genetic background impact in piperaquine resistant and highlight the complex relationship between PfCRT mutations and piperaquine resistance, which does not appear to be explained solely by changes in drug accumulation or efflux. Likewise, our results show that verapamil, a chemical blocker of PfCRT, in the Dd2 *plasmepsin* amplificated lines is enough to confer resistance to piperaquine.

As a blocker of PfCRT function, this result does not support generation of resistance by increased transport. There is possibility that more than one mechanism prevails to give resistance. One alternative mechanism involves piperaquine-mediated binding to and functional inhibition of certain PfCRT isoforms, recalling earlier reports of distinct drug binding sites in this transporter (Bellanca et al., 2014; Callaghan et al., 2015; Lekostaj et al., 2008; Richards et al., 2016). Moreover, due to the similar effect of verapamil and elacridar in the presence of *plasmepsin* duplication, in our study, another possibility is altered vacuolar function that negates the effect of high piperaquine concentrations. Due to the role of both piperaquine and plasmepsins on the hemoglobin degradation pathway it is likely that changes in this pathway affect piperaquine resistance and that PfCRT altered physiological function could lead to disruptions in this process. Further investigations in gene-edited parasites or heterologous expression systems will be important in delineating the relationship between PfCRT mutations, *plasmepsins* copy number, drug accumulation, and piperaquine resistance.

Piperaquine resistance is multifactorial, as seen by the disparity of resistant field samples and their genotypes, which can dispense either *plasmepsins* amplifications or *pfcrt* mutations. As such, conceivably in different genetic backgrounds to those used in this study, *plasmepsins* duplications could confer different degrees of resistance, and could potentiate other loci to mediate resistance in the field. Additionally, other loci might be in the process of selection and fixation that could make PfCRT mutant parasites resistant while dispensing *plasmepsin* duplications. Continuous temporal monitoring of the dynamics of parasite populations in the areas of DHA-PPQ therapy usage is necessary to understand the evolution of resistant parasites and to prevent further fixation of resistant alleles that avoid fitness costs. Deep sampling of contemporary isolates is of particular importance to detect emergence of novel alleles in this propense background and to avoid propagation of piperaquine resistance to neighboring areas. Such fixation could lead to parasite populations to become permanently resistant even after removal of piperaquine based therapies. This could render new therapies based on piperaquine ineffective and compromise strategies of mass drug administration for eradication efforts.

Altogether, our results recapitulate *in vitro* a complementary mechanism between *plasmepsins*, *pfmdr1*, and *pfcrt* involved in piperaquine resistance, supporting the molecular epidemiological data in southeast Asia and furthering understanding of how piperaquine drug resistance evolves.

4.5 Study 4: The *P. falciparum* protein PfMRP1 functions as an influx ABC transporter

Manuscript under submission. Preprint available at Research Square, 13 January 2022. https://doi.org/10.21203/rs.3.rs-1210682/v1

The ABC transporter super-family is known to transport a wide variety of substrates across cellular membranes against a concentration gradient, an action driven by ATP hydrolysis. These transporters are present in multiple life forms, from prokaryotes to the most complex eukaryotes, with importers described so far in prokaryotes and plants (Rees et al., 2009). ABC transporters also serve as drug targets and are important mediators of drug resistance. PfMRP1 has been localized at the parasite plasma membrane (Raj et al., 2009) and was suggested to be a homeostatic modulator of oxidative status and a component of the oxidative response, moreover PfMRP1 was hypothesized as a folate transporter (Atamna and Ginsburg, 1997; Bozdech and Ginsburg, 2004; Raj et al., 2009; Rijpma et al., 2016b). However, the physiological function of this transporter remains largely unexplored.

Early reports have linked PfMRP1 single nucleotide polymorphisms with different susceptibilities to the antimalarial (Mu et al., 2003) and further studies on drug response in clinical isolates and in vivo malaria chemotherapy appeared to support these findings (Dahlström et al., 2009b, 2009a; Phompradit et al., 2014a, 2014b; Raj et al., 2009; Veiga et al., 2011). However, while trying to validate the role of PfMRP1 in drug resistance *in vitro*, conflicting reports have emerged.

Applying ZNF gene editing, this study describes two *pfmrp1*-disrupted lines in the geographically distinct *P. falciparum* 3D7 and Dd2 parasite strains. Our data disclose PfMRP1 as a potential importer can likely transport folate and related compounds, including drugs related to the folate pathway. This is an unusual function of a plasma membrane importer from the ABC transporter family in a eukaryotic organism, aside from plants. A brief introduction to the theme was provided here, but a more detailed description is present on the introduction of this thesis on the importance of ABC transporters in *P. falciparum* including PfMRP1.

4.5.1 *Pfmrp1* disruption

Using customized ZFNs we disrupted the *pfmrp1* gene in *P. falciparum* Dd2 and 3D7 parasites, by single crossover integration of plasmid into the gene locus (Figure 16). These two strains originate from Southeast Asia and Africa respectively, are part of genetically distinct subpopulations of *P. falciparum*,

and harbor very distinct antimalarial drug responses (Antony et al., 2016; Guinet et al., 1987). ZFNs were engineered to bind neighboring sites on opposite strands of *pfmrp1*, producing a double-stranded break 1208 bp upstream of the stop codon. Our homology-driven template consisted of a 2.5 kb *pfmrp1* fragment engineered with three synonymous mutations at the ZFN binding site, to prevent ZFNs from cleaving the plasmid and promoting the integration of the plasmid, disrupting the *pfmrp1*.

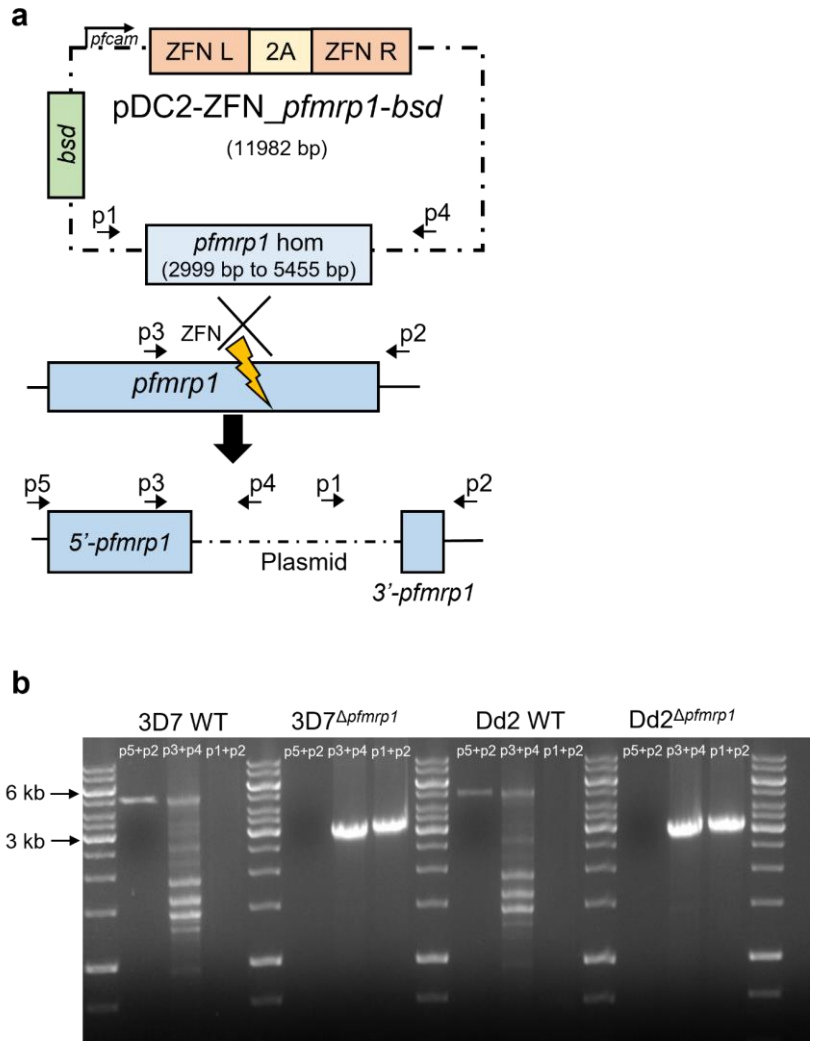


Figure 16. Construction of *pfmrp1* disrupted strains. (a) – Schematic of the ZFN-based strategy. Plasmid expresses the *pfmrp1*-targeting ZFN pair (ZFN L and ZFN R) linked by a 2A peptide from the *calmodulin* promoter (*pfcam*) and *heat shock protein 86* (*hsp86*) 3' UTR, and the *blasticidin S deaminase* (*bsd*) cassette, which confers resistance to blasticidin. The ZFNs create a double-stranded break in *pfmrp1* (thunderbolt), which can be repaired by recombination with the *pfmrp1* homologous region on the plasmid (*pfmrp1* hom) leading to *pfmrp1* disruption (Δ*pfmrp1*) due to introduction of plasmid by single cross-over recombination. **(b)** – PCR genotyping to confirm Δ*pfmrp1* strains. WT – PCR with primers p5+p2, which amplifies the intact gene (~5.7kb). 5' – PCR with primers p3+p4, which amplifies integration of plasmid into *pfmrp1* within the 5' region. 3' – PCR with primers p1+p2, which amplifies integration of plasmid into *pfmrp1* within the 3' region. Amplification confirms integration by a single-crossover event.

Red blood cells infected with ring-stage *P. falciparum* 3D7 or Dd2 parasites were electroporated with the *pfmrp1* ZFN disruption plasmid (Figure 16). This plasmid expresses the *bsd* selection marker,

conferring resistance to BSD. Parasite growth was observable by microscopy after 18-22 days post drug selection. *pfmrp1* disruption was confirmed by PCR, which identified the full-length gene only in non-mutated wild type strains. PCRs spanning both ends of integration were positive for knockout (Δ*pfmrp1*) strains, denoted 3D7$^{\Delta pfmrp1}$ and Dd2$^{\Delta pfmrp1}$, which were then cloned by limiting dilution and one clone was chosen to proceed (Figure 16).

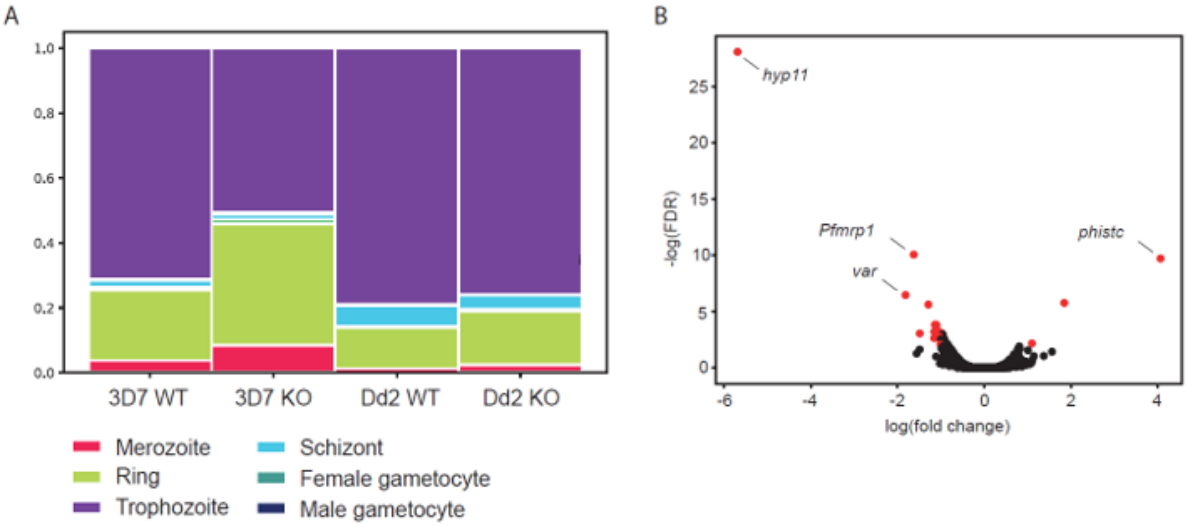


Figure 17. Transcriptomic analysis of mutant parasites. Transcriptome sequencing (RNA-Seq) analysis to test whether the Δ*pfmrp1* mutants showed any differential expression of transcripts and altered metabolic pathways. **(a)** – Deconvolution analysis of the Dd2 and 3D7 bulk RNA-seq samples. The composition of stages was similar between WT and Δ*pfmrp1* for Dd2. The proportion of trophozoites in the 3D7$^{\Delta pfmrp1}$ was ≈20% lower than for WT, making comparison difficult. **(b)** – A volcano plot showing differentially expressed genes (red dots) between WT and mutant Dd2 parasites. *pfmrp1* is clearly downregulated as expected, but other differential expressed genes are limited to clonally variant genes.

To test whether the Δ*pfmrp1* mutants showed any differential expression of transcripts that could lead to altered metabolic pathways, we conducted a transcriptome sequencing (RNA-Seq) analysis. Total RNA was extracted from *P. falciparum* 3D7 and Dd2 strains and their respective 3D7$^{\Delta pfmrp1}$ and Dd2$^{\Delta pfmrp1}$ lines, tightly synchronized at the early trophozoite stage. At this stage, the *pfmrp1* has the highest expression (Bozdech and Ginsburg, 2004; Rovira-Graells et al., 2012; Toenhake et al., 2018; Veiga et al., 2010). Deconvolution analysis of the 3D7 and Dd2 bulk transcriptomes suggests that the 3D7 WT and 3D7Δ^{pfmrp1} parasites were not similarly synchronized, having different mixes of rings and trophozoites, making comparison difficult (Figure 17a). The WT and Δ*pfmrp1* Dd2 samples were very similar in their composition of stages. However, we found few differences in gene expression between them (Figure 15b). *pfmrp1* was expressed at a lower level in the Dd2$^{\Delta pfmrp1}$ as expected, confirming that the disruption influenced the expression of the transcript. However, other differences were largely limited to clonally variant genes, which might have become differentially expressed stochastically during cloning of the mutant line (Figure 17b). This suggests that the *pfmrp1* disruption has little effect on the parasite transcriptome at this point in the lifecycle.

4.5.2 *Pfmrp1* disruption has no impact on quinoline and dihydroartemisinin susceptibility

In vitro drug susceptibility assays were performed on WT and *Δpfmrp1* lines. Tightly synchronized ring-stage parasites were incubated with antimalarials, using a range of 10 compound concentrations diluted 2–fold, for 72 hours. Parasitemias were quantified using flow cytometry. IC_{50} values were determined for chloroquine, mefloquine, lumefantrine, dihydroartemisinin, pyrimethamine, trimethoprim, WR99210, methotrexate and aminopterin. Of note, there was no alteration in IC_{50} for the commonly used quinoline antimalarials chloroquine, mefloquine and lumefantrine, nor to dihydroartemisinin for either $3D7^{\Delta pfmrp1}$ or $Dd2^{\Delta pfmrp1}$ strains when compared to their parental strains (Figure 18a). IC_{50} values were in the range of previous reports. The main differences observed in chloroquine susceptibility between the parental Dd2 and 3D7 strains are explained by known polymorphisms in the PfCRT and PfMDR1 (Johnson et al., 2004; J. Kim et al., 2019; Ross et al., 2018; Veiga et al., 2016).

4.5.3 *Pfmrp1* disruption leads to resistance to folate analogues

$3D7^{\Delta pfmrp1}$ and $Dd2^{\Delta pfmrp1}$ were strikingly less sensitive to the antifolates methotrexate and aminopterin, compared to their respective parental strains. The IC_{50} values for the $3D7^{\Delta pfmrp1}$ and $Dd2^{\Delta pfmrp1}$ lines were increased by 15 and 40-fold when assayed against methotrexate (P=0.001 and P<0.0001, respectively) and 6 and 25-fold against aminopterin (P=0.0016 and P=0.0032, respectively), respectively, compared to their WT parental controls (Figure 18a and Figure **18**d). However, WT and Δ*pfmrp1* lines displayed comparable responses to the antifolates pyrimethamine, trimethoprim and WR99210. Strain-specific differences in susceptibility to pyrimethamine, trimethoprim and WR99210 were observed as expected due to genotypic differences in the *P. falciparum* dihydrofolate reductase (*pfdhfr*) gene (Fidock et al., 1998; Ping Wang et al., 1997). Methotrexate and aminopterin are folate analogs (Figure 18b), while pyrimethamine, trimethoprim and WR99210 are structurally distinct to folate, which might account for the differential pattern of susceptibility changes to these antifolates. These results suggest that loss of *pfmrp1* leads to parasite resistance to folate analogs. This would be incompatible with an exporter function, as the disruption should lead to increased drug concentration in the parasite cytoplasm, leading to heightened drug sensitivity in the Δ*pfmrp1* lines. These findings suggest PfMRP1 as an importer present in the *P. falciparum* plasma membrane.

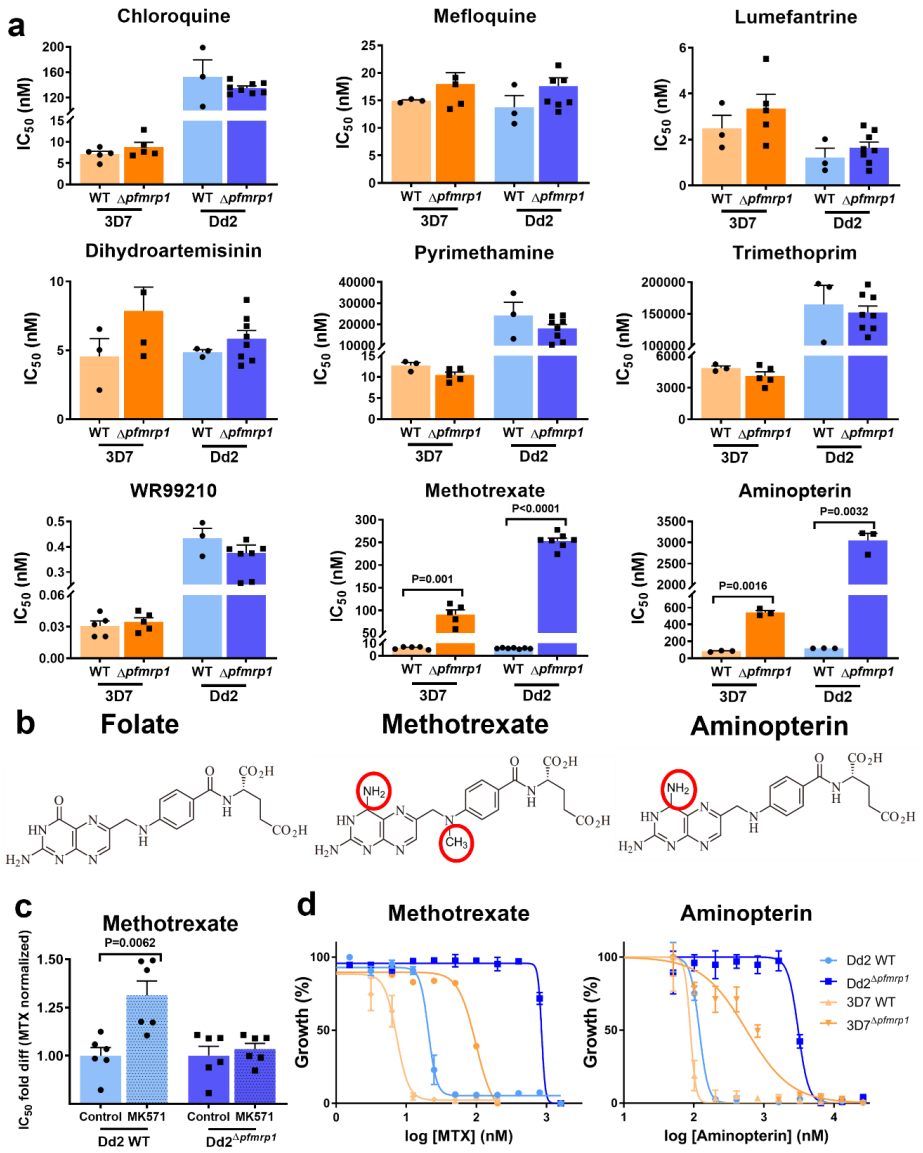


Figure 18. *pfmrp1* disruption leads to decreased susceptibility to folate analogs. (a) – *In vitro* IC_{50} antimalarial responses for Δ*pfmrp1* and wild-type (WT) 3D7 and Dd2 *Plasmodium falciparum* strains. Drug susceptibility assays were performed to obtain values of IC_{50} presented as the mean ± s.e.m. for chloroquine, mefloquine, lumefantrine, dihydroartemisinin, pyrimethamine, trimethoprim, WR99210, methotrexate and aminopterin. Statistically significant differences were observed for methotrexate and aminopterin, manifesting as a significant increase in the IC_{50} for the $3D7^{\Delta pfmrp1}$ (15-fold for methotrexate, P=0.001 and 6-fold for aminopterin, P=0.0016) and $Dd2^{\Delta pfmrp1}$ (40-fold for methotrexate, P<0.0001 and 25-fold for aminopterin, P=0.0032) strains relative to their respective parental control strains. Assays were performed in at least 3 independent biological replicates (black squares and triangles represent the exact number of independent biological replicates). Statistical evaluations comparing WT and Δ*pfmrp1* for each strain were performed using two-tailed unpaired *t* tests with Welch's correction. **(b)** – The PfDHFR inhibitors methotrexate and aminopterin are folate analogs. Red circles represent functional group differences between analogs and folate. **(c)** – *In vitro* IC_{50} responses for Dd2 WT and $Dd2^{\Delta pfmrp1}$ strains in the presence of only MTX (Control) or MTX plus MK571, a PfMRP1 chemical inhibitor. Results are normalized against control condition. Values presented are the mean ± s.e.m. of the ratio of IC_{50} for each assay to the mean of the Control condition for each strain. PfMRP1 chemical inhibition led to a significant decrease in methotrexate susceptibility only for the Dd2 WT strain. Assays were performed in 6 independent biological replicates (black squares and triangles). Statistical evaluation comparing conditions was performed using two-tailed unpaired *t*tests with Welch's correction using non-normalized IC_{50} values. **(d)** – *In vitro* growth response curves for methotrexate and aminopterin of Dd2 and 3D7 WT and Δ*pfmrp1* strains. These response curves represent the increases in the IC_{500} values for the $Dd2^{\Delta pfmrp1}$ and $3D7^{\Delta pfmrp1}$ observed for both folate analogs. Assays were performed in 3 independent biological replicates, each dot represents mean + s.e.m. for each concentration.

To confirm that the observed resistance phenotype was mediated by PfMRP1, the MRP chemical inhibitor MK571 was used to block PfMRP1 transport in the Dd2 strain (da Costa et al., 2018; Gekeler et al., 1995; Tivnan et al., 2015). A small but significant increase (1.3-fold, P=0.0042) in IC_{50} was observed for MTX only in MK571-treated Dd2 WT parasites (24.7 ± 1.4 nM) compared to untreated controls (18.8 ± 0.8 nM), whereas no difference was observed for the MK571-treated Dd2$^{\Delta pfmrp1}$ line, supporting a PfMRP1-mediated effect (Figure 18c). The fold-increase in methotrexate IC_{50} values by chemical inhibition was considerably lower compared to the increase observed with the Dd2$^{\Delta pfmrp1}$ line (Figure 18a and Figure **18**d), which might be explained by incomplete chemical inhibition of PfMRP1-mediated transport by MK571.

Moreover, in the context of understanding the possible crosstalk between antimalarial drug resistance mediators, we examined the effect of inhibiting PfCRT and PfMDR1 in Dd2$^{\Delta pfmrp1}$ parasites with verapamil and elacridar, respectively. Verapamil-mediated inhibition led to a small (1.2-fold, P=0.0315) but significant decrease in methotrexate susceptibility in Dd2$^{\Delta pfmrp1}$ parasites (896 ± 42.4 nM compared to 767 ± 42.4 nM), whereas no effect was observed in Dd2 WT (Figure 19). These results suggest that PfCRT might interplay with PfMRP1 to augment methotrexate resistance. This increased resistance could be due to trapping of residual methotrexate in the digestive vacuole, away from its cytosolic site of action, with methotrexate being unable to be effluxed by PfCRT in the presence of verapamil that is a known blocker of PfCRT-mediated drug transport (Shafik et al., 2020). No effect was observed with elacridar-mediated inhibition (Figure 17).

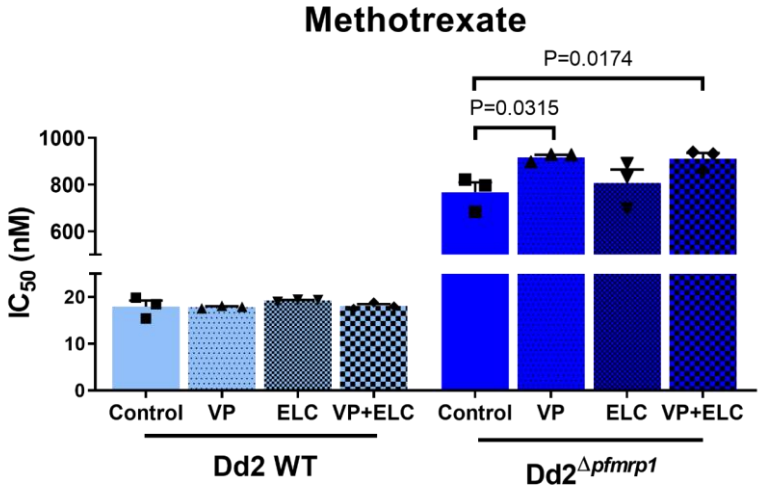


Figure 19. PfCRT inhibition amplifies the decreased susceptibility of Dd2$^{\Delta pfmrp1}$ parasites to folate analogs. *In vitro* IC_{50} responses for Dd2 WT and Dd2$^{\Delta pfmrp1}$ strains in the presence of MTX only (Control) and MTX plus the chemical inhibitors elacridar (ELC) and verapamil (VP). PfCRT chemical inhibition led to significant decrease in MTX susceptibility only for the Dd2$^{\Delta pfmrp1}$ strain. Assays were performed in 3 independent biological replicates (squares). Statistical evaluations comparing conditions for each strain were performed using Kruskal-Wallis test with multiple comparisons performed using Tukey's test.

4.5.4 *Pfmrp1* disrupted parasites accumulate less fluorescein methotrexate

To understand how resistance to folate analogs occurs in the Δ*pfmrp1* parasites, we measured the time-dependent accumulation of fluorescein MTX (F-MTX), a fluorescent derivative of methotrexate, using flow cytometry. Remarkably, the Dd2$^{\Delta_{pfmrp1}}$ line showed a significant lack of F-MTX accumulation over time, with an average of 3.3 times less F-MTX fluorescence intensity after 3 hours of incubation compared to Dd2 WT parasites (Figure 20a). The observed F-MTX phenotype seems specific to PfMRP1-mediated transport (Figure 20b). Fluo-4 is a calcium probe that enters the parasite through passive diffusion, accumulates in the parasite digestive vacuole, and can be modulated by PfMDR1-mediated transport (Calçada et al., 2020; Friedrich et al., 2014). Repeating the assay with Fluo-4 showed no differences in accumulation between Dd2 WT and Δ*pfmrp1* parasites (Figure 20b). These results provide evidence that PfMRP1 can import F-MTX, an effect that is much reduced in Dd2$^{\Delta_{pfmrp1}}$, thus explaining the resistance phenotype. The residual accumulation observed in Dd2$^{\Delta_{pfmrp1}}$ might occur through a distinct transport pathway, passive diffusion, or both.

To confirm the location of F-MTX inside the infected red blood cell, parasites were incubated with F-MTX and visualized using confocal microscopy. After a 3-hour incubation period, F-MTX accumulated in the parasite cytosol, co-localizing with MitoTracker fluorescence that labels mitochondria, but not with the DAPI nuclear stain (Figure 20c). Confocal microscopy confirmed the same accumulation pattern observed in the flow cytometry assay, with Dd2 WT exhibiting more F-MTX accumulation than Dd2$^{\Delta_{pfmrp1}}$ (Figure 20c). The fluorescence was quantified on widefield microscopy and the ratio of F-MTX/MitoTracker fluorescence was significantly increased in Dd2 WT parasites, demonstrating more F-MTX accumulation than Dd2$^{\Delta_{pfmrp1}}$ (Figure 20d).

However, a drug susceptibility assay showed that F-MTX does not retain the antimalarial activity of MTX, most likely due to its bulkier structure preventing binding to PfDHFR, the target of MTX (Figure 21). Since transport proteins are less specific in their substrate binding properties compared to the interaction of a substrate with the less accessible active site of an enzyme, F-MTX might be transported into the parasite cytoplasm via PfMRP1 but unable to bind PfDHFR and impair parasite growth.

Overall, both the flow cytometry and confocal microscopy data on the transport of F-MTX into infected red blood cells point to a putative import function of PfMRP1.

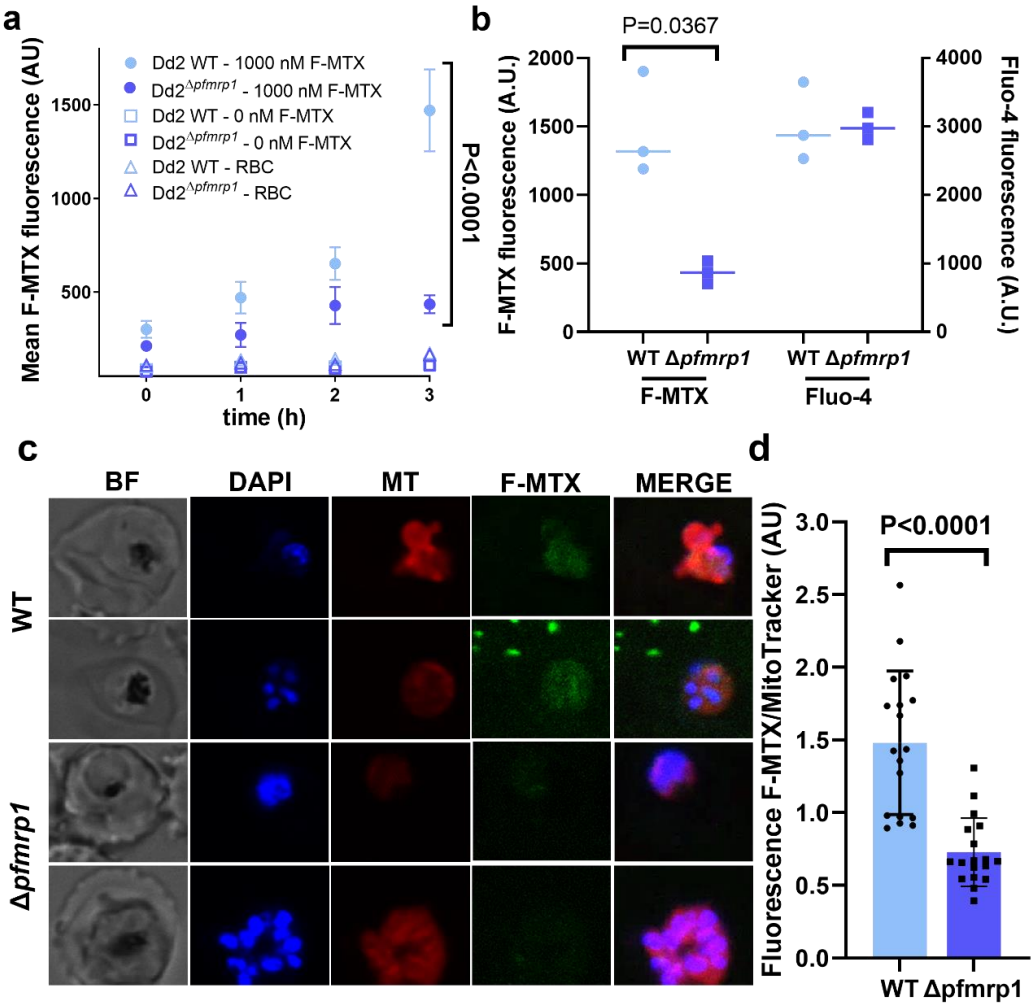


Figure 20. *pfmrp1*-disrupted parasites accumulate less fluorescein methotrexate (F-MTX) than WT parasites. a – Flow cytometry-based F-MTX accumulation assay over time for Dd2 WT and Dd2$^{\Delta pfmrp1}$ strains. F-MTX was used at 1000 nM. 0 nM of F-MTX and uninfected RBCs were used as controls for baseline and autofluorescence. Dd2$^{\Delta pfmrp1}$ parasites accumulated less F-MTX than WT, a trend that was maintained over 3 hours. At the endpoint WT parasites (1470 ± 219 AU) had ≈3-fold more F-MTX fluorescence compared to Δ*pfmrp1* parasites (434 ± 49 AU). Assays were performed in 3 independent replicates. Mean ± s.e.m. values are presented. AU – Arbitrary fluorescence units. Statistical evaluation comparing Dd2 WT and Dd2$^{\Delta pfmrp1}$ was performed using two-way ANOVA with multiple comparisons inside the same time point performed using Šídák's multiple comparisons test. **b** – Quantification from the cytometry-based F-MTX accumulation assays for Dd2$^{\Delta pfmrp1}$ parasites normalized to Dd2 WT fluorescence after 3 hours of incubation. Quantification with Fluo-4 (that enters the parasite cytoplasm through passive diffusion) performed in similar way was used as a control for non-specific transport. Fluo-4 was incorporated to the same degree in WT and Δ*pfmrp1* parasites as expected, while F-MTX was ≈0.3-fold less fluorescent in Δ*pfmrp1* parasites. Assays were performed in 3 independent biological replicates. Statistical evaluation was performed comparing Dd2 WT and Dd2$^{\Delta pfmrp1}$ for each condition using two-tailed unpaired t-test with Welch's correction. c – Confocal microscopy-based MTX accumulation assay after 3 hours of F-MTX incubation for Dd2 WT and Dd2$^{\Delta pfmrp1}$ strains. MitoTracker was used to stain live parasites. Δ*pfmrp1* parasites exhibited less fluorescence than WT (representative parasites). The image was cropped around a single parasite, and the different channels were merged using FlowJ software (MERGE). F-MTX and MERGE images have increased to 40% brightness for better visualization. BF – Bright field; DAPI - 4′,6-diamidino-2-phenylindole; MT – MitoTracker. d – Quantification from widefield microscopy-based F-MTX accumulation assay as a ratio of F-MTX/MitoTracker fluorescence for Dd2 WT and Dd2$^{\Delta pfmrp1}$ strains. 11 and 7 parasites were counted for each condition from 2 independent biological replicates. There is a significant difference of the fluorescence ratio between WT and Δ*pfmrp1* strains (≈2-fold). Statistical evaluation comparing conditions was performed using two-tailed unpaired *t*-tests with Welch's correction. AU – Arbitrary units.

4.5.5 Folate analogues compete for PfMRP1 transport

To further explore the propensity of PfMRP1 to transport antimalarial drugs, the F-MTX probe was used as a proxy of transport capacity. We performed flow cytometry competition assays and measured the fluorescence of F-MTX in infected RBCs, after 3 hours incubation periods with varying concentrations of different compounds and a fixed concentration of F-MTX. These assays tested chloroquine, mefloquine, dihydroartemisinin, and amodiaquine, as well as the antifolates methotrexate, aminopterin, pyrimethamine, trimethoprim and MK571. Competition assays showed that increases in the methotrexate, aminopterin or MK571 concentration caused concentration-dependent decreases in F-MTX accumulation in Dd2 WT parasites but not in Δ*pfmrp1* parasites (Figure 22). These results demonstrate that F-MTX competes for the same transport pathway as methotrexate, aminopterin and MK571. This pathway is mediated by PfMRP1, as evidenced by the lack of competition in the Dd2$^{\Delta pfmrp1}$ line (Figure 22). These assays further suggest that residual F-MTX in the Dd2$^{\Delta pfmrp1}$ strain is probably the result of another transport mechanism or passive transport. The reduction in accumulation was more pronounced for methotrexate and aminopterin compared to MK571. This finding is likely related to MK571 acting as a weak chemical inhibitor of PfMRP1, as supported by the IC_{50} results in the presence of MK571 (Figure 22), compared to PfMRP1 role in direct transport of methotrexate and aminopterin.

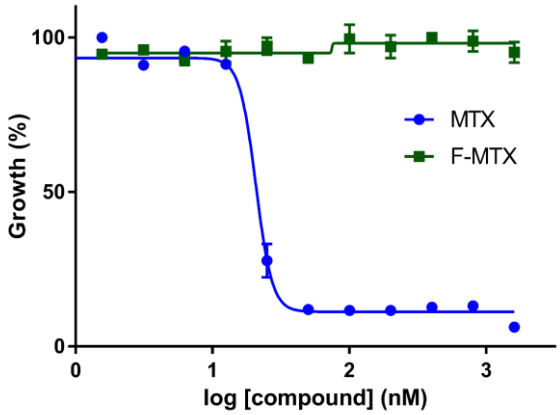


Figure 21. Fluorescein methotrexate (F-MTX) does not impact in parasite growth unlike methotrexate (MTX). *In vitro* growth response curves of the Dd2 WT strain treated with varying concentrations of for MTX and F-MTX. Parasite growth inhibition was achieved with only MTX. Assays were performed in 3 biological replicates. Each point represents mean ± s.e.m. for each concentration.

4.5.6 Folate impacts parasite growth

Parasite growth was monitored to understand the overall impact of Δ*pfmrp1*. Regular levels of folate in human serum are around 6-20 µg/L and folate deficiency in RBCs is considered below 151 µg/L (World

Health Organization, 2015). Standard RPMI medium used for in vitro culture contains 1 mg/L folate, which is 10 times higher than the limit of folate deficiency in RBCs and around 50 times higher than in human serum. Therefore, we modified an assay to limit exogenous folate in order to reduce parasite folate pools and evaluate parasite reliance on external folate sources, and hence PfMRP1 dependency (Ping Wang et al., 1997). Additionally, since Δ*pfmrp1* impacts folate-related transport, growth was evaluated in culture medium lacking an exogenous source of folate. Parasites were cultured for 14 days in medium depleted of folate, after which parasites were tightly synchronized at 0.05% parasitemia and growth was measured in regular medium, medium depleted of folate or medium containing 100 µg/L folate. Both 3D7$^{\Delta_{pfmrp1}}$ and Dd2$^{\Delta_{pfmrp1}}$ parasites were able to achieve high parasitemia like WT control parasites under regular culture conditions, although Dd2$^{\Delta_{pfmrp1}}$ displayed slightly less growth at high parasitemia (>5%) (Figure 23). Without exogenous folate supplementation, the Dd2 strain displayed similar growth in the first cycle but slower growth rate in the second cycle, with around 2% parasitemia compared to 6-8% of regular culture medium. Dd2 parasites were nonetheless able to reach parasitemia of around 8% one cycle later (Figure 23a). In contrast, 3D7 parasites displayed similar growth rates in medium with or without exogenous folate (Figure 23b). There was no difference in growth between WT and Δ*pfmrp1* strains in medium without exogenous folate (Figure 23a and Figure **23**b). To confirm that this was not an artifact due to parasites still having access to folate pools, cultures were diluted multiple times using red blood cells from the same blood donor bag and growth was monitored in medium lacking folate (Wang et al., 1999). Results showed no difference between WT and Δ*pfmrp1* growth for both strains in continuous culture in medium depleted of folate (Figure 23c and Figure **23**d). Overall, no major differences in growth between WT and Δ*pfmrp1* were observable for both 3D7 and Dd2 strains.

The assay with medium containing 100 µg/L folate had results comparable to medium without folate. Addition of a source of exogenous folate partly recovered Dd2 growth compared to medium without folate, with parasites able to grow to 4% on the second cycle compared to 2% in medium without folate (Figure 23a). As with medium lacking folate, there was no impact on the 3D7 growth rate (Figure 23b). These results support the impact of exogenous folate on the growth rate of Dd2, which we observed to be considerably faster than 3D7 under our i*n vitro* conditions. Accordingly, the Dd2 growth rate without folate supplementation resembled the 3D7 growth rate (Figure 23a and Figure **23**b). Although, PfMRP1 might impact parasite growth as observed for Dd2$^{\Delta_{pfmrp1}}$ cultured in standard medium, likely this impact is only observable under very rich *in vitro* growth conditions or as a result of fitness cost at high parasitemia as previously reported (Raj et al., 2009). Furthermore, the effect of folate on the overall Dd2 growth is likely independent of PfMRP1 and mediated by alternative pathways.

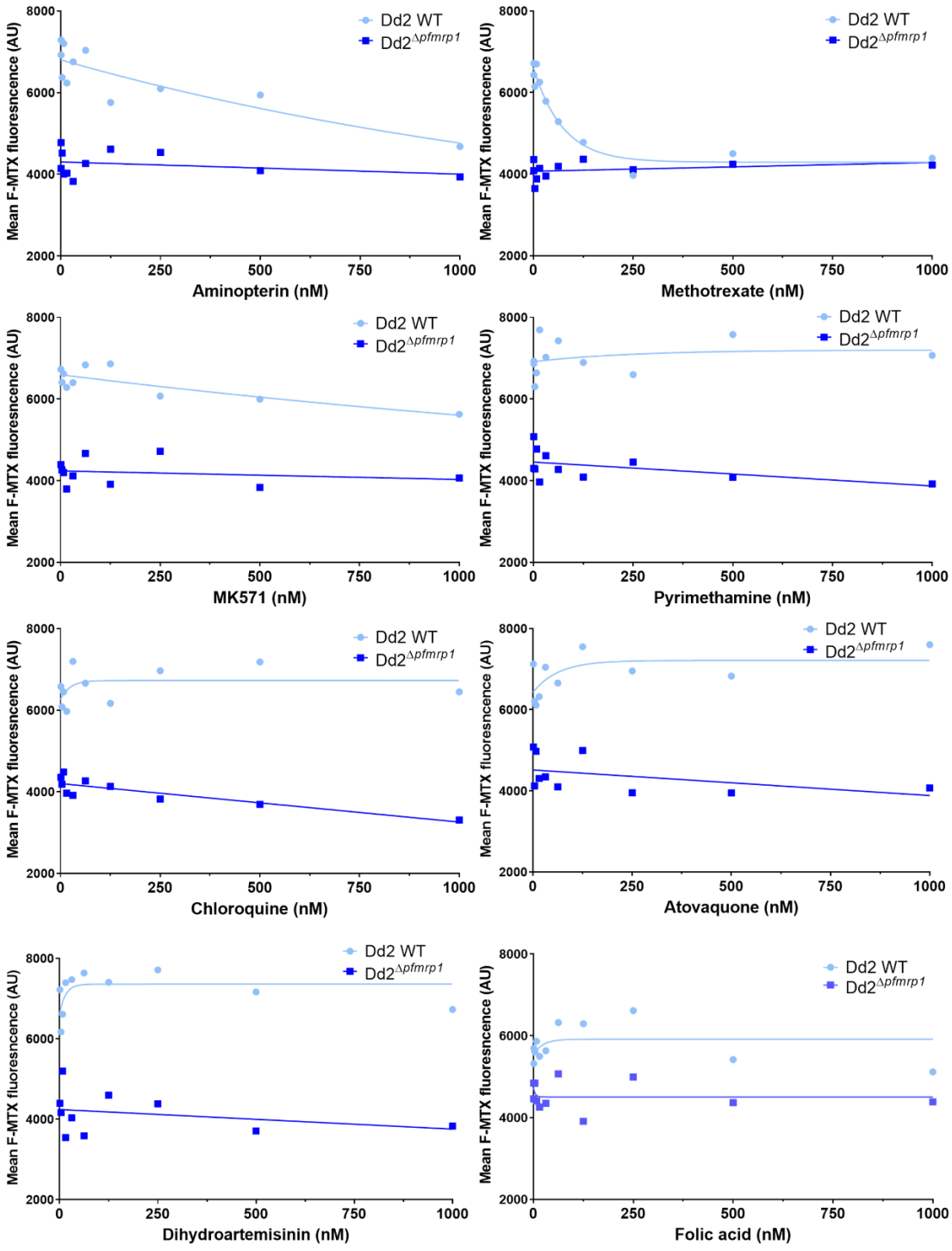


Figure 22. Folate analogs compete with fluorescein methotrexate (F-MTX) for PfMRP1 transport.
Flow cytometry competition assays using a range of compounds concentrations from 0 to 1000 nM with a fixed concentration of 1000 nM F-MTX in Dd2 WT and Dd2$^{\Delta pfmrp1}$ strains. Incubation proceeded for 3 hours prior to detection of F-MTX. Aminopterin, methotrexate and MK571 showed decreasing levels of F-MTX accumulation with increased drug concentrations in the Dd2 WT strain, an effect that was absent in the Dd2$^{\Delta pfmrp1}$ line. No effects were observed for chloroquine, amodiaquine, dihydroartemisinin and folate. Assays were performed in one replicate, except for methotrexate that the phenotype was confirmed by further independent biological replicate. Only one independent replicate is presented due to inherent variations in absolute fluorescence. The second replicate is presented in supplementary figure 7 and presented identical results. Mean values are presented.

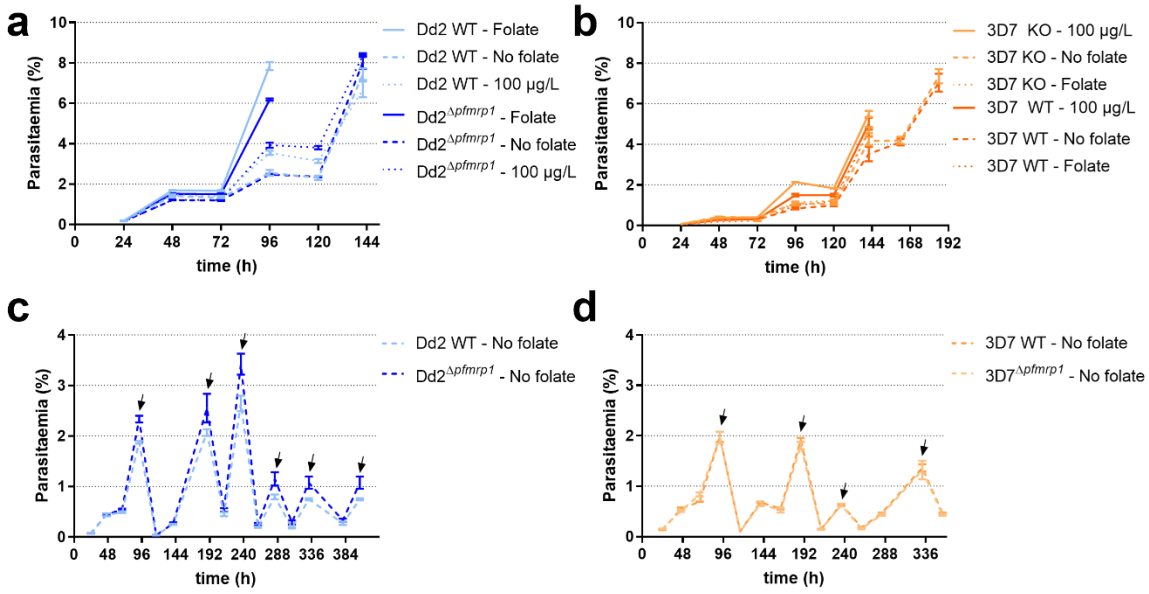


Figure 23. Impact of folate on growth of wild-type (WT) and Δ*pfmrp1* lines. a/b – Parasite growth monitored along time in RPMI-based medium with regular concentration of folate (1 mg/L), 100 µg/L folate, or no folate for Dd2 **(a)** and 3D7 **(b)** *P. falciparum* strains after growth for two weeks under folate starvation. Dd2$^{\Delta pfmrp1}$ showed an impairment of growth compared to Dd2 WT under regular conditions at high parasitemia, an effect that was not detectable in 3D7 parasites. Dd2 propagated more slowly in medium with reduced concentration of folate compared to regular medium, while 3D7 growth was independent of folate. These data need careful interpretation, as the RBCs used for parasite culture naturally contain folate. Mean ± s.e.m. values are presented. Assays were performed in 3 independent biological replicates. **c/d** – Parasite growth was monitored over time in RPMI medium without folic acid supplementation. After parasites grew to near 2% parasitemia, they were diluted multiple times (arrows) to eliminate confounding effects regarding initial parasitemia and folate pools. There was no difference in growth between WT and Δ*pfmrp1* Dd2 **(c)** and 3D7 **(d)** parasites after multiple intraerythrocytic cycles. Mean ± s.e.m. values are presented. Assays were performed with 2 independent biological replicates

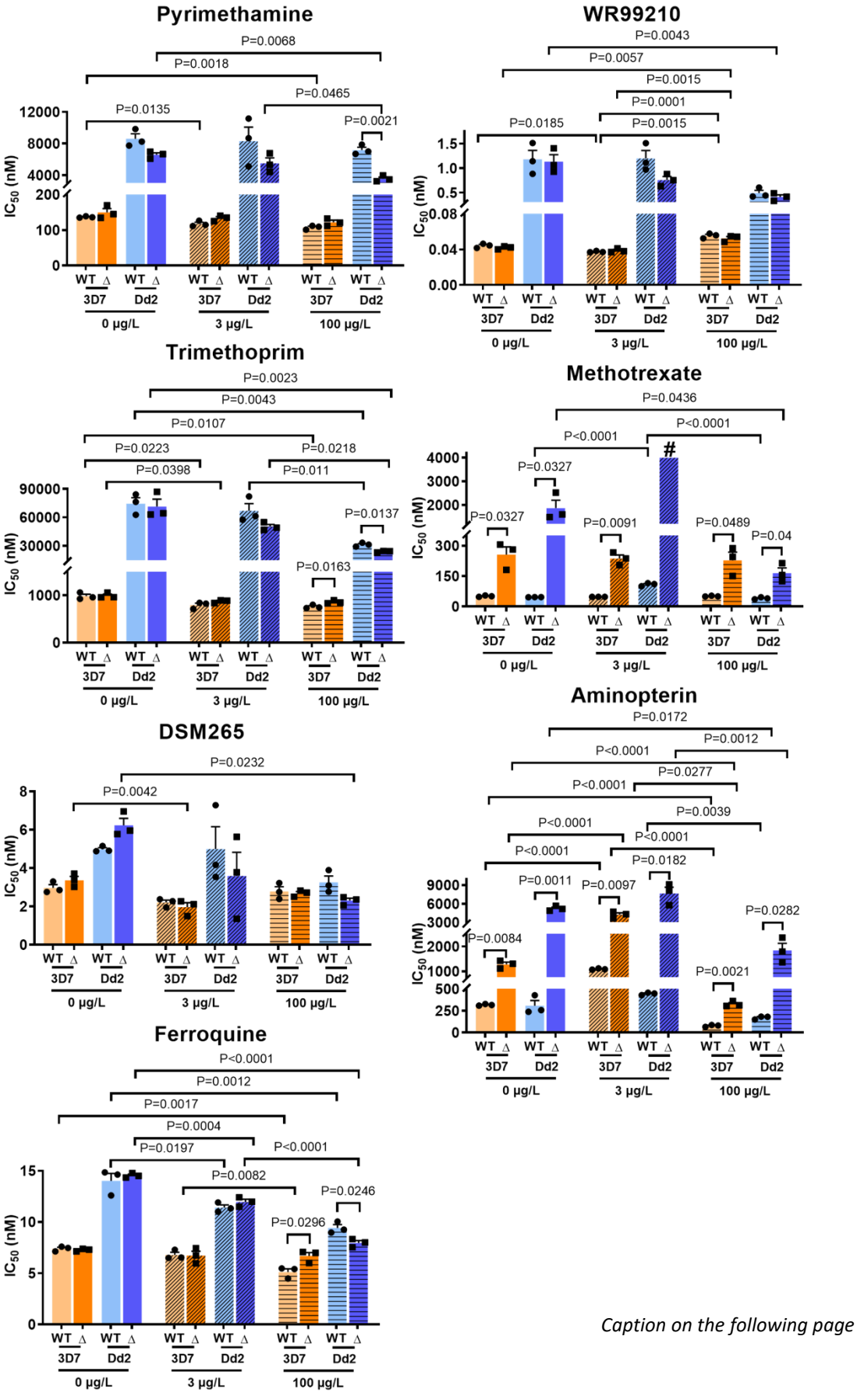
4.5.7 Folic acid impacts the antifolate response

To understand the impact of folate on the antifolate resistance phenotype observed in *Δpfmrp1* parasites, we performed 72-hour drug susceptibility assays under various folate conditions. IC_{50} values were measured for pyrimethamine, trimethoprim, WR99210, aminopterin, methotrexate and the two non-antifolate based compounds ferroquine and DMS265, as controls. Assays were performed in the presence of diminished folate conditions, namely no exogenous folate, and a folate supplementation of 3 µg/L or 100 µg/L folic acid, the last being the folic acid concentration previously tested and that partly recovers Dd2 growth rate. Similarly to growth assays (Figure 24) there was no impact on the overall growth of these strains in the 72-hour period of the assay. Results showed a slight but significant increase in susceptibility of Dd2$^{\Delta pfmrp1}$ relative to WT parasites when assayed against pyrimethamine or trimethoprim, relative to WT parasites, under conditions of 3 µg/L of folate or 100 µg/L of folate (Figure 24). These results suggest that an inefficiency of Dd2$^{\Delta pfmrp1}$ to import folate could render the drugs slightly more effective under limiting exogenous folate conditions, possibly due to lower intracellular dihydrofolate levels,

the natural substrate of PfDHFR. Other folate conditions for pyrimethamine, trimethoprim, and WR99210 did not show significant differences but presented the same trend. An inefficiency to import folate could explain the phenotype of the decreased growth of Dd2$^{\Delta pfmrp1}$ under regular conditions (Figure 23). On 3D7, generally no effect was observed for any of these antifolates, except for a small decrease in susceptibility of 3D7$^{\Delta pfmrp1}$ to trimethoprim under 100 µg/L folic acid.

The resistance phenotype of 3D7$^{\Delta pfmrp1}$ and Dd2$^{\Delta pfmrp1}$ parasites for methotrexate and aminopterin was maintained under all folate conditions (Figure 24). Surprisingly, the susceptibility to aminopterin was drastically reduced under lower folate concentrations, an effect that was even more pronounced for the 3 µg/L folic acid supplementation compared with no exogenous folate (Figure 24). A similar effect was observed for methotrexate, although with a less pronounced difference and evident only in the Dd2$^{\Delta pfmrp1}$ and parental Dd2 lines (Figure 24). This phenotype might be multifactorial, as it seems to be independent of PfMRP1 for aminopterin, as the effect is observable also in WT parasites. A slight increase in IC_{50} was also observed for Dd2 WT at 3 µg/L folic acid for MTX (2-fold, $P<0.0001$), additionally pointing to a PfMRP1-independent effect. These data, with Dd2 drug response being more affected by folate concentration, agree with parasite growth assays, in which Dd2 was generally more impacted by folate concentrations.

Both methotrexate and aminopterin are expected to be competitors of folate for the PfDHFR binding site, and in this case a lower folate concentration was expected to render parasites more susceptible to drug action. However, the opposite was observed where less exogenous folate led to lowered susceptibility to the antifolate analogs methotrexate and aminopterin. This result suggests that this phenotype was likely independent of PfMRP1 and was instead due to another mechanism. Likewise, a similar phenotype was observed for antifolates that are structurally different from folate (pyrimethamine, trimethoprim, WR99210) and even unrelated compounds that are not expected to act on the folate pathway (DSM265 and ferroquine) (Figure 24). Overall, this phenotype was more pronounced in the Dd2 strain in terms of fold variation of drug response, except for aminopterin where similar changes were observed for 3D7 strain.



Caption on the following page

Figure 24. Folic acid impacts parasite drug responses. *In vitro* IC_{50} responses for Δ*pfmrp1* and wild-type (WT) 3D7 and Dd2 *P. falciparum* strains, with varying concentrations of exogenous folate (100 μg/L, 3 μg/L and no exogenous folate). IC_{50} values are presented as mean ± s.e.m. for pyrimethamine, trimethoprim, WR99210, aminopterin, methotrexate, ferroquine and DSM265. All compounds are antifolates, except for ferroquine and DSM265, which are functionally different. #: bar represents a higher IC_{50} value than indicated due to a greatly reduced susceptibility to compound that was beyond the limit of detection in this assay. Assays were performed in 3 independent biological replicates. Statistical evaluations comparing WT and Δ*pfmrp1* for each strain and condition were performed using two-tailed unpaired *t*-tests with Welch's correction. Statistical evaluations comparing same strain between folate conditions were performed using one-way ANOVA with multiple comparisons performed with Tukey's multiple comparisons test.

4.5.8 *Pfmrp1* is phylogenetically unrelated to other eukaryotic ABC transporters

ABC transporters are an ancient family present in a wide variety of species ranging from prokaryotes to eukaryotes. The results suggesting that PfMRP1 can function as an influx ABC transporter prompted us to investigate evolutionary relationships among PfMRP1 and other proteins from the same family and explore potential links between sequence similarity and function. We started by using the PfMRP1 amino acid sequence to BLASTp query the landmark database that includes a taxonomically diverse and non-redundant set of protein sequences. This resulted in 116 protein hits from 11 different species (*Caenorhabditis elegans*, *Arabidopsis thaliana*, *Dictyostelium discoideum*, *Glycine max*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Danio rerio*, *Homo sapiens*, *Schizosaccharomyces pombe*, *Mus musculus* and *Leishmania donovani*). Except for PfMRP2 that shared moderate sequence identity with PfMRP1 (49% identity and 64% query coverage), all hits from other organisms showed low identity (<25%). Phylogenetic analysis using these sequences placed PfMRP1 and PfMRP2 in an isolated branch in the phylogeny (Figure 25). These sequences were inferred to share a node, deep in the phylogeny, only with an ABC transporter from *Leishmania donovani* (XP_003863220.1) but with a low bootstrap support value. In contrast, several other ABC transporters from different species were found to be highly related to each other, branching within well-supported monophyletic clades. This finding suggests that although PfMRP1 harbors transmembrane helices typically found in the ABC transporters that function as exporters, it has a distinct protein sequence and origin. This finding is compatible with the hypothesis that PfMRP1 might have an atypical transport function.

4.5.9 Glutathione-dependent oxidative parasite status

PfMRP1 has been previously associated with glutathione transport (Ballatori et al., 2005; Raj et al., 2009). To interrogate this further, we employed genetically encoded fluorescent probes that are a more accurate method to probe glutathione cytosol redox status compared with chemical analysis of whole-cell extracts (Kasozi et al., 2013; Morgan et al., 2013). We acquired a highly specific and sensitive glutathione biosensor consisting of yeast glutaredoxin-1 (yGrx1) fused to the redox-sensitive variant of green

fluorescent protein (roGFP2) (Kasozi et al., 2013; Morgan et al., 2013), cloned this sensor into the pDC2 expression vector, and successfully transfected episomal plasmid into the Dd2 and Dd2$^{\Delta pfmrp1}$ lines.

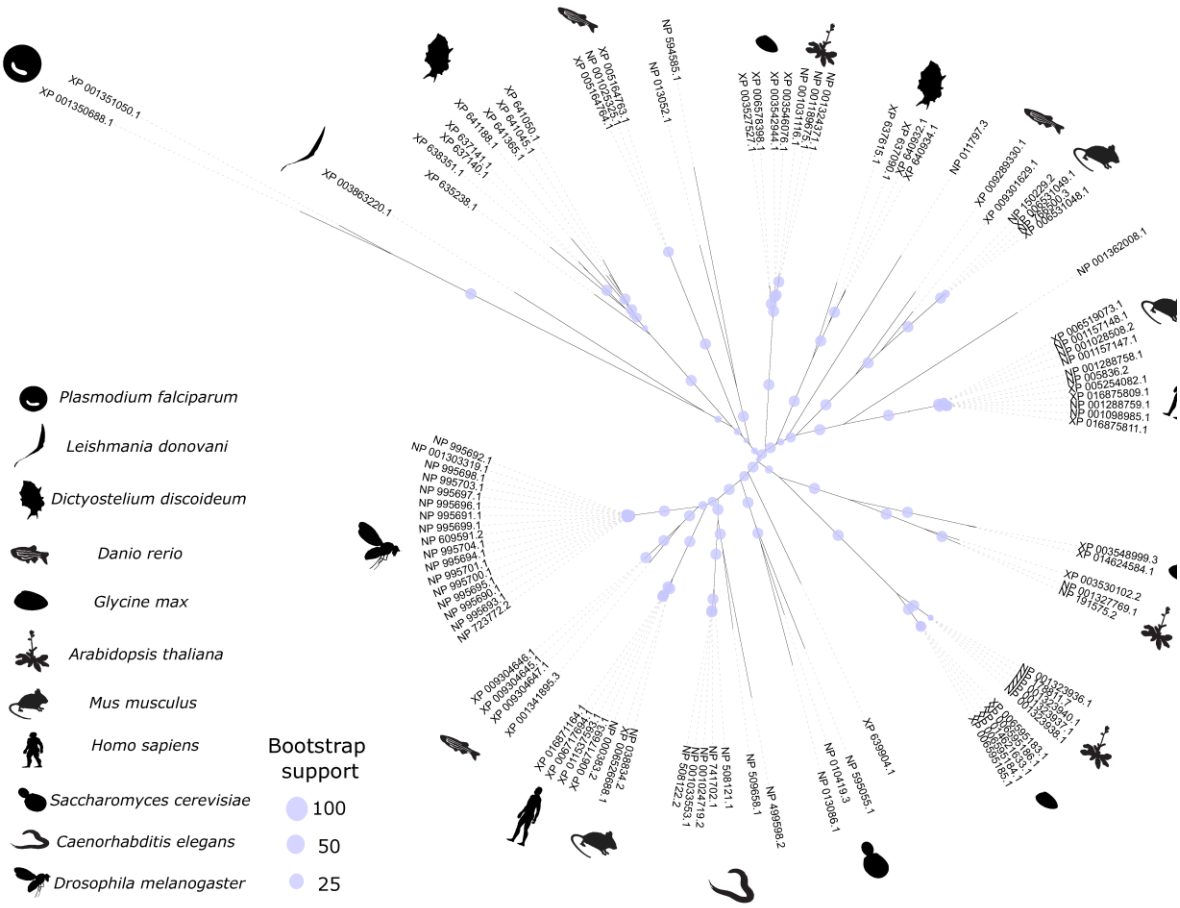


Figure 25. Maximum likelihood phylogenetic representation of *pfmrp1* and other ABC transporters. Sequences were retrieved by a BLASTp query of the Landmark database using the PfMRP1 amino acid sequence. The sequences of ABC transporters spanning a wide taxonomic range (n=117) were aligned with MAFFT and the phylogenetic analysis was performed with IQtree. Tree visualization was done with iTOL using PhyloPic images. Bootstrap support values are indicated in each node with blue circles.

A

cytometer-based assay was performed where the baseline fluorescence was collected for ≈1 minute, after which parasites were submitted to a stimulus and measured for approximately 4 minutes (Figure 24). The collected baselines showed no difference between the Dd2$^{\Delta pfmrp1}$ and parental parasites in their homeostatic redox status (Figure 26b). The oxidative agent H_2O_2 and reducing agent DTT were used to validate the assay and the probe in our system. As expected, H_2O_2 diminished and DTT increased the 488/405 nm fluorescence ratio, an effect that was observed in real time (Figure 26a). Furthermore, the probe detected changes in glutathione disulfide (GSSG) and reduced glutathione (GSH) content (Figure 26b a). Comparing Dd2$^{\Delta pfmrp1}$ and parental parasites, no differences were observed for any of these stimuli. We then subjected parasites to a first stimulus with GSSG or GSH for 1 minute to put the parasites into

a more oxidized or reduced state, respectively (Figure 26a). A second stimulus was then applied to test whether PfMRP1 could impact the return to homeostasis or even cause further stress. A slight effect was observable after GSSG stress, with Dd2 WT being able to recover its reduced status faster after introduction of exogenous GSH; however, this effect was not pronounced (Figure 26b a). Notably, PfMRP1 did not seem to impact any other glutathione redox-dependent phenotype under these conditions (Figure 26a).

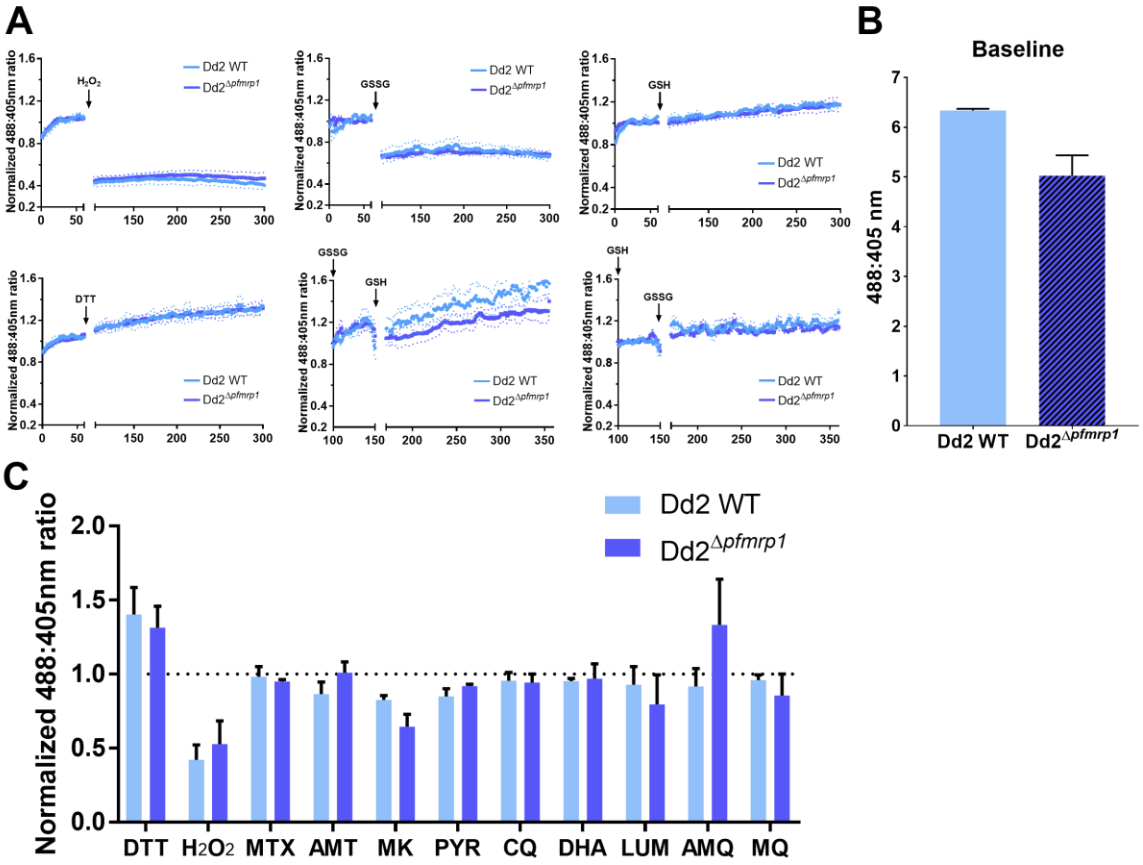


Figure 26. Oxidative stress response of Dd2 wild-type and Δ*pfmrp1* lines. A – Real-time response of Grx1-roGFP probe of Dd2 WT and Dd2$^{\Delta pfmrp1}$ line. After 60 seconds of baseline monitoring, challenges were applied with H_2O_2 as an oxidative stress inducer, DTT as a reducing agent, and oxidized glutathione (GSSG) and reduced glutathione (GSH). Further challenges with the same compounds were induced 60 seconds after first stimuli. Ratio of fluorescence 488:405 was normalized for the mean of initial 60 seconds baseline or after the first stimuli. Assays were run in 3 biological replicates. **B** – Baseline glutathione redox status monitored by pooling the first 60 seconds before stimuli in all testing conditions. **C** – Normalized 488:405 nm ratio of fluorescence after 5 minutes of antimalarial stimuli.

A similar assay was performed using various antimalarials (methotrexate, aminopterin, MK571, pyrimethamine, chloroquine, dihydroartemisinin, lumefantrine, amodiaquine and mefloquine) and fluorescence was collected 5 minutes after the antimalarial stimuli (Figure 26c). Only pyrimethamine and MK571 resulted in a slight oxidative stress, and no impact of PfMRP1 on glutathione redox status was observed for any of these compounds (Figure 26c). This assay constituted a preliminary approach but

indicates no antimalarial or PfMRP1 influence of the short-term impact in the glutathione redox homeostasis. Further assays are necessary to evaluate the impact over larger periods of time.

4.5.10 Discussion

The ABC superfamily is the largest transporter protein family present in virtually all organisms. In eukaryotes, apart from plants, the ABC superfamily has been reported to mainly consist of exporter proteins. This family has been linked to protecting cells against toxic agents, including playing a role in drug resistance phenotypes. PfMRP1 is an ABC transporter whose biological role remains elusive in *P. falciparum*. *pfmrp1* has been suggested to be under natural selective drug pressure but no compelling evidence has shown this transporter to mediate a resistance phenotype (Dahlström et al., 2009a, 2009b; Mu et al., 2003; Ursing et al., 2006). Our study of genetically engineered *P. falciparum* lines with disrupted *pfmrp1* in two different genetic backgrounds, derived from Africa and Asia phylogeographic subpopulations, supports that PfMRP1 does not substantially impact the *in vitro* susceptibility to commonly used antimalarials such as chloroquine, dihydroartemisinin, lumefantrine, and mefloquine (Figure 18). Moreover, this transporter is dispensable for asexual blood stage parasite growth, provided the right *in vitro* conditions.

Interestingly, in agreement with an earlier report (Rijpma et al., 2016b), we found that Δ*pfmrp1* provided markedly increased resistance to the folate analog methotrexate, but not to other structurally unrelated antifolates such as pyrimethamine, WR99210 or trimethoprim (Rijpma et al., 2016b). Additionally, we observed that *Δpfmrp1* also provides resistance to aminopterin, another folate analog, pinpointing this phenotype to be specific for folate-related compounds. Using MK571, a MRP inhibitor (Gekeler et al., 1995), we observed a decrease in methotrexate susceptibility only in the WT strain and not in *Δpfmrp1* parasites, demonstrating this phenotype to be specific for PfMRP1 (Figure 18). The lowered sensitivity to folate analogs observed in the *Δpfmrp1* lines suggests reduced drug accumulation in the parasite cytoplasm, where the target enzyme PfDHFR is located. Once in the cytoplasm, the antifolates compete with folate for binding to the PfDHFR active site, thereby inhibiting the folate pathway by blocking reduction of dihydrofolic acid to tetrahydrofolic acid, a key cofactor used in carbon transfer reactions. Using labelled fluorescein F-MTX we showed through microscopy and flow cytometry, that F-MTX accumulates in the cytoplasm of WT parasites in contrast to *Δpfmrp1* parasites, which only showed residual F-MTX accumulation (Figure 19). This suggests that in WT parasites, PfMRP1 is responsible for transporting F-MTX and likely other folate analogs into the parasite cytosol.

MRP proteins are folate transporters in diverse organisms, and in humans they are associated with transport of the anticancer agent methotrexate (Chen et al., 2002; Hooijberg et al., 1999; Zeng et al., 2001). In *P. falciparum*, PfMRP1 has been associated with folate transport *in vitro* and *in vivo* (Dahlström et al., 2009b; Rijpma et al., 2016b). Previous *pfmrp1* knockout experiments suggested apparently contradictory results of PfMRP1-mediated export of folate from the parasite cytoplasm, while concomitantly Δ*pfmrp1* parasites displayed reduced susceptibility to the antifolate methotrexate (Rijpma et al., 2016b). Due to the proposed exporter function of PfMRP1, this resistance phenotype was hypothesized to result from increased folate accumulation within the Δ*pfmrp1* parasites that could compete with methotrexate due to lack of export. However, our data, including drug assays with varying concentrations of folate, demonstrated that folate supplementation within the culture medium increases susceptibility to a panel of antimalarials. This included the folate analogs methotrexate and aminopterin, making it unlikely that folate competition is the main driver of the observed resistance phenotype (Figure 24). Another proposed hypothesis based on structural similarity is that PfMRP1 directly transports methotrexate (Rijpma et al., 2016b). Under the new proposed importer function, a decrease in cytoplasmic MTX concentration in *Δpfmrp1* parasites due to the absence of PfMRP1-mediated transport would explain reduced susceptibility. This hypothesis can further explain the reduction in folate concentration on the system of infected red blood cells previously observed (Rijpma et al., 2016b). Our data using labelled methotrexate supports the direct methotrexate transport hypothesis, implicating PfMRP1 as an importer protein.

Earlier studies suggested that the main folate uptake pathway in *P. falciparum* is dependent on the proton gradient maintained by proton symporters at the parasite plasma membrane. The ATPase inhibitor concanamycin A greatly reduced folate uptake, linking folate transport with ATP-powered proton transport (Salcedo-Sora et al., 2011; Wang et al., 2007). Considering our results, the mechanism of concanamycin A-mediated inhibition of folate uptake is also compatible with blockage of the ATP-dependent PfMRP1 transporter potentially involved in parasite folate import. Moreover, methotrexate has been shown to reduce folate import, suggesting a similar pathway of uptake (Wang et al., 2007). Accordingly, our competition assays indicate that methotrexate and aminopterin are transported by PfMRP1. Nevertheless, although folate, methotrexate and aminopterin share very similar structures and are likely to share the same transport pathway, we could not directly demonstrate that folate competes with F-MTX for PfMRP1-mediated transport (Figure 22). *P. falciparum* is cultured in red blood cells that contain folate from the host, thereby making the folate competition assay challenging to interpret since the folate import system

could be saturated even without exogenous supplementation of folate. Furthermore, there was no major impact on parasite growth in folate-depleted medium.

Our growth assays suggest that 3D7 is less dependent on an external folate source compared to Dd2. Since, the lack of folate was shown to reduce the Dd2 parasite growth rate, which in regular conditions is comparably higher to 3D7 (Figure 23). This could possibly be explained by the presence of *para*-aminobenzoic acid in the medium, which can be used for *de novo* folate synthesis and bypass the lack of folate import. *P. falciparum* synthesizes folate *de novo*, and as such this pathway might be more functional in 3D7. The higher Dd2 growth rates, with folate supplementation, indicates additional mechanisms to import or use exogenous folate independently of PfMRP1, as Δ*pfmrp1* lines were not differentially impacted by the lack of exogenous folate (Figure 23). The Δ*pfmrp1* impact in Dd2 that causes slightly slower growth than WT, on regular culture conditions, might be related to the fact that this strain is more susceptible to the absence of folate. Consequently, less folate import due to Δ*pfmrp1* might render the parasite less fit. Additionally, other folate transporters have been described, such as *P. falciparum* folate transporter 1 and 2 (PfFT1 and PfFT2) and might account for strain variability. Although, these transporters were hypothesized to be able to only transport pABA at physiological concentrations (Salcedo-Sora et al., 2011).

The PfMRP1 import properties herein observed are not commonly observed in many eukaryotic organisms. Recently, a novel structure of an *E. coli* ABC importer, YbtPQ, was described that has an ABC type IV exporter structure (Wang et al., 2020). This transporter shows significant sequence similarity to Sav1866, a homolog of multidrug ABC transporters including MDR1 (Wang et al., 2020). Different ABC classifications are currently in use, including newer groupings based on the more translatable TMD folds and topologies resulting from advances in cryo-electron microscopy and X-ray crystallography (Thomas et al., 2020). However, even structural reclassification does not enable predictions of transporter functionality. For example, type IV ABCs share a single structural scaffold yet have great functional diversity consisting of importers, exporters, lipid floppases, ion channels and regulators. PfMRP1 is predicted to be in the same structural class as the exporters Sav1866 and MDR1, but also importers such as YbtPQ and ABC Subfamily D Member 4 (ABCD4) (Dahlström et al., 2009a; Ward et al., 2007). Generally, this scaffold, which includes PfMRP1, allows a wide range of functions across multiple organisms. Obtaining the crystal structure of PfMRP1 would help to explore its transporter properties mechanistically.

Horizontal transfer has played a role in the evolution of apicomplexan and evidence of prokaryote horizontal transfer has been found for the peroxiredoxins, a family of thiol-dependent hydroperoxidases

that function as detoxifying enzymes, redox sensors and chaperones (Djuika et al., 2015; Mu et al., 2002). Conceivably, the PfMRP1 transporter could also have resulted from horizontal transfer (Huang et al., 2004b, 2004a; Striepen et al., 2004). Based on our phylogenetic analysis, p*fmrp1* and *pfmrp2* have identifiable ABC sequence motifs but have low overall sequence identity with the ABCs of other eukaryotic organisms and thus a distinct origin. Moreover, *P. falciparum,* as an intracellular parasite, encodes far fewer enzymes and transporters compared to other free-living eukaryotic microbes, which might lead the available transporters to have less substrate specificity and wider functionality (Gardner et al., 2002).

MRPs are known to transport glutathione and conjugates (Jedlitschky et al., 1996; Rappa et al., 1997). MRPs have been shown as low affinity GSH transporters, while others do not transport GSH but can transport GSH conjugates (Ballatori et al., 2005). Also, GSH might be required for transport of other substrates or even stimulating the transport system, while not transported across the membrane (Ballatori et al., 2005). In *P. falciparum*, PfMRP1 was shown to potentially efflux GSH, as radioactive GSH accumulated in PfMRP1 parasites. Furthermore, GSH was shown to reduce chloroquine and quinine accumulation in W2 and 3D7 parasites and according decrease in susceptibility. However, such effects were also observed in W2 *pfmrp1* disrupted parasites, which suggests a mechanism independent of PfMRP1 (Raj et al., 2009). Our studies using the glutathione biosensor yGRX1-roGFP2 only show effects in glutathione homeostasis for pyrimethamine and MK571, but no differences in behavior were observed between WT and Δ*pfmrp1*, further suggesting these effects to be PfMRP1 independent. Furthermore, using diverse stimuli with H_2O_2, DTT, GSH and GSSG there was only an observable difference between WT and Δ*pfmrp1* in response to GSH after GSSG oxidative stress in returning to homeostasis, which occurred faster in Dd2 WT. This difference was not robust but hints at a possible impact of PfMRP1 in modulating GSH homeostasis (Figure 26). However, more robust assays are needed to determine the full extent of PfMRP1 involvement in modulating parasite glutathione redox status of the parasite.

Overall, our data provide evidence that PfMRP1 functions as an ABC importer protein that can mediate import of folate analogs into the parasite cytoplasm. As one of the few ABC importers identified to date in the plasma membrane of a eukaryotic organism, this study highlights the importance of functional studies to elucidate the molecular basis of importer function in *P. falciparum*.

5. CONCLUSIONS

Plasmodium falciparum is a very complex organism in which very high replication rates allow fast adaption to the environment, which lead to very complex resistance mechanisms. The past view of a direct relationship between a specific mutation to a resistance phenotype, as observed for chloroquine, is now outdated. Drug resistance phenotypes should now be explored and analyzed in a view of multifactorial causes (Figure 27).

The work on this thesis explores critical alterations on parasite genome and transcriptome to unravel factors that contribute to resistance to ACT therapies. Study 1 and Study 2 explored in Africa, the continent most ravaged by malaria, genetic and transcriptomic factors that could be predicative of development of resistance to artemisinin and piperaquine. Even though reports of ACT resistance in Africa are still sparse compared to Southeast Asia, there is a fundamental importance to keep monitoring the emergence of resistance factors before these become fixated and drug resistance becomes harder to reverse.

Study 1 explored the genetic background of parasites circulating in Mali from a DHA-PPQ trial and determined that *plasmepsin2* copy number is present in 10% of recurrent infections. This increased copy number is frequently associated in Southeast Asia with therapy failure. Despite not identifying any trend with earlier recurrence for this subset, these findings highlight the importance to further evaluate parasites in the region for the presence of mutations associated with DHA-PPQ therapy failure and moreover evaluate the susceptibility of field isolates to piperaquine before large-scale implementation of DHA-PPQ in the area.

Despite partner drug resistance being the most predicative marker of ACT failure, the artemisinin component of the therapy is also under significant pressure, which further increases pressure on already subpar partner drugs. As such, Study 2 evaluates in Tanzania the transcriptional response of *pfk13* in patients treated with AM-LF. In this study, parasite clearance data had a negative correlation with *pfk13* expression, suggesting that *pfk13* reduced expression could lead to longer parasite clearance times. PfK13 has been demonstrated to be the most predicative gene related to artemisinin resistance. In accordance with this study, recently PfK13 decreased protein levels associated with SNPs were shown to result in less susceptibility to artemisinin (Figure 27). Importantly, our study highlights the importance to monitor not only the genomic background of field circulating parasites, but also to monitor *pfk13* expression levels. Importantly, this study opens new opportunity to explore mechanisms behind *pfk13* expression that could impact artemisinin resistance. Epigenetic changes or alterations on regulatory

regions of *pfk13* could also be predicative of artemisinin decreased susceptibility. Further elucidation of these mechanisms, as well as continuous monitoring of field parasites, are key topics for future advances.

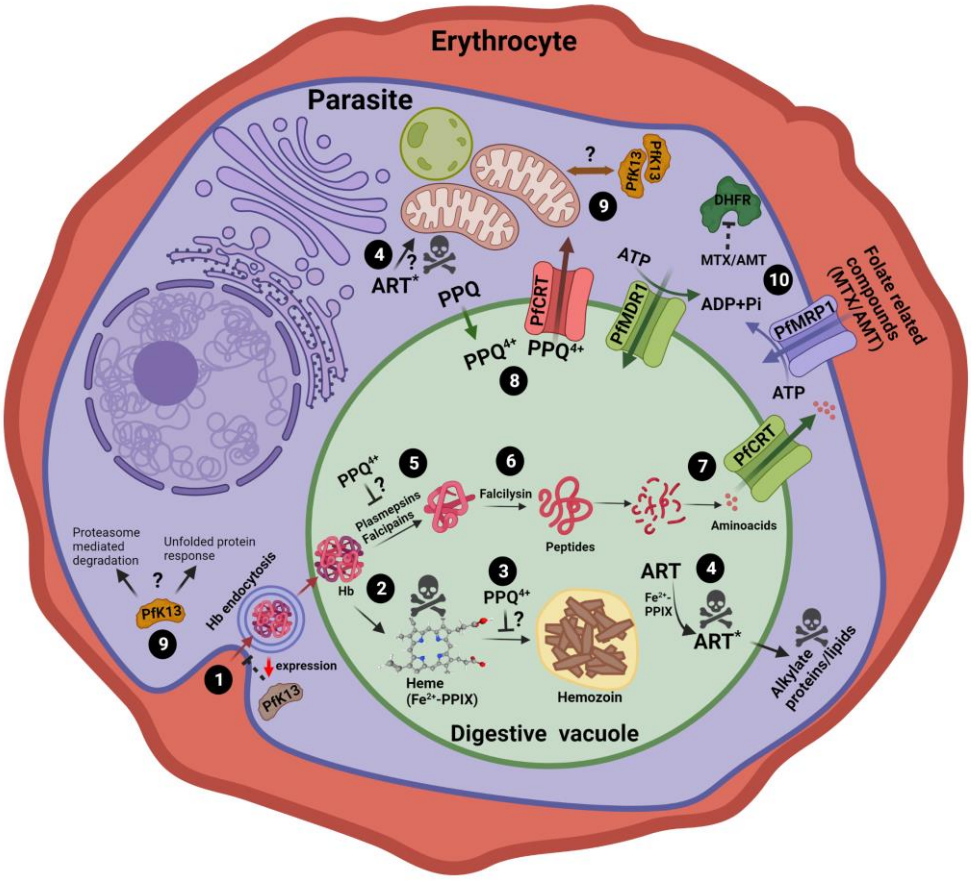
In vitro studies are fundamental to complement the field studies in order to understand the key factors and the mechanisms leading to drug resistance. Study 3 focused on exploring the factors that lead to resistance to piperaquine having particular focus in understanding the *plasmepsins* amplifications impact in resistance, which Study 1 identified as being present in Africa (Figure 27). This study corroborated former observations that *plasmepsin* copy number amplification leads to an intriguing phenotype of resistance to high concentrations of piperaquine in a bimodal effect. Furthermore, PfCRT and PfMDR1 were identified as being able to augment the bimodal phenotype (Figure 27). Understanding how all these factors interact to promote resistance is of most interest in the future. This study highlights the interplay of various factors in generating resistance and gives insights into the possible way that resistance emerged previously in Southeast Asia.

This knowledge is fundamental to now monitor African parasites in order to detect earlier signs of resistance and adapt local policies.

As observed in Study 3 and in the history of research on drug resistance, malaria transporter proteins are fundamental in resistance phenotypes as either main drivers of resistance or even as secondary players augmenting drug resistance phenotypes. There are transporters with an established role in antimalarial resistance such as PfMDR1 and PfCRT. The PfMRP1 had early studies pointing to an impact in antimalarial resistance, however, the role of this transporter has been unclear with even apparent contradictory reports. Study 4 explored the influence of PfMRP1 in drug resistance by creating *in vitro* strains lacking this transporter (Figure 27). This study demonstrated PfMRP1 to not have an apparent impact on the response to classic antimalarials. This study does not exclude the impact of PfMRP1 in modulating drug response in certain resistant background but indicates that PfMRP1 is not a main driver for resistance. Nevertheless, resistance to folate analogs was evident, corroborating a previous study that demonstrated knockout of PfMRP1 led to methotrexate resistance. Study 4 further expanded on that, pinpointing to be a specific effect to folate analogs. The cause of this phenotype was deciphered as a decrease in accumulation of these compounds in the parasite. Interestingly, this evidence points for PfMRP1 to act as an importer protein able to transport folate related compounds. This function is highly unusual for ABC transporters in eukaryotic organisms. The PfMRP1 was postulated as an exporter based on phylogenic comparison with other organisms and predicted 2D structure. However, even 3D structure seems not sufficient to predict functionality with transporters of similar 3D structures having different functions (Kavishe et al., 2009; Klokouzas et al., 2004; Martin et al., 2005; Veiga et al., 2011). This

study highlights a need to carefully interpret data regarding previously unidentified transporters and the importance to explore protein function to then infer about mechanisms of drug resistance. Moreover, Study 4 identified PfMRP1 as a potential folate transporter, in accordance with earlier reports of folate transport being linked with active transport (Salcedo-Sora et al., 2011; Wang et al., 2007). Despite that, folate transport might not be the main physiological function of PfMRP1. Putatively PfMRP1 could be just a redundant transporter transporting folate in lower rates compared to other primary transporters. In the future, it is of interest to explore other metabolic alterations within the cell to PfMRP1 even though this might prove challenging due to compensatory mechanisms, even potentially by the close homolog PfMRP2. Future studies could take advantage to disrupt both transporters at the same time.

Overall, the work on this dissertation provides new advances into drug resistance phenotypes, particularly to ACTs giving new knowledge about artemisinin tolerance and piperaquine partner drug resistance. Furthermore, unravels PfMRP1 as having potential import functions able to influence folate related compounds transport.



Caption on the following page

Figure 27. Graphical scheme of mechanisms explored and unraveled in the thesis. (1) - Parasite uptakes hemoglobin (Hb) via endocytosis dependent on PfK13 and associated proteins. Decreased PfK13 expression, resultant from mutations or gene expression, can lead to reduced Hb uptake. **(2)** - Hb is degraded in the digestive vacuole, releasing toxic free heme by-product. **(3)** - Parasite catalyzes bio polymerization of toxic heme into hemozoin inert crystals. One of the possible mechanisms of action of piperaquine (PPQ) is inhibition of this process. **(4)** - Artemisinin (ART) class of drugs are activated via Fe^{2+}-PPIX dependent cleavage of endoperoxide bridge, this process can generate oxygen reactive species. Activated ART (ART*) can lead to cell death by alkylating proteins, lipids and heme. Moreover, ART* might influence mitochondria potential. Reduced Hb uptake, via reduced PfK13 expression **(1)**, consequently leads to reduced ART* which in turn makes the parasite less susceptible to the drug. This mechanism of resistance comes at a fitness cost for the parasite, as parasite breakdown Hb to obtain nutrients for survival. **(5)** - Plasmepsins are one of the first enzymes in the Hb digestion process and a potential target for PPQ action. Overexpression of *plasmepsins* led to bimodal resistance to high concentrations of PPQ, a process still not completely understood mechanistically. Moreover, overexpression of *plasmepsins* can help parasites to metabolize faster Hb for higher efficiency to obtain amino acids, potentially counteracting PfK13 reduced expression. **(6)** - Falcilysin participate in further degradation of Hb into small peptides. **(7)** - Small peptides are further broken down into amino acids, which are transported to cytosol. PfCRT has been recently identified as transporter of small peptides and amino acids. **(8)** - Additionally, mutated PfCRT forms can transport antimalarials, such as PPQ, reducing concentration of compound in digestive vacuole. Moreover, PfMDR1 can also transport antimalarials differentially dependent on specific mutations. By interfering on the PfCRT and PfMDR1 function by using chemical blockers, PPQ resistance phenotype was augmented. This phenotype is unlikely to be due to direct transport, since both import and export transport displayed similar phenotype. While the mechanism is uncertain for now, the altered subtract transport and homeostasis in this organelle renders the organelle more sensible to PPQ action. **(9)** - PfK13 mutations, due to disruption of protein function, have been suggested to also lead to resistance by augmented proteasome mediated degradation, unfolded protein response and affecting mitochondrial physiology and maintain potential during drug-induced ring stage quiescence. PfK13 reduced expression could act similarly and can affect artemisinin response. **(10)** - PfMRP1 is an active transporter that Study 4 indicates to be able to import folate related compounds, including the drugs methotrexate (MTX) and aminopterin (AMT). MTX and AMT act on dihydrofolate reductase (DHFR) blocking folate biosynthesis pathway, and disruption of PfMRP1 leads to parasite resistance to these compounds by blocking access to cytosol.

6. REFERENCES

Abu Bakar, N., Klonis, N., Hanssen, E., Chan, C., Tilley, L., 2010. Digestive-vacuole genesis and endocytic processes in the early intraerythrocytic stages of *Plasmodium falciparum*. J. Cell Sci. 123, 441–450. https://doi.org/10.1242/jcs.061499

Achan, J., Talisuna, A.O., Erhart, A., Yeka, A., Tibenderana, J.K., Baliraine, F.N., Rosenthal, P.J., D'Alessandro, U., 2011. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. Malar. J. 10, 144. https://doi.org/10.1186/1475-2875-10-144

Adam, I., Ibrahim, Y., Gasim, G.I., 2018. Efficacy and safety of artemisinin-based combination therapy for uncomplicated *Plasmodium falciparum* malaria in Sudan: a systematic review and meta-analysis. Malar. J. 17, 110. https://doi.org/10.1186/s12936-018-2265-x

Adjalley, S.H., Lee, M.C.S., Fidock, D.A., 2010. A method for rapid genetic integration into *Plasmodium falciparum* utilizing mycobacteriophage Bxb1 integrase. Methods Mol. Biol. 634, 87–100. https://doi.org/10.1007/978-1-60761-652-8_6

Adjuik, M., Babiker, A., Garner, P., Olliaro, P., Taylor, W., White, N., International Artemisinin Study Group, 2004. Artesunate combinations for treatment of malaria: meta-analysis. Lancet 363, 9–17. https://doi.org/10.1016/s0140-6736(03)15162-8

Aikawa, M., Hepler, P.K., Huff, C.G., Sprinz, H., 1966. The feeding mechanism of avian malarial parasites. J. Cell Biol. 28, 355–373. https://doi.org/10.1083/jcb.28.2.355

Alonso, A., Marsal, S., Tortosa, R., Canela-Xandri, O., Julià, A., 2013. GStream: Improving SNP and CNV coverage on genome-wide association studies. PLoS One 8, e68822. https://doi.org/10.1371/journal.pone.0068822

Aly, A.S.I., Vaughan, A.M., Kappe, S.H.I., 2009. Malaria parasite development in the mosquito and infection of the mammalian host. Annu. Rev. Microbiol. 63, 195–221. https://doi.org/10.1146/annurev.micro.091208.073403

Amaratunga, C., Lim, P., Suon, S., Sreng, S., Mao, S., Sopha, C., Sam, B., Dek, D., Try, V., Amato, R., Blessborn, D., Song, L., Tullo, G.S., Fay, M.P., Anderson, J.M., Tarning, J., Fairhurst, R.M., 2016. Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. Lancet Infect. Dis. 16, 357–365. https://doi.org/10.1016/S1473-3099(15)00487-9

Amaratunga, C., Neal, A.T., Fairhurst, R.M., 2014a. Flow cytometry-based analysis of artemisinin-resistant *Plasmodium falciparum* in the ring-stage survival assay. Antimicrob. Agents Chemother. 58, 4938–4940. https://doi.org/10.1128/AAC.02902-14

Amaratunga, C., Sreng, S., Suon, S., Phelps, E.S., Stepniewska, K., Lim, P., Zhou, C., Mao, S., Anderson, J.M., Lindegardh, N., Jiang, H., Song, J., Su, X., White, N.J., Dondorp, A.M., Anderson, T.J.C., Fay, M.P., Mu, J., Duong, S., Fairhurst, R.M., 2012. Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. Lancet Infect. Dis. 12, 851–858. https://doi.org/10.1016/S1473-3099(12)70181-0

Amaratunga, C., Witkowski, B., Khim, N., Menard, D., Fairhurst, R.M., 2014b. Artemisinin resistance in *Plasmodium falciparum*. Lancet Infect. Dis. 14, 449–450. https://doi.org/10.1016/S1473-3099(14)70777-7

Amato, R., Lim, P., Miotto, O., Amaratunga, C., Dek, D., Pearson, R.D., Almagro-Garcia, J., Neal, A.T., Sreng, S., Suon, S., Drury, E., Jyothi, D., Stalker, J., Kwiatkowski, D.P., Fairhurst, R.M., 2017. Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. Lancet Infect. Dis. 17, 164–173. https://doi.org/10.1016/S1473-3099(16)30409-1

Amato, R., Pearson, R.D., Almagro-Garcia, J., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Drury, E., Stalker, J., Miotto, O., Fairhurst, R.M., Kwiatkowski, D.P., 2018. Origins of the current outbreak of multidrug-resistant malaria in southeast Asia: a retrospective genetic study. Lancet Infect. Dis. 18, 337–345. https://doi.org/10.1016/S1473-3099(18)30068-9

Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J.-F., Prévost, M.-C., Ishino, T., Yuda, M., Ménard, R., 2008. Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. Cell Host Microbe 3, 88–96. https://doi.org/10.1016/j.chom.2007.12.007

Amukoye, E., Winstanley, P.A., Watkins, W.M., Snow, R.W., Hatcher, J., Mosobo, M., Ngumbao, E., Lowe, B., Ton, M., Minyiri, G., Marsh, K., 1997. Chlorproguanil-dapsone: effective treatment for uncomplicated falciparum malaria. Antimicrob. Agents Chemother. 41, 2261–2264. https://doi.org/10.1128/AAC.41.10.2261

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq–a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169. https://doi.org/10.1093/bioinformatics/btu638

Anderson, T.J.C., Nair, S., McDew-White, M., Cheeseman, I.H., Nkhoma, S., Bilgic, F., McGready, R., Ashley, E., Pyae Phyo, A., White, N.J., Nosten, F., 2017. Population parameters underlying an ongoing soft sweep in Southeast Asian malaria parasites. Mol. Biol. Evol. 34, 131–144. https://doi.org/10.1093/molbev/msw228

Ansbro, M.R., Jacob, C.G., Amato, R., Kekre, M., Amaratunga, C., Sreng, S., Suon, S., Miotto, O., Fairhurst, R.M., Wellems, T.E., Kwiatkowski, D.P., 2020. Development of copy number assays for detection and surveillance of piperaquine resistance associated *plasmepsin 2/3* copy number variation in *Plasmodium falciparum*. Malar. J. 19, 181. https://doi.org/10.1186/s12936-020-03249-x

Antoine, T., Fisher, N., Amewu, R., O'Neill, P.M., Ward, S.A., Biagini, G.A., 2014. Rapid kill of malaria parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent depolarization of the membrane potential. J. Antimicrob. Chemother. 69, 1005–1016. https://doi.org/10.1093/jac/dkt486

Antony, H.A., Parija, S.C., 2016. Antimalarial drug resistance: An overview. Trop. Parasitol. 6, 30–41. https://doi.org/10.4103/2229-5070.175081

Antony, H.A., Pathak, V., Parija, S.C., Ghosh, K., Bhattacherjee, A., 2016. Transcriptomic analysis of chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*: toward malaria diagnostics and therapeutics for global health. OMICS 20, 424–432. https://doi.org/10.1089/omi.2016.0058

Archibald, H.M., 1951. Preliminary field trials on a new schizonticide. Br. Med. J. 2, 821–823. https://doi.org/10.1136/bmj.2.4735.821

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C.M., Bout, D.M., Ménard, S., Rogers, W.O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Le Bras, J., Berry, A., Barale, J.C., Fairhurst, R.M., Benoit-Vical, F., Mercereau-Puijalon, O., Ménard, D., 2014. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature 505, 50–55. https://doi.org/10.1038/nature12876

Arnold, F.M., Weber, M.S., Gonda, I., Gallenito, M.J., Adenau, S., Egloff, P., Zimmermann, I., Hutter, C.A.J., Hürlimann, L.M., Peters, E.E., Piel, J., Meloni, G., Medalia, O., Seeger, M.A., 2020. The ABC exporter IrtAB imports and reduces mycobacterial siderophores. Nature 580, 413–417. https://doi.org/10.1038/s41586-020-2136-9

Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J.M., Mao, S., Sam, B., Sopha, C., Chuor, C.M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T.T.,

Thuy-Nhien, N.T., Thanh, N.V., Phu, N.H., Htut, Y., Han, K.-T., Aye, K.H., Mokuolu, O.A., Olaosebikan, R.R., Folaranmi, O.O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P.N., Onyamboko, M.A., Fanello, C.I., Tshefu, A.K., Mishra, N., Valecha, N., Phyo, A.P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M.A., Ghose, A., Hossain, M.A., Samad, R., Rahman, M.R., Hasan, M.M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D.P., Bozdech, Z., Jeeyapant, A., Cheah, P.Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S.J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W.J., Yeung, S., Woodrow, C.J., Flegg, J.A., Das, D., Smith, J., Venkatesan, M., Plowe, C.V., Stepniewska, K., Guerin, P.J., Dondorp, A.M., Day, N.P., White, N.J., Tracking Resistance to Artemisinin Collaboration (TRAC), 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N. Engl. J. Med. 371, 411–423. https://doi.org/10.1056/NEJMoa1314981

Ashley, E.A., White, N.J., 2005. Artemisinin-based combinations. Curr. Opin. Infect. Dis. 18, 531–536. https://doi.org/10.1097/01.qco.0000186848.46417.6c

Ashton, M., Hai, T.N., Sy, N.D., Huong, D.X., Van Huong, N., Niêu, N.T., Công, L.D., 1998. Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. Drug. Metab. Dispos. 26, 25–27.

Asimus, S., Elsherbiny, D., Hai, T.N., Jansson, B., Huong, N.V., Petzold, M.G., Simonsson, U.S.H., Ashton, M., 2007. Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects. Fundam. Clin. Pharmacol. 21, 307–316. https://doi.org/10.1111/j.1472-8206.2007.00471.x

Atamna, H., Ginsburg, H., 1997. The malaria parasite supplies glutathione to its host cell - Investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*. Eur. J. Biochem. 250, 670–679. https://doi.org/10.1111/j.1432-1033.1997.00670.x

Aunin, E., Böhme, U., Sanderson, T., Simons, N.D., Goldberg, T.L., Ting, N., Chapman, C.A., Newbold, C.I., Berriman, M., Reid, A.J., 2020. Genomic and transcriptomic evidence for descent from *Plasmodium* and loss of blood schizogony in Hepatocystis parasites from naturally infected red colobus monkeys. PLoS Pathog. 16, e1008717. https://doi.org/10.1371/journal.ppat.1008717

Baca, A.M., Sirawaraporn, R., Turley, S., Sirawaraporn, W., Hol, W.G., 2000. Crystal structure of Mycobacterium tuberculosis 7,8-dihydropteroate synthase in complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. J. Mol. Biol. 302, 1193–1212. https://doi.org/10.1006/jmbi.2000.4094

Bakouh, N., Bellanca, S., Nyboer, B., Moliner Cubel, S., Karim, Z., Sanchez, C.P., Stein, W.D., Planelles, G., Lanzer, M., 2017. Iron is a substrate of the *Plasmodium falciparum* chloroquine resistance transporter PfCRT in *Xenopus oocytes*. J. Biol. Chem. 292, 16109–16121. https://doi.org/10.1074/jbc.M117.805200

Balabaskaran-Nina, P., Desai, S.A., 2018. Diverse target gene modifications in *Plasmodium falciparum* using Bxb1 integrase and an intronic *attB*. Parasites Vectors 11. https://doi.org/10.1186/s13071-018-3129-5

Balikagala, B., Fukuda, N., Ikeda, M., Katuro, O.T., Tachibana, S.-I., Yamauchi, M., Opio, W., Emoto, S., Anywar, D.A., Kimura, E., Palacpac, N.M.Q., Odongo-Aginya, E.I., Ogwang, M., Horii, T., Mita, T., 2021. Evidence of Artemisinin-Resistant Malaria in Africa. N. Engl. J. Med. 385, 1163–1171. https://doi.org/10.1056/NEJMoa2101746

Ballatori, N., Hammond, C.L., Cunningham, J.B., Krance, S.M., Marchan, R., 2005. Molecular mechanisms of reduced glutathione transport: Role of the MRP/CFTR/ABCC and OATP/SLC21A

families of membrane proteins. Toxicol. Appl. Pharmacol. 204, 238–255. https://doi.org/10.1016/j.taap.2004.09.008

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., Mitchell, G.H., 2000. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. Parasitol. Today 16, 427–433. https://doi.org/10.1016/s0169-4758(00)01755-5

Bansal, A., Molina-Cruz, A., Brzostowski, J., Mu, J., Miller, L.H., 2017. *Plasmodium falciparum* calcium-dependent protein kinase 2 is critical for male gametocyte exflagellation but not essential for asexual proliferation. mBio 8. https://doi.org/10.1128/mBio.01656-17

Basco, L.K., Eldin de Pécoulas, P., Wilson, C.M., Le Bras, J., Mazabraud, A., 1995. Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 69, 135–138. https://doi.org/10.1016/0166-6851(94)00207-4

Batty, K.T., Anh Thu, L.T., Davis, T.M.E., Ilett, K.F., Xuan Mai, T., Canh Hung, N., Phuc Tien, N., Powell, S.M., Van Thien, H., Quang Binh, T., Kim, N.V., 1998. A pharmacokinetic and pharmacodynamic study of intravenous vs oral artesunate in uncomplicated falciparum malaria. Br. J. Clin. Pharmacol. 45, 123–129. https://doi.org/10.1046/j.1365-2125.1998.00655.x

Bellanca, S., Summers, R.L., Meyrath, M., Dave, A., Nash, M.N., Dittmer, M., Sanchez, C.P., Stein, W.D., Martin, R.E., Lanzer, M., 2014. Multiple Drugs Compete for Transport via the *Plasmodium falciparum* Chloroquine Resistance Transporter at Distinct but Interdependent Sites. J. Biol. Chem. 289, 36336–36351. https://doi.org/10.1074/jbc.M114.614206

Belz, S., Nau, H., 1998. Determination of folate patterns in mouse plasma, erythrocytes, and embryos by HPLC coupled with a microbiological assay. Anal. Biochem. 265, 157–166. https://doi.org/10.1006/abio.1998.2865

Beshir, K.B., Diallo, N., Somé, F.A., Sombie, S., Zongo, I., Fofana, B., Traore, A., Dama, S., Bamadio, A., Traore, O.B., Coulibaly, S.A., Maurice, O.S., Diarra, A., Kaboré, J.M., Kodio, A., Togo, A.H., Dara, N., Coulibaly, M., Dao, F., Nikiema, F., Compaore, Y.D., Kabore, N.T., Barry, N., Soulama, I., Sagara, I., Sirima, S.B., Ouédraogo, J.-B., Djimde, A., Sutherland, C.J., 2021. Persistent submicroscopic *Plasmodium falciparum* parasitemia 72 hours after treatment with artemether-lumefantrine predicts 42-day treatment failure in Mali and Burkina Faso. Antimicrob. Agents Chemother. 65, e0087321. https://doi.org/10.1128/AAC.00873-21

Bhattacharjee, S., Coppens, I., Mbengue, A., Suresh, N., Ghorbal, M., Slouka, Z., Safeukui, I., Tang, H.-Y., Speicher, D.W., Stahelin, R.V., Mohandas, N., Haldar, K., 2018. Remodeling of the malaria parasite and host human red cell by vesicle amplification that induces artemisinin resistance. Blood 131, 1234–1247. https://doi.org/10.1182/blood-2017-11-814665

Birnbaum, J., Flemming, S., Reichard, N., Soares, A.B., Mesén-Ramírez, P., Jonscher, E., Bergmann, B., Spielmann, T., 2017. A genetic system to study *Plasmodium falciparum* protein function. Nat. Methods 14, 450–456. https://doi.org/10.1038/nmeth.4223

Birnbaum, J., Scharf, S., Schmidt, S., Jonscher, E., Hoeijmakers, W.A.M., Flemming, S., Toenhake, C.G., Schmitt, M., Sabitzki, R., Bergmann, B., Fröhlke, U., Mesén-Ramírez, P., Soares, A.B., Herrmann, H., Bártfai, R., Spielmann, T., 2020. A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. Science 367, 51–59. https://doi.org/10.1126/science.aax4735

Blanshard, A., Hine, P., 2021. Atovaquone-proguanil for treating uncomplicated *Plasmodium falciparum* malaria. Cochrane Database Syst. Rev. 1, CD004529. https://doi.org/10.1002/14651858.CD004529.pub3

Blasco, B., Leroy, D., Fidock, D.A., 2017. Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. Nat. Med. 23, 917–928. https://doi.org/10.1038/nm.4381

Böhme, U., Otto, T.D., Sanders, M., Newbold, C.I., Berriman, M., 2019. Progression of the canonical reference malaria parasite genome from 2002-2019. Wellcome Open Res. 4, 58. https://doi.org/10.12688/wellcomeopenres.15194.2

Bonn, D., 2004. Artekin: an affordable antimalarial. Lancet Infect. Dis. 4, 256. https://doi.org/10.1016/S1473-3099(04)01017-5

Boonyalai, N., Vesely, B.A., Thamnurak, C., Praditpol, C., Fagnark, W., Kirativanich, K., Saingam, P., Chaisatit, C., Lertsethtakarn, P., Gosi, P., Kuntawunginn, W., Vanachayangkul, P., Spring, M.D., Fukuda, M.M., Lon, C., Smith, P.L., Waters, N.C., Saunders, D.L., Wojnarski, M., 2020. Piperaquine resistant Cambodian *Plasmodium falciparum* clinical isolates: *in vitro* genotypic and phenotypic characterization. Malar. J. 19, 269. https://doi.org/10.1186/s12936-020-03339-w

Bopp, S., Magistrado, P., Wong, W., Schaffner, S.F., Mukherjee, A., Lim, P., Dhorda, M., Amaratunga, C., Woodrow, C.J., Ashley, E.A., White, N.J., Dondorp, A.M., Fairhurst, R.M., Ariey, F., Menard, D., Wirth, D.F., Volkman, S.K., 2018. *Plasmepsin II–III* copy number accounts for bimodal piperaquine resistance among Cambodian *Plasmodium falciparum*. Nat. Commun. 9, 1769. https://doi.org/10.1038/s41467-018-04104-z

Bozdech, Z., Ginsburg, H., 2004. Antioxidant defense in *Plasmodium falciparum* – data mining of the transcriptome. Malar. J. 3, 23. https://doi.org/10.1186/1475-2875-3-23

Bozdech, Z., Llinás, M., Pulliam, B.L., Wong, E.D., Zhu, J., DeRisi, J.L., 2003. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. PLOS Biol. 1, e5. https://doi.org/10.1371/journal.pbio.0000005

Brasil, P., Zalis, M.G., Pina-Costa, A. de, Siqueira, A.M., Júnior, C.B., Silva, S., Areas, A.L.L., Pelajo-Machado, M., Alvarenga, D.A.M. de, Santelli, A.C.F. da S., Albuquerque, H.G., Cravo, P., Abreu, F.V.S. de, Peterka, C.L., Zanini, G.M., Mutis, M.C.S., Pissinatti, A., Lourenço-de-Oliveira, R., Brito, C.F.A. de, Ferreira-da-Cruz, M. de F., Culleton, R., Daniel-Ribeiro, C.T., 2017. Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. Lancet Glob. Health 5, e1038–e1046. https://doi.org/10.1016/S2214-109X(17)30333-9

Bray, P.G., Ward, S.A., O'Neill, P.M., 2005. Quinolines and Artemisinin: Chemistry, Biology and History, in: Compans, R.W., Cooper, M.D., Honjo, T., Koprowski, H., Melchers, F., Oldstone, M.B.A., Olsnes, S., Potter, M., Vogt, P.K., Wagner, H., Sullivan, D.J., Krishna, S. (Eds.), Malaria: Drugs, Disease and Post-Genomic Biology, Current Topics in Microbiology and Immunology. Springer-Verlag, Berlin/Heidelberg, pp. 3–38. https://doi.org/10.1007/3-540-29088-5_1

Breglio, K.F., Rahman, R.S., Sá, J.M., Hott, A., Roberts, D.J., Wellems, T.E., 2018. Kelch mutations in *Plasmodium falciparum* protein K13 do not modulate dormancy after artemisinin exposure and sorbitol selection *In Vitro*. Antimicrob. Agents Chemother. 62. https://doi.org/10.1128/AAC.02256-17

Bridgford, J.L., Xie, S.C., Cobbold, S.A., Pasaje, C.F.A., Herrmann, S., Yang, T., Gillett, D.L., Dick, L.R., Ralph, S.A., Dogovski, C., Spillman, N.J., Tilley, L., 2018. Artemisinin kills malaria parasites by damaging proteins and inhibiting the proteasome. Nat. Commun. 9, 3801. https://doi.org/10.1038/s41467-018-06221-1

Briolant, S., Bogreau, H., Gil, M., Bouchiba, H., Baret, E., Amalvict, R., Rogier, C., Pradines, B., 2012. The F423Y Mutation in the *pfmdr2* gene and mutations N51I, C59R, and S108N in the pfdhfr gene are independently associated with pyrimethamine resistance in *Plasmodium falciparum* isolates. Antimicrob. Agents Chemother. 56, 2750–2752. https://doi.org/10.1128/AAC.05618-11

Brockman, A., Price, R.N., van Vugt, M., Heppner, D.G., Walsh, D., Sookto, P., Wimonwattrawatee, T., Looareesuwan, S., White, N.J., Nosten, F., 2000. *Plasmodium falciparum* antimalarial drug susceptibility on the north-western border of Thailand during five years of extensive use of

artesunate-mefloquine. Trans. R. Soc. Trop. Med. Hyg. 94, 537–544. https://doi.org/10.1016/s0035-9203(00)90080-4

Brooks, D.R., Wang, P., Read, M., Watkins, W.M., Sims, P.F., Hyde, J.E., 1994. Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine. Eur. J. Biochem. 224, 397–405. https://doi.org/10.1111/j.1432-1033.1994.00397.x

Buck, E., Finnigan, N.A., 2020. Malaria, in: StatPearls. StatPearls Publishing, Treasure Island (FL).

Bunnag, D., Harinasuta, T., Pinichpongse, S., Suntharasami, P., 1980. Effect of primaquine on gametocytes of *Plasmodium falciparum* in Thailand. Lancet 2, 91. https://doi.org/10.1016/s0140-6736(80)92970-0

Burk, O., Arnold, K.A., Nussler, A.K., Schaeffeler, E., Efimova, E., Avery, B.A., Avery, M.A., Fromm, M.F., Eichelbaum, M., 2005. Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. Mol. Pharmacol. 67, 1954–1965. https://doi.org/10.1124/mol.104.009019

Bushell, E., Gomes, A.R., Sanderson, T., Anar, B., Girling, G., Herd, C., Metcalf, T., Modrzynska, K., Schwach, F., Martin, R.E., Mather, M.W., McFadden, G.I., Parts, L., Rutledge, G.G., Vaidya, A.B., Wengelnik, K., Rayner, J.C., Billker, O., 2017. Functional profiling of a *Plasmodium* genome reveals an abundance of essential genes. Cell 170, 260-272.e8. https://doi.org/10.1016/j.cell.2017.06.030

Butterworth, A.S., Skinner-Adams, T.S., Gardiner, D.L., Trenholme, K.R., 2013. *Plasmodium falciparum* gametocytes: with a view to a kill. Parasitology 140, 1718–1734. https://doi.org/10.1017/S0031182013001236

Bzik, D.J., Li, W.B., Horii, T., Inselburg, J., 1987. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc. Natl. Acad. Sci. 84, 8360–8364. https://doi.org/10.1073/pnas.84.23.8360

Calçada, C., Silva, M., Baptista, V., Thathy, V., Silva-Pedrosa, R., Granja, D., Ferreira, P.E., Gil, J.P., Fidock, D.A., Veiga, M.I., 2020. Expansion of a specific *Plasmodium falciparum* PfMDR1 haplotype in Southeast Asia with increased substrate transport. mBio 11, e02093-20. https://doi.org/10.1128/mBio.02093-20

Callaghan, P.S., Hassett, M.R., Roepe, P.D., 2015. Functional comparison of 45 naturally occurring isoforms of the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT). Biochemistry 54, 5083–5094. https://doi.org/10.1021/acs.biochem.5b00412

Carlsson, A.M., Ngasala, B.E., Dahlström, S., Membi, C., Veiga, I.M., Rombo, L., Abdulla, S., Premji, Z., Gil, J.P., Björkman, A., Mårtensson, A., 2011. *Plasmodium falciparum* population dynamics during the early phase of anti-malarial drug treatment in Tanzanian children with acute uncomplicated malaria. Malar. J. 10, 380. https://doi.org/10.1186/1475-2875-10-380

Carrara, V.I., Zwang, J., Ashley, E.A., Price, R.N., Stepniewska, K., Barends, M., Brockman, A., Anderson, T., McGready, R., Phaiphun, L., Proux, S., Vugt, M. van, Hutagalung, R., Lwin, K.M., Phyo, A.P., Preechapornkul, P., Imwong, M., Pukrittayakamee, S., Singhasivanon, P., White, N.J., Nosten, F., 2009. Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. PLoS One 4, e4551. https://doi.org/10.1371/journal.pone.0004551

Cerqueira, G.C., Cheeseman, I.H., Schaffner, S.F., Nair, S., McDew-White, M., Phyo, A.P., Ashley, E.A., Melnikov, A., Rogov, P., Birren, B.W., Nosten, F., Anderson, T.J.C., Neafsey, D.E., 2017. Longitudinal genomic surveillance of *Plasmodium falciparum* malaria parasites reveals complex genomic architecture of emerging artemisinin resistance. Genome Biol. 18, 78. https://doi.org/10.1186/s13059-017-1204-4

Chan, C.W., Lynch, D., Spathis, R., Hombhanje, F.W., Kaneko, A., Garruto, R.M., Lum, J.K., 2006. Flashback to the 1960s: utility of archived sera to explore the origin and evolution of *Plasmodium falciparum* chloroquine resistance in the Pacific. Acta Trop. 99, 15–22. https://doi.org/10.1016/j.actatropica.2006.05.011

Chan, C.W., Spathis, R., Reiff, D.M., McGrath, S.E., Garruto, R.M., Lum, J.K., 2012. Diversity of *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) exon 2 haplotypes in the Pacific from 1959 to 1979. PLoS One 7, e30213. https://doi.org/10.1371/journal.pone.0030213

Chappell, L., Ross, P., Orchard, L., Russell, T.J., Otto, T.D., Berriman, M., Rayner, J.C., Llinás, M., 2020. Refining the transcriptome of the human malaria parasite *Plasmodium falciparum* using amplification-free RNA-seq. BMC Genom. 21, 395. https://doi.org/10.1186/s12864-020-06787-5

Chavchich, M., Van Breda, K., Rowcliffe, K., Diagana, T.T., Edstein, M.D., 2016. The spiroindolone KAE609 does not induce dormant ring stages in *Plasmodium falciparum* Parasites. Antimicrob. Agents Chemother. 60, 5167–5174. https://doi.org/10.1128/AAC.02838-15

Cheeseman, I.H., Gomez-Escobar, N., Carret, C.K., Ivens, A., Stewart, L.B., Tetteh, K.K., Conway, D.J., 2009. Gene copy number variation throughout the *Plasmodium falciparum* genome. BMC Genom. 10, 353. https://doi.org/10.1186/1471-2164-10-353

Cheeseman, I.H., Miller, B., Tan, J.C., Tan, A., Nair, S., Nkhoma, S.C., De Donato, M., Rodulfo, H., Dondorp, A., Branch, O.H., Mesia, L.R., Newton, P., Mayxay, M., Amambua-Ngwa, A., Conway, D.J., Nosten, F., Ferdig, M.T., Anderson, T.J.C., 2016. Population structure shapes copy number variation in malaria parasites. Mol. Biol. Evol. 33, 603–620. https://doi.org/10.1093/molbev/msv282

Chen, M.Z., Moily, N.S., Bridgford, J.L., Wood, R.J., Radwan, M., Smith, T.A., Song, Z., Tang, B.Z., Tilley, L., Xu, X., Reid, G.E., Pouladi, M.A., Hong, Y., Hatters, D.M., 2017. A thiol probe for measuring unfolded protein load and proteostasis in cells. Nat. Commun. 8, 474. https://doi.org/10.1038/s41467-017-00203-5

Chen, P.Q., Li, G.Q., Guo, X.B., He, K.R., Fu, Y.X., Fu, L.C., Song, Y.Z., 1994. The infectivity of gametocytes of *Plasmodium falciparum* from patients treated with artemisinin. Chin. Med. J. 107, 709–711.

Chen, Z.S., Lee, K., Walther, S., Raftogianis, R.B., Zeng, H., Kruh, G.D., Kuwano, M., 2002. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res. 62, 3144–3150.

Chin, W., Contacos, P.G., Coatney, G.R., King, H.K., 1966. The evaluation of sulfonamides, alone or in combination with pyrimethamine, in the treatment of multi-resistant falciparum malaria. Am. J. Trop. Med. Hyg. 15, 823–829. https://doi.org/10.4269/ajtmh.1966.15.823

Chulay, J.D., Watkins, W.M., Sixsmith, D.G., 1984. Synergistic antimalarial activity of pyrimethamine and sulfadoxine against *Plasmodium falciparum in vitro*. Am. J. Trop. Med. Hyg. 33, 325–330. https://doi.org/10.4269/ajtmh.1984.33.325

Clyde, D.F., Shute, G.T., 1954. Resistance of East African varieties of *Plasmodium falciparum* to pyrimethamine. Trans. R. Soc. Trop. Med. Hyg. 48, 495–500. https://doi.org/10.1016/0035-9203(54)90085-1

Coggeshall, L.T., Maier, J., Best, C.A., 1941. The effectiveness of two new types of chemotherapeutic agents in malaria: sodium p,p′-diaminodiphenylsulfone n,n′-didextrosesulfonate (promin) and 2-sulfanilamido pyrimidine (sulfadiazine). JAMA 117, 1077–1081. https://doi.org/10.1001/jama.1941.02820390019006

Combrinck, J.M., Mabotha, T.E., Ncokazi, K.K., Ambele, M.A., Taylor, D., Smith, P.J., Hoppe, H.C., Egan, T.J., 2013. Insights into the role of heme in the mechanism of action of antimalarials. ACS Chem. Biol. 8, 133–137. https://doi.org/10.1021/cb300454t

Conrad, D.F., Hurles, M.E., 2007. The population genetics of structural variation. Nat. Genet. 39, S30-36. https://doi.org/10.1038/ng2042

Conrad, M.D., LeClair, N., Arinaitwe, E., Wanzira, H., Kakuru, A., Bigira, V., Muhindo, M., Kamya, M.R., Tappero, J.W., Greenhouse, B., Dorsey, G., Rosenthal, P.J., 2014. Comparative impacts over 5 years of artemisinin-based combination therapies on *Plasmodium falciparum* polymorphisms that modulate drug sensitivity in Ugandan children. J. Infect. Dis. 210, 344–353. https://doi.org/10.1093/infdis/jiu141

Conrad, M.D., Rosenthal, P.J., 2019. Antimalarial drug resistance in Africa: the calm before the storm? Lancet Infect. Dis. 19, e338–e351. https://doi.org/10.1016/S1473-3099(19)30261-0

Cooper, R.A., Ferdig, M.T., Su, X.-Z., Ursos, L.M.B., Mu, J., Nomura, T., Fujioka, H., Fidock, D.A., Roepe, P.D., Wellems, T.E., 2002. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Mol. Pharmacol. 61, 35–42. https://doi.org/10.1124/mol.61.1.35

Coronado, L.M., Nadovich, C.T., Spadafora, C., 2014. Malarial hemozoin: from target to tool. Biochim. Biophys. Acta 1840, 2032–2041. https://doi.org/10.1016/j.bbagen.2014.02.009

Cowell, A.N., Winzeler, E.A., 2019. The genomic architecture of antimalarial drug resistance. Brief. Funct. Genomics 18, 314–328. https://doi.org/10.1093/bfgp/elz008

Cowman, A.F., Berry, D., Baum, J., 2012. The cellular and molecular basis for malaria parasite invasion of the human red blood cell. J. Cell Biol. 198, 961–971. https://doi.org/10.1083/jcb.201206112

Cowman, A.F., Galatis, D., Thompson, J.K., 1994. Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. Proc. Natl. Acad. Sci. 91, 1143–1147. https://doi.org/10.1073/pnas.91.3.1143

Cowman, A.F., Morry, M.J., Biggs, B.A., Cross, G.A., Foote, S.J., 1988. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. 85, 9109–9113. https://doi.org/10.1073/pnas.85.23.9109

Cox, F.E., 2010. History of the discovery of the malaria parasites and their vectors. Parasites Vectors 3. https://doi.org/10.1186/1756-3305-3-5

Cox-Singh, J., Davis, T.M.E., Lee, K.S., Shamsul, S.S.G., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J., Singh, B., 2008. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. Clin. Infect. Dis. 46, 165–171. https://doi.org/10.1086/524888

Crabb, B.S., Cowman, A.F., 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. 93, 7289–7294.

Crawford, E.D., Quan, J., Horst, J.A., Ebert, D., Wu, W., DeRisi, J.L., 2017. Plasmid-free CRISPR/Cas9 genome editing in *Plasmodium falciparum* confirms mutations conferring resistance to the dihydroisoquinolone clinical candidate SJ733. PLoS One 12. https://doi.org/10.1371/journal.pone.0178163

Crompton, P.D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L.S., Miller, L.H., Barillas-Mury, C., Pierce, S.K., 2014. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annu. Rev. Immunol. 32, 157–187. https://doi.org/10.1146/annurev-immunol-032713-120220

Cui, L., Su, X., 2009. Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev. Anti Infect. Ther. 7, 999–1013. https://doi.org/10.1586/eri.09.68

Cunha, C.B., Cunha, B.A., 2008. Brief history of the clinical diagnosis of malaria: from Hippocrates to Osler. J. Vector. Borne Dis. 45, 194–199.

da Costa, K.M., Valente, R.C., Salustiano, E.J., Gentile, L.B., Freire-de-Lima, L., Mendonça-Previato, L., Previato, J.O., 2018. Functional characterization of ABCC proteins from *Trypanosoma cruzi* and their involvement with thiol transport. Front. Microbiol. 9, 205. https://doi.org/10.3389/fmicb.2018.00205

Dahlström, S., Ferreira, P.E., Veiga, M.I., Sedighi, N., Wiklund, L., Mårtensson, A., Färnert, A., Sisowath, C., Osório, L., Darban, H., Andersson, B., Kaneko, A., Conseil, G., Björkman, A., Gill, J.P., 2009a. *Plasmodium falciparum* multidrug resistance protein 1 and artemisinin-based combination therapy in Africa. J. Infect. Dis. 200, 1456–1464. https://doi.org/10.1086/606009

Dahlström, S., Veiga, M.I., Mårtensson, A., Björkman, A., Gil, J.P., 2009b. Polymorphism in *Pfmrp1* (*Plasmodium falciparum* multidrug resistance protein 1) amino acid 1466 associated with resistance to sulfadoxine-pyrimethamine treatment. Antimicrob. Agents Chemother. 53, 2553–2556. https://doi.org/10.1128/AAC.00091-09

Dallas, W.S., Gowen, J.E., Ray, P.H., Cox, M.J., Dev, I.K., 1992. Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of *Escherichia coli* MC4100. J. Bacteriol. 174, 5961–5970. https://doi.org/10.1128/jb.174.18.5961-5970.1992

Davis, T.M.E., Hung, T.-Y., Sim, I.-K., Karunajeewa, H.A., Ilett, K.F., 2005. Piperaquine: a resurgent antimalarial drug. Drugs 65, 75–87. https://doi.org/10.2165/00003495-200565010-00004

De Koning-Ward, T.F., Gilson, P.R., Crabb, B.S., 2015. Advances in molecular genetic systems in malaria. Nat. Rev. Microbiol. 13, 373–387. https://doi.org/10.1038/nrmicro3450

de Villiers, K.A., Marques, H.M., Egan, T.J., 2008. The crystal structure of halofantrine-ferriprotoporphyrin IX and the mechanism of action of arylmethanol antimalarials. J. Inorg. Biochem. 102, 1660–1667. https://doi.org/10.1016/j.jinorgbio.2008.04.001

Dean, M., Allikmets, R., 2001. Complete characterization of the human ABC gene family. J. Bioenerg. Biomembr. 33, 475–479. https://doi.org/10.1023/a:1012823120935

Deeley, R.G., Cole, S.P.C., 2006. Substrate recognition and transport by multidrug resistance protein 1 (ABCC1). FEBS Lett. 580, 1103–1111. https://doi.org/10.1016/j.febslet.2005.12.036

Delfino, R.T., Santos-Filho, O.A., Figueroa-Villar, J.D., 2002. Molecular modeling of wild-type and antifolate resistant mutant *Plasmodium falciparum* DHFR. Biophys. Chem. 98, 287–300. https://doi.org/10.1016/s0301-4622(02)00077-7

Demas, A.R., Sharma, A.I., Wong, W., Early, A.M., Redmond, S., Bopp, S., Neafsey, D.E., Volkman, S.K., Hartl, D.L., Wirth, D.F., 2018. Mutations in *Plasmodium falciparum* actin-binding protein coronin confer reduced artemisinin susceptibility. Proc. Natl. Acad. Sci. 115, 12799–12804. https://doi.org/10.1073/pnas.1812317115

Denis, M.B., Tsuyuoka, R., Lim, P., Lindegardh, N., Yi, P., Top, S.N., Socheat, D., Fandeur, T., Annerberg, A., Christophel, E.M., Ringwald, P., 2006. Efficacy of artemether-lumefantrine for the treatment of uncomplicated *falciparum* malaria in northwest Cambodia. Trop. Med. Int. Health 11, 1800–1807. https://doi.org/10.1111/j.1365-3156.2006.01739.x

Denisov, E., 2011. An important role of intramolecular free radical reactions in antimalarial activity of artemisinin and its analogs. Org. Biomol. Chem. 9, 4219–4225. https://doi.org/10.1039/C0OB01150A

Derbie, A., Mekonnen, D., Adugna, M., Yeshitela, B., Woldeamanuel, Y., Abebe, T., 2020. Therapeutic efficacy of artemether-lumefantrine (Coartem®) for the treatment of uncomplicated *Falciparum* malaria in Africa: A systematic review. J. Parasitol. Res. 2020. https://doi.org/10.1155/2020/7371681

Dery, V., Duah, N.O., Ayanful-Torgby, R., Matrevi, S.A., Anto, F., Quashie, N.B., 2015. An improved SYBR Green-1-based fluorescence method for the routine monitoring of *Plasmodium falciparum* resistance to anti-malarial drugs. Malar. J. 14, 481. https://doi.org/10.1186/s12936-015-1011-x

Dhingra, S.K., Redhi, D., Combrinck, J.M., Yeo, T., Okombo, J., Henrich, P.P., Cowell, A.N., Gupta, P., Stegman, M.L., Hoke, J.M., Cooper, R.A., Winzeler, E., Mok, S., Egan, T.J., Fidock, D.A., 2017. A variant PfCRT isoform can contribute to *Plasmodium falciparum* resistance to the first-line partner drug piperaquine. mBio 8. https://doi.org/10.1128/mBio.00303-17

Dhingra, S.K., Small-Saunders, J.L., Ménard, D., Fidock, D.A., 2019. *Plasmodium falciparum* resistance to piperaquine driven by PfCRT. Lancet Infect. Dis. 19, 1168–1169. https://doi.org/10.1016/S1473-3099(19)30543-2

Dieckmann, A., Jung, A., 1986. Stage-specific sensitivity of *Plasmodium falciparum* to antifolates. Z. Parasitenkd. 72, 591–594. https://doi.org/10.1007/BF00925479

Dimbu, P.R., Horth, R., Cândido, A.L.M., Ferreira, C.M., Caquece, F., Garcia, L.E.A., André, K., Pembele, G., Jandondo, D., Bondo, B.J., Nieto Andrade, B., Labuda, S., Ponce de León, G., Kelley, J., Patel, D., Svigel, S.S., Talundzic, E., Lucchi, N., Morais, J.F.M., Fortes, F., Martins, J.F., Pluciński, M.M., 2021. Continued low efficacy of artemether-lumefantrine in Angola in 2019. Antimicrob. Agents Chemother. 65, e01949-20. https://doi.org/10.1128/AAC.01949-20

Diourté, Y., Djimdé, A., Doumbo, O.K., Sagara, I., Coulibaly, Y., Dicko, A., Diallo, M., Diakité, M., Cortese, J.F., Plowe, C.V., 1999. Pyrimethamine-sulfadoxine efficacy and selection for mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase in Mali. Am. J. Trop. Med. Hyg. 60, 475–478. https://doi.org/10.4269/ajtmh.1999.60.475

Djimde, A.A., Makanga, M., Kuhen, K., Hamed, K., 2015. The emerging threat of artemisinin resistance in malaria: focus on artemether-lumefantrine. Expert Rev. Anti Infect. Ther. 13, 1031–1045. https://doi.org/10.1586/14787210.2015.1052793

Djuika, C.F., Huerta-Cepas, J., Przyborski, J.M., Deil, S., Sanchez, C.P., Doerks, T., Bork, P., Lanzer, M., Deponte, M., 2015. Prokaryotic ancestry and gene fusion of a dual localized peroxiredoxin in malaria parasites. Microb. Cell 2, 5–13. https://doi.org/10.15698/mic2015.01.182

Dogovski, C., Xie, S.C., Burgio, G., Bridgford, J., Mok, S., McCaw, J.M., Chotivanich, K., Kenny, S., Gnädig, N., Straimer, J., Bozdech, Z., Fidock, D.A., Simpson, J.A., Dondorp, A.M., Foote, S., Klonis, N., Tilley, L., 2015. Targeting the cell stress response of *Plasmodium falciparum* to overcome artemisinin resistance. PLoS Biol. 13, e1002132. https://doi.org/10.1371/journal.pbio.1002132

Dondorp, A.M., Fairhurst, R.M., Slutsker, L., Macarthur, J.R., Breman, J.G., Guerin, P.J., Wellems, T.E., Ringwald, P., Newman, R.D., Plowe, C.V., 2011. The threat of artemisinin-resistant malaria. N. Engl. J. Med. 365, 1073–1075. https://doi.org/10.1056/NEJMp1108322

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P.J., Lindegardh, N., Socheat, D., White, N.J., 2009. Artemisinin Resistance in *Plasmodium falciparum* Malaria. N. Engl. J. Med. 361, 455–467. https://doi.org/10.1056/NEJMoa0808859

Duffy, P.E., Patrick Gorres, J., 2020. Malaria vaccines since 2000: progress, priorities, products. NPJ Vaccines 5, 1–9. https://doi.org/10.1038/s41541-020-0196-3

Duraisingh, M.T., Cowman, A.F., 2005. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. Acta Trop. 94, 181–190. https://doi.org/10.1016/j.actatropica.2005.04.008

Duraisingh, M.T., Jones, P., Sambou, I., von Seidlein, L., Pinder, M., Warhurst, D.C., 2000. The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity

to the anti-malarials mefloquine and artemisinin. Mol. Biochem. Parasitol. 108, 13–23. https://doi.org/10.1016/s0166-6851(00)00201-2

Ebong, C., Sserwanga, A., Namuganga, J.F., Kapisi, J., Mpimbaza, A., Gonahasa, S., Asua, V., Gudoi, S., Kigozi, R., Tibenderana, J., Bwanika, J.B., Bosco, A., Rubahika, D., Kyabayinze, D., Opigo, J., Rutazana, D., Sebikaari, G., Belay, K., Niang, M., Halsey, E.S., Moriarty, L.F., Lucchi, N.W., Souza, S.S.S., Nsobya, S.L., Kamya, M.R., Yeka, A., 2021. Efficacy and safety of artemether-lumefantrine and dihydroartemisinin-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria and prevalence of molecular markers associated with artemisinin and partner drug resistance in Uganda. Malar. J. 20, 484. https://doi.org/10.1186/s12936-021-04021-5

Ecker, A., Lehane, A.M., Clain, J., Fidock, D.A., 2012. PfCRT and its role in antimalarial drug resistance. Trends Parasitol. 28, 504–514. https://doi.org/10.1016/j.pt.2012.08.002

Edeson, J.F.B., Field, J.W., 1950. Proguanil-resistant falciparum malaria. Br. Med. J. 1, 147–151.

Eisele, T.P., 2019. Mass drug administration can be a valuable addition to the malaria elimination toolbox. Malar. J. 18, 281. https://doi.org/10.1186/s12936-019-2906-8

Ezzet, F., van Vugt, M., Nosten, F., Looareesuwan, S., White, N.J., 2000. Pharmacokinetics and pharmacodynamics of lumefantrine (Benflumetol) in acute *Falciparum* malaria. Antimicrob. Agents Chemother. 44, 697–704. https://doi.org/doi.org/10.1128/AAC.44.3.697-704.2000

Ferlan, J.T., Mookherjee, S., Okezie, I.N., Fulgence, L., Sibley, C.H., 2001. Mutagenesis of dihydrofolate reductase from *Plasmodium falciparum*: analysis in Saccharomyces cerevisiae of triple mutant alleles resistant to pyrimethamine or WR99210. Mol. Biochem. Parasitol. 113, 139–150. https://doi.org/10.1016/s0166-6851(01)00207-9

Ferone, R., 1977. Folate metabolism in malaria. Bull. World Health Organ. 55, 291–298.

Ferreira, P.E., Holmgren, G., Veiga, M.I., Uhlén, P., Kaneko, A., Gil, J.P., 2011. PfMDR1: mechanisms of transport modulation by functional polymorphisms. PLoS One 6, e23875. https://doi.org/10.1371/journal.pone.0023875

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naudé, B., Deitsch, K.W., Su, X.Z., Wootton, J.C., Roepe, P.D., Wellems, T.E., 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell 6, 861–871. https://doi.org/10.1016/s1097-2765(05)00077-8

Fidock, D.A., Nomura, T., Wellems, T.E., 1998. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. Mol. Pharmacol. 54, 1140–1147. https://doi.org/10.1124/mol.54.6.1140

Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., Moch, J.K., Muster, N., Sacci, J.B., Tabb, D.L., Witney, A.A., Wolters, D., Wu, Y., Gardner, M.J., Holder, A.A., Sinden, R.E., Yates, J.R., Carucci, D.J., 2002. A proteomic view of the *Plasmodium falciparum* life cycle. Nature 419, 520–526. https://doi.org/10.1038/nature01107

Folarin, O.A., Bustamante, C., Gbotosho, G.O., Sowunmi, A., Zalis, M.G., Oduola, A.M.J., Happi, C.T., 2011. *In vitro* Amodiaquine resistance and its association with mutations in *pfcrt* and *pfmdr1* genes of *Plasmodium falciparum* isolates from Nigeria. Acta Trop. 120, 224–230. https://doi.org/10.1016/j.actatropica.2011.08.013

Foote, S.J., Galatis, D., Cowman, A.F., 1990. Amino acids in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. Proc. Natl. Acad. Sci. 87, 3014–3017. https://doi.org/10.1073/pnas.87.8.3014

Friedrich, O., Reiling, S., Wunderlich, J., Rohrbach, P., 2014. Assessment of Plasmodium falciparum PfMDR1 transport rates using Fluo-4. J. Cell. Mol. Med. 18, 1851–1862. https://doi.org/10.1111/jcmm.12313

Fröberg, G., Ferreira, P.E., Mårtensson, A., Ali, A., Björkman, A., Gil, J.P., 2013. Assessing the cost-benefit effect of a *Plasmodium falciparum* drug resistance mutation on parasite growth *in vitro*. Antimicrob. Agents Chemother. 57, 887–892. https://doi.org/10.1128/AAC.00950-12

Frosch, A.E.P., Laufer, M.K., Mathanga, D.P., Takala-Harrison, S., Skarbinski, J., Claassen, C.W., Dzinjalamala, F.K., Plowe, C.V., 2014. Return of widespread chloroquine-sensitive *Plasmodium falciparum* to Malawi. J. Infect. Dis. 210, 1110–1114. https://doi.org/10.1093/infdis/jiu216

Gabryszewski, S.J., Dhingra, S.K., Combrinck, J.M., Lewis, I.A., Callaghan, P.S., Hassett, M.R., Siriwardana, A., Henrich, P.P., Lee, A.H., Gnädig, N.F., Musset, L., Llinás, M., Egan, T.J., Roepe, P.D., Fidock, D.A., 2016a. Evolution of Fitness Cost-Neutral Mutant PfCRT Conferring P. falciparum 4-Aminoquinoline Drug Resistance Is Accompanied by Altered Parasite Metabolism and Digestive Vacuole Physiology. PLOS Pathogens 12, e1005976. https://doi.org/10.1371/journal.ppat.1005976

Gabryszewski, S.J., Modchang, C., Musset, L., Chookajorn, T., Fidock, D.A., 2016b. Combinatorial genetic modeling of *pfcrt*-mediated drug resistance evolution in *Plasmodium falciparum*. Mol. Biol. Evol. 33, 1554–1570. https://doi.org/10.1093/molbev/msw037

Gansané, A., Moriarty, L.F., Ménard, D., Yerbanga, I., Ouedraogo, E., Sondo, P., Kinda, R., Tarama, C., Soulama, E., Tapsoba, M., Kangoye, D., Compaore, C.S., Badolo, O., Dao, B., Tchwenko, S., Tinto, H., Valea, I., 2021. Anti-malarial efficacy and resistance monitoring of artemether-lumefantrine and dihydroartemisinin-piperaquine shows inadequate efficacy in children in Burkina Faso, 2017–2018. Malar. J. 20, 48. https://doi.org/10.1186/s12936-021-03585-6

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M.A., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Davis, R.W., Fraser, C.M., Barrell, B., 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419, 498–511. https://doi.org/10.1038/nature01097

Gekeler, V., Ise, W., Sanders, K.H., Ulrich, W.R., Beck, J., 1995. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem. Biophys. Res. Commun. 208, 345–352. https://doi.org/10.1006/bbrc.1995.1344

Gendrot, M., Diawara, S., Madamet, M., Kounta, M.B., Briolant, S., Wade, K.A., Fall, M., Benoit, N., Nakoulima, A., Amalvict, R., Diémé, Y., Fall, B., Wade, B., Diatta, B., Pradines, B., 2017. Association between polymorphisms in the *Pfmdr6* gene and ex vivo susceptibility to quinine in *Plasmodium falciparum* parasites from Dakar, Senegal. Antimicrob. Agents Chemother. 61. https://doi.org/10.1128/AAC.01183-16

Gendrot, M., Wague Gueye, M., Tsombeng Foguim, F., Madamet, M., Wade, K.A., Bou Kounta, M., Fall, M., Diawara, S., Benoit, N., Lo, G., Bercion, R., Amalvict, R., Mosnier, J., Fall, B., Briolant, S., Diatta, B., Pradines, B., 2019. Modulation of *in vitro* antimalarial responses by polymorphisms in *Plasmodium falciparum* ABC transporters (*pfmdr1* and *pfmdr5*). Acta Trop. 196, 126–134. https://doi.org/10.1016/j.actatropica.2019.05.020

Gerardin, J., Eckhoff, P., Wenger, E.A., 2015. Mass campaigns with antimalarial drugs: a modelling comparison of artemether-lumefantrine and DHA-piperaquine with and without primaquine as tools for malaria control and elimination. BMC Infect. Dis. 15, 144. https://doi.org/10.1186/s12879-015-0887-y

Ghorbal, M., Gorman, M., Macpherson, C.R., Martins, R.M., Scherf, A., Lopez-Rubio, J.-J., 2014. Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. Nat. Biotechnol. 32, 819–821. https://doi.org/10.1038/nbt.2925

Ghosh, P., Bibb, L.A., Hatfull, G.F., 2008. Two-step site selection for serine-integrase-mediated excision: DNA-directed integrase conformation and central dinucleotide proofreading. Proc. Natl. Acad. Sci. 105, 3238–3243. https://doi.org/10.1073/pnas.0711649105

Giao, P.T., de Vries, P.J., Hung, L.Q., Binh, T.Q., Nam, N.V., Kager, P.A., 2004. CV8, a new combination of dihydroartemisinin, piperaquine, trimethoprim and primaquine, compared with atovaquone–proguanil against falciparum malaria in Vietnam. Trop. Med. Int. Health 9, 209–216. https://doi.org/10.1046/j.1365-3156.2003.01180.x

Gibbons, J., Button-Simons, K.A., Adapa, S.R., Li, S., Pietsch, M., Zhang, M., Liao, X., Adams, J.H., Ferdig, M.T., Jiang, R.H.Y., 2018. Altered expression of K13 disrupts DNA replication and repair in *Plasmodium falciparum*. BMC Genom. 19, 849. https://doi.org/10.1186/s12864-018-5207-7

Gil, J.P., Krishna, S., 2017. *pfmdr1* (*Plasmodium falciparum multidrug* drug resistance gene 1): a pivotal factor in malaria resistance to artemisinin combination therapies. Expert Rev. Anti Infect. Ther. 15, 527–543. https://doi.org/10.1080/14787210.2017.1313703

Glickman, M.H., Ciechanover, A., 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428. https://doi.org/10.1152/physrev.00027.2001

Goodwin, L.G., 1952. Daraprim (B.W. 50-63) - A New Antimalarial. Br. Med. J. 1, 732–734. https://doi.org/10.1136/bmj.1.4761.732

Gordi, T., Huong, D.X., Hai, T.N., Nieu, N.T., Ashton, M., 2002. Artemisinin pharmacokinetics and efficacy in uncomplicated-malaria patients treated with two different dosage regimens. Antimicrob. Agents Chemother. 46, 1026–1031. https://doi.org/10.1128/AAC.46.4.1026-1031.2002

Greenberg, J., Richeson, E.M., 1950. Potentiation of the antimalarial activity of sulfadiazine by 2,4-diamino-5-aryloxypyrimidines. J. Pharmacol. Exp. Ther. 99, 444–9.

Gritzmacher, C.A., Reese, R.T., 1984. Protein and nucleic acid synthesis during synchronized growth of *Plasmodium falciparum*. J. Bacteriol. 160, 1165–1167. https://doi.org/10.1128/jb.160.3.1165-1167.1984

Guinet, F., Dvorak, J.A., Fujioka, H., Keister, D.B., Muratova, O., Kaslow, D.C., Aikawa, M., Vaidya, A.B., Wellems, T.E., 1996. A developmental defect in *Plasmodium falciparum* male gametogenesis. J. Cell Biol. 135, 269–278. https://doi.org/10.1083/jcb.135.1.269

Gupta, A., Balabaskaran-Nina, P., Nguitragool, W., Saggu, G.S., Schureck, M.A., Desai, S.A., 2018. CLAG3 self-associates in malaria parasites and quantitatively determines nutrient uptake channels at the host Membrane. mBio 9. https://doi.org/10.1128/mBio.02293-17

Gutman, J., Kovacs, S., Dorsey, G., Stergachis, A., Ter Kuile, F.O., 2017. Safety, tolerability, and efficacy of repeated doses of dihydroartemisinin-piperaquine for prevention and treatment of malaria: a systematic review and meta-analysis. Lancet Infect. Dis. 17, 184–193. https://doi.org/10.1016/S1473-3099(16)30378-4

Gutteridge, W.E., Trigg, P.I., 1971. Action of pyrimethamine and related drugs against *Plasmodium knowlesi* in vitro. Parasitology 62, 431–444. https://doi.org/10.1017/S0031182000077581

Hale, V., Keasling, J.D., Renninger, N., Diagana, T.T., 2007. Microbially derived artemisinin: A biotechnology solution to the global problem of access to affordable antimalarial drugs, Defining and Defeating the Intolerable Burden of Malaria III: Progress and Perspectives: Supplement to Volume 77(6) of American Journal of Tropical Medicine and Hygiene. American Society of Tropical Medicine and Hygiene.

Hamed, K., Grueninger, H., 2012. Coartem(®): a decade of patient-centric malaria management. Expert Rev. Anti Infect. Ther. 10, 645–659. https://doi.org/10.1586/eri.12.51

Hamilton, W.L., Amato, R., van der Pluijm, R.W., Jacob, C.G., Quang, H.H., Thuy-Nhien, N.T., Hien, T.T., Hongvanthong, B., Chindavongsa, K., Mayxay, M., Huy, R., Leang, R., Huch, C., Dysoley, L., Amaratunga, C., Suon, S., Fairhurst, R.M., Tripura, R., Peto, T.J., Sovann, Y., Jittamala, P., Hanboonkunupakarn, B., Pukrittayakamee, S., Chau, N.H., Imwong, M., Dhorda, M., Vongpromek, R., Chan, X.H.S., Maude, R.J., Pearson, R.D., Nguyen, T., Rockett, K., Drury, E., Gonçalves, S., White, N.J., Day, N.P., Kwiatkowski, D.P., Dondorp, A.M., Miotto, O., 2019. Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. Lancet Infect. Dis. 19, 943–951. https://doi.org/10.1016/S1473-3099(19)30392-5

Harinasuta, T., Viravan, C., Reid, H.A., 1967. Sulphormethoxine in chloroquine-resistant falciparum malaria in Thailand. Lancet 1, 1117–1119. https://doi.org/10.1016/s0140-6736(67)91703-5

Hartmeyer, G.N., Stensvold, C.R., Fabricius, T., Marmolin, E.S., Hoegh, S.V., Nielsen, H.V., Kemp, M., Vestergaard, L.S., 2019. *Plasmodium cynomolgi* as cause of malaria in tourist to Southeast Asia, 2018. Emerg. Infect. Dis. 25, 1936–1939. https://doi.org/10.3201/eid2510.190448

Hastings, I.M., Ward, S.A., 2005. Coartem (Artemether-Lumefantrine) in Africa: the beginning of the end? J. Glob. Infect. Dis. 192, 1303–1304. https://doi.org/10.1086/432554

Hayward, R., Saliba, K.J., Kirk, K., 2005. *pfmdr1* mutations associated with chloroquine resistance incur a fitness cost in *Plasmodium falciparum*. Mol. Microbiol. 55, 1285–1295. https://doi.org/10.1111/j.1365-2958.2004.04470.x

Heinberg, A., Kirkman, L., 2015. The molecular basis of antifolate resistance in *Plasmodium falciparum*: looking beyond point mutations. Ann. N. Y. Acad. Sci. 1342, 10–18. https://doi.org/10.1111/nyas.12662

Henriques, G., Hallett, R.L., Beshir, K.B., Gadalla, N.B., Johnson, R.E., Burrow, R., van Schalkwyk, D.A., Sawa, P., Omar, S.A., Clark, T.G., Bousema, T., Sutherland, C.J., 2014. Directional selection at the *pfmdr1*, *pfcrt*, *pfubp1*, and *pfap2mu* Loci of *Plasmodium falciparum* in Kenyan children treated with ACT. J. Infect. Dis. 210, 2001–2008. https://doi.org/10.1093/infdis/jiu358

Henriques, G., Martinelli, A., Rodrigues, L., Modrzynska, K., Fawcett, R., Houston, D.R., Borges, S.T., d'Alessandro, U., Tinto, H., Karema, C., Hunt, P., Cravo, P., 2013. Artemisinin resistance in rodent malaria - mutation in the AP2 adaptor μ-chain suggests involvement of endocytosis and membrane protein trafficking. Malar. J. 12, 118. https://doi.org/10.1186/1475-2875-12-118

Henriques, G., van Schalkwyk, D.A., Burrow, R., Warhurst, D.C., Thompson, E., Baker, D.A., Fidock, D.A., Hallett, R., Flueck, C., Sutherland, C.J., 2015. The Mu subunit of *Plasmodium falciparum* clathrin-associated adaptor protein 2 modulates in vitro parasite response to artemisinin and quinine. Antimicrob. Agents Chemother. 59, 2540–2547. https://doi.org/10.1128/AAC.04067-14

Henry, B., Roussel, C., Carucci, M., Brousse, V., Ndour, P.A., Buffet, P., 2020. The human spleen in malaria: filter or shelter? Trends Parasitol. 36, 435–446. https://doi.org/10.1016/j.pt.2020.03.001

Hien, T.T., Thuy-Nhien, N.T., Phu, N.H., Boni, M.F., Thanh, N.V., Nha-Ca, N.T., Thai, L.H., Thai, C.Q., Van Toi, P., Thuan, P.D., Long, L.T., Dong, L.T., Merson, L., Dolecek, C., Stepniewska, K., Ringwald, P., White, N.J., Farrar, J., Wolbers, M., 2012. *In vivo* susceptibility of *Plasmodium falciparum* to artesunate in Binh Phuoc Province, Vietnam. Malar. J. 11, 355. https://doi.org/10.1186/1475-2875-11-355

Higgins, C.F., 2007. Multiple molecular mechanisms for multidrug resistance transporters. Nature 446, 749–757. https://doi.org/10.1038/nature05630

Hill, R.A., Goodwin, M.H., 1937. Prontosil in treatment of malaria report of 100 cases. South. Med. J. 30, 1170–1172. https://doi.org/10.1097/00007611-193712000-00007

Holmgren, G., Gil, J.P., Ferreira, P.M., Veiga, M.I., Obonyo, C.O., Björkman, A., 2006. Amodiaquine resistant *Plasmodium falciparum* malaria *in vivo* is associated with selection of *pfcrt* 76T and *pfmdr1* 86Y. Infect. Genet. Evol. 6, 309–314. https://doi.org/10.1016/j.meegid.2005.09.001

Hoo, R., Bruske, E., Dimonte, S., Zhu, L., Mordmüller, B., Sim, B.K.L., Kremsner, P.G., Hoffman, S.L., Bozdech, Z., Frank, M., Preiser, P.R., 2019. Transcriptome profiling reveals functional variation in *Plasmodium falciparum* parasites from controlled human malaria infection studies. EBioMedicine 48, 442–452. https://doi.org/10.1016/j.ebiom.2019.09.001

Hooijberg, J.H., Broxterman, H.J., Kool, M., Assaraf, Y.G., Peters, G.J., Noordhuis, P., Scheper, R.J., Borst, P., Pinedo, H.M., Jansen, G., 1999. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res. 59, 2532–2535.

Howes, R.E., Battle, K.E., Mendis, K.N., Smith, D.L., Cibulskis, R.E., Baird, J.K., Hay, S.I., 2016. Global epidemiology of *Plasmodium vivax*. Am. J. Trop. Med. Hyg. 95, 15–34. https://doi.org/10.4269/ajtmh.16-0141

Hsu, E., 2006. Reflections on the 'discovery' of the antimalarial qinghao. Br. J. Clin. Pharmacol. 61, 666–670. https://doi.org/10.1111/j.1365-2125.2006.02673.x

Huang, F., Takala-Harrison, S., Jacob, C.G., Liu, H., Sun, X., Yang, H., Nyunt, M.M., Adams, M., Zhou, S., Xia, Z., Ringwald, P., Bustos, M.D., Tang, L., Plowe, C.V., 2015. A single mutation in K13 predominates in Southern China and Is associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment. J. Infect. Dis. 212, 1629–1635. https://doi.org/10.1093/infdis/jiv249

Huang, J., Mullapudi, N., Lancto, C.A., Scott, M., Abrahamsen, M.S., Kissinger, J.C., 2004a. Phylogenomic evidence supports past endosymbiosis, intracellular and horizontal gene transfer in *Cryptosporidium parvum*. Genome Biol. 5, R88. https://doi.org/10.1186/gb-2004-5-11-r88

Huang, J., Mullapudi, N., Sicheritz-Ponten, T., Kissinger, J.C., 2004b. A first glimpse into the pattern and scale of gene transfer in the Apicomplexa. Int. J. Parasitol. 34, 265–274. https://doi.org/10.1016/j.ijpara.2003.11.025

Hurly, M.G.D., 1959. Potentiation of pyrimethamine by sulphadiazine in human malaria. Trans. R. Soc. Trop. Med. Hyg. 53, 412–413. https://doi.org/10.1016/0035-9203(59)90044-6

Hyde, J.E., 2005. Exploring the folate pathway in *Plasmodium falciparum*. Acta Trop. 94, 191–206. https://doi.org/10.1016/j.actatropica.2005.04.002

Hyde, J.E., Sims, P.F.G., 2001. Sulfa-drug resistance in *Plasmodium falciparum*. Trends Parasitol. 17, 265–266. https://doi.org/10.1016/S1471-4922(01)01949-3

Ibraheem, Z.O., Abd Majid, R., Noor, S.Mohd., Sedik, H.Mohd., Basir, R., 2014. Role of different *Pf*crt and *Pf*mdr-1 mutations in conferring resistance to antimalaria drugs in *Plasmodium falciparum*. Malar. Res. Treat. 2014. https://doi.org/10.1155/2014/950424

Ikeda, M., Kaneko, M., Tachibana, S.-I., Balikagala, B., Sakurai-Yatsushiro, M., Yatsushiro, S., Takahashi, N., Yamauchi, M., Sekihara, M., Hashimoto, M., Katuro, O.T., Olia, A., Obwoya, P.S., Auma, M.A., Anywar, D.A., Odongo-Aginya, E.I., Okello-Onen, J., Hirai, M., Ohashi, J., Palacpac, N.M.Q., Kataoka, M., Tsuboi, T., Kimura, E., Horii, T., Mita, T., 2018. Artemisinin-Resistant *Plasmodium falciparum* with high survival rates, Uganda, 2014-2016. Emerg. Infect. Dis. 24, 718–726. https://doi.org/10.3201/eid2404.170141

Imwong, M., Dhorda, M., Tun, K.M., Thu, A.M., Phyo, A.P., Proux, S., Suwannasin, K., Kunasol, C., Srisutham, S., Duanguppama, J., Vongpromek, R., Promnarate, C., Saejeng, A., Khantikul, N., Sugaram, R., Thanapongpichat, S., Sawangjaroen, N., Sutawong, K., Han, K.T., Htut, Y., Linn, K., Win, A.A., Hlaing, T.M., Pluijm, R.W. van der, Mayxay, M., Pongvongsa, T., Phommasone, K., Tripura, R., Peto, T.J., Seidlein, L. von, Nguon, C., Lek, D., Chan, X.H.S., Rekol, H., Leang, R., Huch, C., Kwiatkowski, D.P., Miotto, O., Ashley, E.A., Kyaw, M.P., Pukrittayakamee, S., Day, N.P.J., Dondorp, A.M., Smithuis, F.M., Nosten, F.H., White, N.J., 2020. Molecular epidemiology

of resistance to antimalarial drugs in the Greater Mekong subregion: an observational study. Lancet Infect. Dis. 20, 1470–1480. https://doi.org/10.1016/S1473-3099(20)30228-0

Imwong, M., Hien, T.T., Thuy-Nhien, N.T., Dondorp, A.M., White, N.J., 2017a. Spread of a single multidrug resistant malaria parasite lineage (*PfPailin*) to Vietnam. Lancet Infect. Dis. 17, 1022–1023. https://doi.org/10.1016/S1473-3099(17)30524-8

Imwong, M., Suwannasin, K., Kunasol, C., Sutawong, K., Mayxay, M., Rekol, H., Smithuis, F.M., Hlaing, T.M., Tun, K.M., van der Pluijm, R.W., Tripura, R., Miotto, O., Menard, D., Dhorda, M., Day, N.P.J., White, N.J., Dondorp, A.M., 2017b. The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: a molecular epidemiology observational study. Lancet Infect. Dis. 17, 491–497. https://doi.org/10.1016/S1473-3099(17)30048-8

Inselburg, J., Bzik, D.J., Horii, T., 1987. Pyrimethamine resistant *Plasmodium falciparum*: overproduction of dihydrofolate reductase by a gene duplication. Mol. Biochem. Parasitol. 26, 121–134. https://doi.org/10.1016/0166-6851(87)90136-8

Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs, Arrow, K.J., Panosian, C., Gelband, H., 2004. A brief history of malaria, Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance. National Academies Press (US).

Ismail, H.M., Barton, V., Phanchana, M., Charoensutthivarakul, S., Wong, M.H.L., Hemingway, J., Biagini, G.A., O'Neill, P.M., Ward, S.A., 2016. Artemisinin activity-based probes identify multiple molecular targets within the asexual stage of the malaria parasites *Plasmodium falciparum* 3D7. Proc. Natl. Acad. Sci. 113, 2080–2085. https://doi.org/10.1073/pnas.1600459113

Iyer, J.K., Milhous, W.K., Cortese, J.F., Kublin, J.G., Plowe, C.V., 2001. *Plasmodium falciparum* cross-resistance between trimethoprim and pyrimethamine. Lancet 358, 1066–1067. https://doi.org/10.1016/S0140-6736(01)06201-8

Jedlitschky, G., Leier, I., Buchholz, U., Barnouin, K., Kurz, G., Keppler, D., 1996. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res. 56, 988–994.

Jelinek, T., Kilian, A.H., Curtis, J., Duraisingh, M.T., Kabagambe, G., von Sonnenburg, F., Warhurst, D.C., 1999. *Plasmodium falciparum*: selection of serine 108 of dihydrofolate reductase during treatment of uncomplicated malaria with co-trimoxazole in Ugandan children. Am. J. Trop. Med. Hyg. 61, 125–130. https://doi.org/10.4269/ajtmh.1999.61.125

Jianfang, Z., 2013. A detailed chronological record of project 523 and the discovery and development of Qinghaosu. Strategic Book Publishing & Rights Agency, LLC.

Johnson, D.J., Fidock, D.A., Mungthin, M., Lakshmanan, V., Sidhu, A.B.S., Bray, P.G., Ward, S.A., 2004. Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol. Cell 15, 867–877. https://doi.org/10.1016/j.molcel.2004.09.012

Joice, R., Nilsson, S.K., Montgomery, J., Dankwa, S., Egan, E., Morahan, B., Seydel, K.B., Bertuccini, L., Alano, P., Williamson, K.C., Duraisingh, M.T., Taylor, T.E., Milner, D.A., Marti, M., 2014. *Plasmodium falciparum* transmission stages accumulate in the human bone marrow. Sci. Transl. Med. 6, 244re5. https://doi.org/10.1126/scitranslmed.3008882

Jones, R., Pullman, T.N., Whorton, C.M., Craige, B., Alving, A.S., Eichelberger, L., 1948. The therapeutic effectiveness of large doses of paludrine in acute attacks of sporozoite induced vivax malaria (chesson strain). J. Clin. Invest. 27, 51–55. https://doi.org/10.1172/JCI101971

Jones, S.A., 1954. Resistance of *P. falciparum* and *P. malariae* to pyrimethamine (daraprim) following mass treatment with this drug; a preliminary note. East Afr. Med. J. 31, 47–49.

Josling, G.A., Llinás, M., 2015. Sexual development in *Plasmodium parasites*: knowing when it's time to commit. Nat. Rev. Microbiol. 13, 573–587. https://doi.org/10.1038/nrmicro3519

Juge, N., Moriyama, S., Miyaji, T., Kawakami, M., Iwai, H., Fukui, T., Nelson, N., Omote, H., Moriyama, Y., 2015. *Plasmodium falciparum* chloroquine resistance transporter is a H+-coupled polyspecific

nutrient and drug exporter. Proc. Natl. Acad. Sci. 112, 3356–3361. https://doi.org/10.1073/pnas.1417102112

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K., Mumba, D., Kekre, M., Yavo, W., Mead, D., Bouyou-Akotet, M., Apinjoh, T., Golassa, L., Randrianarivelojosia, M., Andagalu, B., Maiga-Ascofare, O., Amambua-Ngwa, A., Tindana, P., Ghansah, A., MacInnis, B., Kwiatkowski, D., Djimde, A.A., 2015. K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. J. Infect. Dis. 211, 1352–1355. https://doi.org/10.1093/infdis/jiu608

Kapishnikov, S., Staalsø, T., Yang, Y., Lee, J., Pérez-Berná, A.J., Pereiro, E., Yang, Y., Werner, S., Guttmann, P., Leiserowitz, L., Als-Nielsen, J., 2019. Mode of action of quinoline antimalarial drugs in red blood cells infected by *Plasmodium falciparum* revealed in vivo. Proc. Natl. Acad. Sci. 116, 22946–22952. https://doi.org/10.1073/pnas.1910123116

Kasozi, D., Mohring, F., Rahlfs, S., Meyer, A.J., Becker, K., 2013. Real-Time imaging of the intracellular glutathione redox potential in the malaria parasite *Plasmodium falciparum*. PLoS Pathog. 9, 1–18. https://doi.org/10.1371/journal.ppat.1003782

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010

Kavishe, R.A., van den Heuvel, J.M.W., van de Vegte-Bolmer, M., Luty, A.J.F., Russel, F.G.M., Koenderink, J.B., 2009. Localization of the ATP-binding cassette (ABC) transport proteins PfMRP1, PfMRP2, and PfMDR5 at the Plasmodium falciparum plasma membrane. Malar. J. 8, 205. https://doi.org/10.1186/1475-2875-8-205

Khalil, I.F., Alifrangis, M., Tarimo, D.S., Staalsø, T., Satti, G.M.H., Theander, T.G., Rønn, A.M., Bygbjerg, I.C., 2005. The roles of the *pfcrt* 76T and *pfmdr1* 86Y mutations, immunity and the initial level of parasitaemia, in predicting the outcome of chloroquine treatment in two areas with different transmission intensities. Ann. Trop. Med. Parasitol. 99, 441–448. https://doi.org/10.1179/136485905X46441

Kho, S., Qotrunnada, L., Leonardo, L., Andries, B., Wardani, P.A.I., Fricot, A., Henry, B., Hardy, D., Margyaningsih, N.I., Apriyanti, D., Puspitasari, A.M., Prayoga, P., Trianty, L., Kenangalem, E., Chretien, F., Safeukui, I., Del Portillo, H.A., Fernandez-Becerra, C., Meibalan, E., Marti, M., Price, R.N., Woodberry, T., Ndour, P.A., Russell, B.M., Yeo, T.W., Minigo, G., Noviyanti, R., Poespoprodjo, J.R., Siregar, N.C., Buffet, P.A., Anstey, N.M., 2021. Hidden biomass of intact malaria parasites in the human spleen. N. Engl. J. Med. 384, 2067–2069. https://doi.org/10.1056/NEJMc2023884

Kim, A.I., Ghosh, P., Aaron, M.A., Bibb, L.A., Jain, S., Hatfull, G.F., 2003. Mycobacteriophage Bxb1 integrates into the Mycobacterium smegmatis groEL1 gene. Mol. Microbiol. 50, 463–473. https://doi.org/10.1046/j.1365-2958.2003.03723.x

Kim, D., Paggi, J.M., Park, C., Bennett, C., Salzberg, S.L., 2019. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat. Biotechnol. 37, 907–915. https://doi.org/10.1038/s41587-019-0201-4

Kim, J., Tan, Y.Z., Wicht, K.J., Erramilli, S.K., Dhingra, S.K., Okombo, J., Vendome, J., Hagenah, L.M., Giacometti, S.I., Warren, A.L., Nosol, K., Roepe, P.D., Potter, C.S., Carragher, B., Kossiakoff, A.A., Quick, M., Fidock, D.A., Mancia, F., 2019. Structure and drug resistance of the *Plasmodium falciparum* transporter PfCRT. Nature 576, 315–320. https://doi.org/10.1038/s41586-019-1795-x

Kirkman, L.A., Lawrence, E.A., Deitsch, K.W., 2014. Malaria parasites utilize both homologous recombination and alternative end joining pathways to maintain genome integrity. Nucleic Acids Res. 42, 370–379. https://doi.org/10.1093/nar/gkt881

Kitchen, L.W., Vaughn, D.W., Skillman, D.R., 2006. Role of US military research programs in the development of US Food and Drug Administration–approved antimalarial drugs. Clin. Infect. Dis. 43, 67–71. https://doi.org/10.1086/504873

Klokouzas, A., Tiffert, T., van Schalkwyk, D., Wu, C.-P., van Veen, H.W., Barrand, M.A., Hladky, S.B., 2004. *Plasmodium falciparum* expresses a multidrug resistance-associated protein. Biochem. Biophys. Res. Commun. 321, 197–201. https://doi.org/10.1016/j.bbrc.2004.06.135

Klonis, N., Crespo-Ortiz, M.P., Bottova, I., Abu-Bakar, N., Kenny, S., Rosenthal, P.J., Tilley, L., 2011. Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. Proc. Natl. Acad. Sci. 108, 11405–11410. https://doi.org/10.1073/pnas.1104063108

Klonis, N., Xie, S.C., McCaw, J.M., Crespo-Ortiz, M.P., Zaloumis, S.G., Simpson, J.A., Tilley, L., 2013. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. Proc. Natl. Acad. Sci. 110, 5157–5162. https://doi.org/10.1073/pnas.1217452110

Kone, A., Sissoko, S., Fofana, B., Sangare, C.O., Dembele, D., Haidara, A.S., Diallo, N., Coulibaly, A., Traore, A., Toure, S., Haidara, K., Sanogo, K., Sagara, I., Beshir, K.B., Gil, J.P., Doumbo, O.K., Djimde, A.A., 2020. Different *Plasmodium falciparum* clearance times in two Malian villages following artesunate monotherapy. Int. J. Infect. Dis. 95, 399–405. https://doi.org/10.1016/j.ijid.2020.03.082

Kremsner, P.G., Krishna, S., 2004. Antimalarial combinations. Lancet 364, 285–294. https://doi.org/10.1016/S0140-6736(04)16680-4

Krugliak, M., Zhang, J., Ginsburg, H., 2002. Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. Mol. Biochem. Parasitol. 119, 249–256. https://doi.org/10.1016/S0166-6851(01)00427-3

Krungkrai, J., Webster, H.K., Yuthavong, Y., 1989. De novo and salvage biosynthesis of pteroylpentaglutamates in the human malaria parasite, *Plasmodium falciparum*. Mol. Biochem. Parasitol. 32, 25–37. https://doi.org/10.1016/0166-6851(89)90126-6

Kublin, J.G., Cortese, J.F., Njunju, E.M., Mukadam, R.A.G., Wirima, J.J., Kazembe, P.N., Djimdé, A.A., Kouriba, B., Taylor, T.E., Plowe, C.V., 2003. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J. Infect. Dis. 187, 1870–1875. https://doi.org/10.1086/375419

Kumar, N., Zheng, H., 1990. Stage-specific gametocytocidal effect in vitro of the antimalaria drug qinghaosu on *Plasmodium falciparum*. Parasitol. Res. 76, 214–218. https://doi.org/10.1007/BF00930817

Kung, S.H., Lund, S., Murarka, A., McPhee, D., Paddon, C.J., 2018. Approaches and recent developments for the commercial production of semi-synthetic artemisinin. Front. Plant Sci. 9. https://doi.org/10.3389/fpls.2018.00087

Kyaw, M.P., Nyunt, M.H., Chit, K., Aye, M.M., Aye, K.H., Aye, M.M., Lindegardh, N., Tarning, J., Imwong, M., Jacob, C.G., Rasmussen, C., Perin, J., Ringwald, P., Nyunt, M.M., 2013. Reduced susceptibility of *Plasmodium falciparum* to artesunate in southern Myanmar. PLoS One 8, e57689. https://doi.org/10.1371/journal.pone.0057689

Laing, A.B., 1970. Studies on the chemotherapy of malaria: (II) Pyrimethamine resistance in The Gambia. Trans. R. Soc. Trop. Med. Hyg. 64, 569–80. https://doi.org/doi.org/10.1016/0035-9203(70)90079-9

Laing, A.B., 1968. Antimalarial effects of sulphormethoxine, diaphenylsulphone and separate combinations of these with pyrimethamine: a review of preliminary investigations carried out in Tanzania. J. Trop. Med. Hyg. 71, 27–35.

Laing, A.B.G., 1965. Treatment of acute falciparum malaria with Sulphorthodimethoxine (Fanasil). Brit. Med. J. 1, 905–907. https://doi.org/10.1136/bmj.1.5439.905

Lakshmanan, V., Bray, P.G., Verdier-Pinard, D., Johnson, D.J., Horrocks, P., Muhle, R.A., Alakpa, G.E., Hughes, R.H., Ward, S.A., Krogstad, D.J., Sidhu, A.B.S., Fidock, D.A., 2005. A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. EMBO J. 24, 2294–2305. https://doi.org/10.1038/sj.emboj.7600681

Laufer, M.K., Takala-Harrison, S., Dzinjalamala, F.K., Stine, O.C., Taylor, T.E., Plowe, C.V., 2010. Return of chloroquine-susceptible *Falciparum* malaria in Malawi was a reexpansion of diverse susceptible parasites. J. Infect. Dis. 202, 801–808. https://doi.org/10.1086/655659

Laufer, M.K., Thesing, P.C., Eddington, N.D., Masonga, R., Dzinjalamala, F.K., Takala, S.L., Taylor, T.E., Plowe, C.V., 2006. Return of chloroquine antimalarial efficacy in Malawi. N. Engl. J. Med. 355, 1959–1966. https://doi.org/10.1056/NEJMoa062032

Laurens, M.B., 2019. RTS,S/AS01 vaccine (Mosquirix™): an overview. Hum. Vaccin. Immunother. 16, 480–489. https://doi.org/10.1080/21645515.2019.1669415

Leang, R., Barrette, A., Bouth, D.M., Menard, D., Abdur, R., Duong, S., Ringwald, P., 2013a. Efficacy of dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010. Antimicrob. Agents Chemother. 57, 818–826. https://doi.org/10.1128/AAC.00686-12

Leang, R., Ros, S., Duong, S., Navaratnam, V., Lim, P., Ariey, F., Kiechel, J.-R., Ménard, D., Taylor, W.R., 2013b. Therapeutic efficacy of fixed dose artesunate-mefloquine for the treatment of acute, uncomplicated *Plasmodium falciparum* malaria in Kampong Speu, Cambodia. Malar. J. 12, 343. https://doi.org/10.1186/1475-2875-12-343

Lee, A.H., Symington, L.S., Fidock, D.A., 2014. DNA repair mechanisms and their biological roles in the malaria parasite *Plasmodium falciparum*. Microbiol. Mol. Biol. Rev. 78, 469–486. https://doi.org/10.1128/MMBR.00059-13

Lee, M., Choi, Y., Burla, B., Kim, Y.Y., Jeon, B., Maeshima, M., Yoo, J.Y., Martinoia, E., Lee, Y., 2008. The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO2. Nat. Cell Biol. 10, 1217–1223. https://doi.org/10.1038/ncb1782

Lee, M.C.S., Lindner, S.E., Lopez-Rubio, J.-J., Llinás, M., 2019. Cutting back malaria: CRISPR/Cas9 genome editing of *Plasmodium*. Brief. Funct. Genomics 18, 281–289. https://doi.org/10.1093/bfgp/elz012

Lee, R.S., Waters, A.P., Brewer, J.M., 2018. A cryptic cycle in haematopoietic niches promotes initiation of malaria transmission and evasion of chemotherapy. Nat. Commun. 9, 1689. https://doi.org/10.1038/s41467-018-04108-9

Lehane, A.M., Hayward, R., Saliba, K.J., Kirk, K., 2008. A verapamil-sensitive chloroquine-associated H+ leak from the digestive vacuole in chloroquine-resistant malaria parasites. J. Cell Sci. 121, 1624–1632. https://doi.org/10.1242/jcs.016758

Lehane, A.M., Kirk, K., 2008. Chloroquine resistance-conferring mutations in *pfcrt* give rise to a chloroquine-associated H+ leak from the malaria parasite's digestive vacuole. Antimicrob. Agents Chemother. 52, 4374–4380. https://doi.org/10.1128/AAC.00666-08

Lekostaj, J.K., Natarajan, J.K., Paguio, M.F., Wolf, C., Roepe, P.D., 2008. Photoaffinity labeling of the *Plasmodium falciparum* chloroquine resistance transporter with a novel perfluorophenylazido chloroquine. Biochemistry 47, 10394–10406. https://doi.org/10.1021/bi8010658

Leroy, D., Macintyre, F., Adoke, Y., Ouoba, S., Barry, A., Mombo-Ngoma, G., Ndong Ngomo, J.M., Varo, R., Dossou, Y., Tshefu, A.K., Duong, T.T., Phuc, B.Q., Laurijssens, B., Klopper, R., Khim, N., Legrand, E., Ménard, D., 2019. African isolates show a high proportion of multiple copies of the *Plasmodium falciparum plasmepsin-2 gene*, a piperaquine resistance marker. Malar. J. 18, 126. https://doi.org/10.1186/s12936-019-2756-4

Levin, B.R., Perrot, V., Walker, N., 2000. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics 154, 985–997. https://doi.org/10.1093/genetics/154.3.985

Lewinson, O., Livnat-Levanon, N., 2017. Mechanism of Action of ABC Importers: Conservation, Divergence, and Physiological Adaptations. J. Mol. Biol. 429, 606–619. https://doi.org/10.1016/j.jmb.2017.01.010

Lewis, I.A., Wacker, M., Olszewski, K.L., Cobbold, S.A., Baska, K.S., Tan, A., Ferdig, M.T., Llinás, M., 2014. Metabolic QTL analysis links chloroquine resistance in *Plasmodium falciparum* to impaired hemoglobin catabolism. PLoS Genet. 10, e1004085. https://doi.org/10.1371/journal.pgen.1004085

Li, Y., Wu, Y.-L., 2003. An over four millennium story behind qinghaosu (artemisinin)–a fantastic antimalarial drug from a traditional chinese herb. Curr. Med. Chem. 10, 2197–2230. https://doi.org/10.2174/0929867033456710

Li, Y., Wu, Y.L., 1998. How Chinese scientists discovered qinghaosu (artemisinin) and developed its derivatives? What are the future perspectives? Med. Trop. 58, 9–12.

Liu, W., Li, Y., Learn, G.H., Rudicell, R.S., Robertson, J.D., Keele, B.F., Ndjango, J.B.N., Sanz, C.M., Morgan, D.B., Locatelli, S., Gonder, M.K., Kranzusch, P.J., Walsh, P.D., Delaporte, E., Mpoudi-Ngole, E., Georgiev, A.V., Muller, M.N., Shaw, G.M., Peeters, M., Sharp, P.M., Rayner, J.C., Hahn, B.H., 2010. Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. Nature 467, 420–425. https://doi.org/10.1038/nature09442

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta_{CT}}$ Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Llinás, M., Bozdech, Z., Wong, E.D., Adai, A.T., DeRisi, J.L., 2006. Comparative whole genome transcriptome analysis of three *Plasmodium falciparum* strains. Nucleic Acids Res. 34, 1166–1173. https://doi.org/10.1093/nar/gkj517

Loesbanluechai, D., Kotanan, N., de Cozar, C., Kochakarn, T., Ansbro, M.R., Chotivanich, K., White, N.J., Wilairat, P., Lee, M.C.S., Gamo, F.J., Sanz, L.M., Chookajorn, T., Kümpornsin, K., 2019. Overexpression of *plasmepsin II* and *plasmepsin III* does not directly cause reduction in *Plasmodium falciparum* sensitivity to artesunate, chloroquine and piperaquine. Int. J. Parasitol. 9, 16–22. https://doi.org/10.1016/j.ijpddr.2018.11.004

Lubell, Y., Dondorp, A., Guérin, P.J., Drake, T., Meek, S., Ashley, E., Day, N.P., White, N.J., White, L.J., 2014. Artemisinin resistance – modelling the potential human and economic costs. Malar. J. 13, 452. https://doi.org/10.1186/1475-2875-13-452

Luchavez, J., Espino, F., Curameng, P., Espina, R., Bell, D., Chiodini, P., Nolder, D., Sutherland, C., Lee, K.-S., Singh, B., 2008. Human infections with *Plasmodium knowlesi*, the Philippines. Emerg. Infect. Dis. 14, 811–813. https://doi.org/10.3201/eid1405.071407

Macintyre, F., Adoke, Y., Tiono, A.B., Duong, T.T., Mombo-Ngoma, G., Bouyou-Akotet, M., Tinto, H., Bassat, Q., Issifou, S., Adamy, M., Demarest, H., Duparc, S., Leroy, D., Laurijssens, B.E., Biguenet, S., Kibuuka, A., Tshefu, A.K., Smith, M., Foster, C., Leipoldt, I., Kremsner, P.G., Phuc, B.Q., Ouedraogo, A., Ramharter, M., OZ-Piperaquine Study Group, 2017. A randomised, double-blind clinical phase II trial of the efficacy, safety, tolerability and pharmacokinetics of a single dose combination treatment with artefenomel and piperaquine in adults and children with uncomplicated *Plasmodium falciparum* malaria. BMC Med. 15, 181. https://doi.org/10.1186/s12916-017-0940-3

Maegraith, B.G., Adams, A.R.D., King, J.D., Tottey, M.M., Rigby, D.J., Sladden, R.A., 1946. Paludrine in the treatment of malaria. Brit. Med. J. 1.

Maegraith, B.G., Adams, A.R.D., King, J.D., Townshend, R.H., Davey, T.H., Havard, R.E., 1945. Studies on synthetic antimalarial drugs xiv results of a preliminary investigation of the therapeutic action

of 4888 (paludrine) on acute attacks of malignant tertian malaria. Ann. Trop. Med. Parasitol. 39, 232–236. https://doi.org/doi.org/10.1080/00034983.1945.11685241

Magallón-Tejada, A., Machevo, S., Cisteró, P., Lavstsen, T., Aide, P., Rubio, M., Jiménez, A., Turner, L., Valmaseda, A., Gupta, H., Salas, B.D.L., Mandomando, I., Wang, C.W., Petersen, J.E.V., Muñoz, J., Gascón, J., Macete, E., Alonso, P.L., Chitnis, C.E., Bassat, Q., Mayor, A., 2016. Cytoadhesion to gC1qR through *Plasmodium falciparum* erythrocyte membrane protein 1 in severe malaria. PLoS Pathog. 12, e1006011. https://doi.org/10.1371/journal.ppat.1006011

Maiga, A.W., Fofana, B., Sagara, I., Dembele, D., Dara, A., Traore, O.B., Toure, S., Sanogo, K., Dama, S., Sidibe, B., Kone, A., Thera, M.A., Plowe, C.V., Doumbo, O.K., Djimde, A.A., 2012. No evidence of delayed parasite clearance after oral artesunate treatment of uncomplicated *falciparum* malaria in Mali. Am. J. Trop. Med. Hyg. 87, 23–28. https://doi.org/10.4269/ajtmh.2012.12-0058

Makanga, M., Krudsood, S., 2009. The clinical efficacy of artemether/lumefantrine (Coartem®). Malar. J. 8, S5. https://doi.org/10.1186/1475-2875-8-S1-S5

Malmberg, M., Ferreira, P.E., Tarning, J., Ursing, J., Ngasala, B., Björkman, A., Mårtensson, A., Gil, J.P., 2013. *Plasmodium falciparum* drug resistance phenotype as assessed by patient antimalarial drug levels and its association with *pfmdr1* polymorphisms. J. Infect. Dis. 207, 842–847. https://doi.org/10.1093/infdis/jis747

Martin, R.E., 2020. The transportome of the malaria parasite. Biol. Rev. 95, 305–332. https://doi.org/10.1111/brv.12565

Martin, R.E., Henry, R.I., Abbey, J.L., Clements, J.D., Kirk, K., 2005. The "permeome" of the malaria parasite: an overview of the membrane transport proteins of *Plasmodium falciparum*. Genome Biol. 6, R26. https://doi.org/10.1186/gb-2005-6-3-r26

Martin, R.E., Marchetti, R.V., Cowan, A.I., Howitt, S.M., Bröer, S., Kirk, K., 2009. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science 325, 1680–1682. https://doi.org/10.1126/science.1175667

Martin, R.E., Shafik, S.H., Richards, S.N., 2018. Mechanisms of resistance to the partner drugs of artemisinin in the malaria parasite. Curr. Opin. Pharmacol. 42, 71–80. https://doi.org/10.1016/j.coph.2018.07.010

Martin, S.K., Oduola, A.M., Milhous, W.K., 1987. Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science 235, 899–901. https://doi.org/10.1126/science.3544220

Martiney, J.A., Cerami, A., Slater, A.F., 1995. Verapamil reversal of chloroquine resistance in the malaria parasite *Plasmodium falciparum* is specific for resistant parasites and independent of the weak base effect. J. Biol. Chem. 270, 22393–22398. https://doi.org/10.1074/jbc.270.38.22393

Mathieu, L.C., Cox, H., Early, A.M., Mok, S., Lazrek, Y., Paquet, J.-C., Ade, M.-P., Lucchi, N.W., Grant, Q., Udhayakumar, V., Alexandre, J.S., Demar, M., Ringwald, P., Neafsey, D.E., Fidock, D.A., Musset, L., 2020. Local emergence in Amazonia of *Plasmodium falciparum k13* C580Y mutants associated with *in vitro* artemisinin resistance. Elife 9. https://doi.org/10.7554/eLife.51015

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R.V., Rizk, S.S., Njimoh, D.L., Ryan, Y., Chotivanich, K., Nguon, C., Ghorbal, M., Lopez-Rubio, J.-J., Pfrender, M., Emrich, S., Mohandas, N., Dondorp, A.M., Wiest, O., Haldar, K., 2015. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. Nature 520, 683–687. https://doi.org/10.1038/nature14412

McGinnis, S., Madden, T.L., 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 32, W20-25. https://doi.org/10.1093/nar/gkh435

McKie, J.H., Douglas, K.T., Chan, C., Roser, S.A., Yates, R., Read, M., Hyde, J.E., Dascombe, M.J., Yuthavong, Y., Sirawaraporn, W., 1998. Rational Drug Design Approach for Overcoming Drug

Resistance: Application to Pyrimethamine Resistance in Malaria. J. Med. Chem. 41, 1367–1370. https://doi.org/10.1021/jm970845u

Mehlotra, R.K., Mattera, G., Bhatia, K., Reeder, J.C., Stoneking, M., Zimmerman, P.A., 2005. Insight into the early spread of chloroquine-resistant *Plasmodium falciparum* infections in Papua New Guinea. J. Infect. Dis. 192, 2174–2179. https://doi.org/10.1086/497694

Meibalan, E., Marti, M., 2017. Biology of malaria transmission. Cold Spring Harb. Perspect. Med. 7. https://doi.org/10.1101/cshperspect.a025452

Ménard, D., Khim, N., Beghain, J., Adegnika, A.A., Shafiul-Alam, M., Amodu, O., Rahim-Awab, G., Barnadas, C., Berry, A., Boum, Y., Bustos, M.D., Cao, J., Chen, J.-H., Collet, L., Cui, L., Thakur, G.-D., Dieye, A., Djallé, D., Dorkenoo, M.A., Eboumbou-Moukoko, C.E., Espino, F.-E.-C.J., Fandeur, T., Ferreira-da-Cruz, M.-F., Fola, A.A., Fuehrer, H.-P., Hassan, A.M., Herrera, S., Hongvanthong, B., Houzé, S., Ibrahim, M.L., Jahirul-Karim, M., Jiang, L., Kano, S., Ali-Khan, W., Khanthavong, M., Kremsner, P.G., Lacerda, M., Leang, R., Leelawong, M., Li, M., Lin, K., Mazarati, J.-B., Ménard, S., Morlais, I., Muhindo-Mavoko, H., Musset, L., Na-Bangchang, K., Nambozi, M., Niaré, K., Noedl, H., Ouédraogo, J.-B., Pillai, D.R., Pradines, B., Quang-Phuc, B., Ramharter, M., Randrianarivelojosia, M., Sattabongkot, J., Sheikh-Omar, A., Silué, K.D., Sirima, S.B., Sutherland, C., Syafruddin, D., Tahar, R., Tang, L.-H., Touré, O.A., Tshibangu-wa-Tshibangu, P., Vigan-Womas, I., Warsame, M., Wini, L., Zakeri, S., Kim, S., Eam, R., Berne, L., Khean, C., Chy, S., Ken, M., Loch, K., Canier, L., Duru, V., Legrand, E., Barale, J.-C., Stokes, B., Straimer, J., Witkowski, B., Fidock, D.A., Rogier, C., Ringwald, P., Ariey, F., Mercereau-Puijalon, O., KARMA Consortium, 2016. A worldwide map of *Plasmodium falciparum* K13-propeller polymorphisms. N. Engl. J. Med. 374, 2453–2464. https://doi.org/10.1056/NEJMoa1513137

Menard, D., Matsika-Claquin, M.D., Djalle, D., Yapou, F., Manirakiza, A., Dolmazon, V., Sarda, J., Talarmin, A., 2005. Association of failures of seven-day courses of artesunate in a non-immune population in Bangui, Central African Republic with decreased sensitivity of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 73, 616–621.

Merrick, C.J., 2021. Hypnozoites in *Plasmodium*: Do parasites parallel plants? Trends Parasitol. 37, 273–282. https://doi.org/10.1016/j.pt.2020.11.001

Meshnick, S.R., Taylor, T.E., Kamchonwongpaisan, S., 1996. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol. Rev. 60, 301–315. https://doi.org/10.1128/mr.60.2.301-315.1996

Meshnick, S.R., Yang, Y.Z., Lima, V., Kuypers, F., Kamchonwongpaisan, S., Yuthavong, Y., 1993. Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). Antimicrob. Agents Chemother. 37, 1108–1114. https://doi.org/10.1128/AAC.37.5.1108

Meunier, B., Robert, A., 2010. Heme as trigger and target for trioxane-containing antimalarial drugs. Acc. Chem. Res. 43, 1444–1451. https://doi.org/10.1021/ar100070k

Milani, K.J., Schneider, T.G., Taraschi, T.F., 2015. Defining the morphology and mechanism of the hemoglobin transport pathway in *Plasmodium falciparum*-infected erythrocytes. Eukaryot. Cell 14, 415–426. https://doi.org/10.1128/EC.00267-14

Milhous, W.K., Weatherly, N.F., Bowdre, J.H., Desjardins, R.E., 1985. *In vitro* activities of and mechanisms of resistance to antifol antimalarial drugs. Antimicrob. Agents Chemother. 27, 525–530. https://doi.org/10.1128/AAC.27.4.525

Miller, K.D., Lobel, H.O., Pappaioanou, M., Patchen, L.C., Churchill, F.C., 1986. Failures of combined chloroquine and Fansidar prophylaxis in American travelers to East Africa. J. Infect. Dis. 154, 689–691. https://doi.org/10.1093/infdis/154.4.689

Miller, L.H., Baruch, D.I., Marsh, K., Doumbo, O.K., 2002. The pathogenic basis of malaria. Nature 415, 673–679. https://doi.org/10.1038/415673a

Minh, B.Q., Nguyen, M.A.T., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. 30, 1188–1195. https://doi.org/10.1093/molbev/mst024

Miotto, O., Almagro-Garcia, J., Manske, M., Macinnis, B., Campino, S., Rockett, K.A., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J.M., Duong, S., Nguon, C., Chuor, C.M., Saunders, D., Se, Y., Lon, C., Fukuda, M.M., Amenga-Etego, L., Hodgson, A.V.O., Asoala, V., Imwong, M., Takala-Harrison, S., Nosten, F., Su, X.-Z., Ringwald, P., Ariey, F., Dolecek, C., Hien, T.T., Boni, M.F., Thai, C.Q., Amambua-Ngwa, A., Conway, D.J., Djimdé, A.A., Doumbo, O.K., Zongo, I., Ouedraogo, J.-B., Alcock, D., Drury, E., Auburn, S., Koch, O., Sanders, M., Hubbart, C., Maslen, G., Ruano-Rubio, V., Jyothi, D., Miles, A., O'Brien, J., Gamble, C., Oyola, S.O., Rayner, J.C., Newbold, C.I., Berriman, M., Spencer, C.C.A., McVean, G., Day, N.P., White, N.J., Bethell, D., Dondorp, A.M., Plowe, C.V., Fairhurst, R.M., Kwiatkowski, D.P., 2013. Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. Nat. Genet. 45, 648–655. https://doi.org/10.1038/ng.2624

Miotto, O., Amato, R., Ashley, E.A., MacInnis, B., Almagro-Garcia, J., Amaratunga, C., Lim, P., Mead, D., Oyola, S.O., Dhorda, M., Imwong, M., Woodrow, C., Manske, M., Stalker, J., Drury, E., Campino, S., Amenga-Etego, L., Thanh, T.-N.N., Tran, H.T., Ringwald, P., Bethell, D., Nosten, F., Phyo, A.P., Pukrittayakamee, S., Chotivanich, K., Chuor, C.M., Nguon, C., Suon, S., Sreng, S., Newton, P.N., Mayxay, M., Khanthavong, M., Hongvanthong, B., Htut, Y., Han, K.T., Kyaw, M.P., Faiz, M.A., Fanello, C.I., Onyamboko, M., Mokuolu, O.A., Jacob, C.G., Takala-Harrison, S., Plowe, C.V., Day, N.P., Dondorp, A.M., Spencer, C.C.A., McVean, G., Fairhurst, R.M., White, N.J., Kwiatkowski, D.P., 2015. Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. Nat. Genet. 47, 226–234. https://doi.org/10.1038/ng.3189

Miotto, O., Sekihara, M., Tachibana, S.-I., Yamauchi, M., Pearson, R.D., Amato, R., Gonçalves, S., Mehra, S., Noviyanti, R., Marfurt, J., Auburn, S., Price, R.N., Mueller, I., Ikeda, M., Mori, T., Hirai, M., Tavul, L., Hetzel, M.W., Laman, M., Barry, A.E., Ringwald, P., Ohashi, J., Hombhanje, F., Kwiatkowski, D.P., Mita, T., 2020. Emergence of artemisinin-resistant *Plasmodium falciparum* with kelch13 C580Y mutations on the island of New Guinea. PLoS Pathog. 16, e1009133. https://doi.org/10.1371/journal.ppat.1009133

Mohring, F., Hart, M.N., Rawlinson, T.A., Henrici, R., Charleston, J.A., Diez Benavente, E., Patel, A., Hall, J., Almond, N., Campino, S., Clark, T.G., Sutherland, C.J., Baker, D.A., Draper, S.J., Moon, R.W., 2019. Rapid and iterative genome editing in the malaria parasite *Plasmodium knowlesi* provides new tools for *P. vivax* research. ELife 8. https://doi.org/10.7554/elife.45829

Mok, S., Ashley, E.A., Ferreira, P.E., Zhu, L., Lin, Z., Yeo, T., Chotivanich, K., Imwong, M., Pukrittayakamee, S., Dhorda, M., Nguon, C., Lim, P., Amaratunga, C., Suon, S., Hien, T.T., Htut, Y., Faiz, M.A., Onyamboko, M.A., Mayxay, M., Newton, P.N., Tripura, R., Woodrow, C.J., Miotto, O., Kwiatkowski, D.P., Nosten, F., Day, N.P.J., Preiser, P.R., White, N.J., Dondorp, A.M., Fairhurst, R.M., Bozdech, Z., 2015. Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science 347, 431–435. https://doi.org/10.1126/science.1260403

Mok, S., Stokes, B.H., Gnädig, N.F., Ross, L.S., Yeo, T., Amaratunga, C., Allman, E., Solyakov, L., Bottrill, A.R., Tripathi, J., Fairhurst, R.M., Llinás, M., Bozdech, Z., Tobin, A.B., Fidock, D.A., 2021. Artemisinin-resistant K13 mutations rewire *Plasmodium falciparum*'s intra-erythrocytic metabolic program to enhance survival. Nat. Commun. 12, 530. https://doi.org/10.1038/s41467-020-20805-w

Molina-Franky, J., Cuy-Chaparro, L., Camargo, A., Reyes, C., Gómez, M., Salamanca, D.R., Patarroyo, M.A., Patarroyo, M.E., 2020. *Plasmodium falciparum* pre-erythrocytic stage vaccine development. Malar. J. 19, 56. https://doi.org/10.1186/s12936-020-3141-z

Moraes Barros, R.R., Straimer, J., Sa, J.M., Salzman, R.E., Melendez-Muniz, V.A., Mu, J., Fidock, D.A., Wellems, T.E., 2015. Editing the *Plasmodium vivax* genome, using zinc-finger nucleases. J. Infect. Dis. 211, 125–129. https://doi.org/10.1093/infdis/jiu423

Morgan, B., Ezerina, D., Amoako, T.N.E., Riemer, J., Seedorf, M., Dick, T.P., 2013. Multiple glutathione disulfide removal pathways mediate cytosolic redox homeostasis. Nat. Chem. Biol. 9, 119–125. https://doi.org/10.1038/nchembio.1142

Moser, K.A., Drábek, E.F., Dwivedi, A., Stucke, E.M., Crabtree, J., Dara, A., Shah, Z., Adams, M., Li, T., Rodrigues, P.T., Koren, S., Phillippy, A.M., Munro, J.B., Ouattara, A., Sparklin, B.C., Dunning Hotopp, J.C., Lyke, K.E., Sadzewicz, L., Tallon, L.J., Spring, M.D., Jongsakul, K., Lon, C., Saunders, D.L., Ferreira, M.U., Nyunt, M.M., Laufer, M.K., Travassos, M.A., Sauerwein, R.W., Takala-Harrison, S., Fraser, C.M., Sim, B.K.L., Hoffman, S.L., Plowe, C.V., Silva, J.C., 2020. Strains used in whole organism *Plasmodium falciparum* vaccine trials differ in genome structure, sequence, and immunogenic potential. Genome Med. 12, 6. https://doi.org/10.1186/s13073-019-0708-9

Mota, M.M., Hafalla, J.C.R., Rodriguez, A., 2002. Migration through host cells activates *Plasmodium* sporozoites for infection. Nat. Med. 8, 1318–1322. https://doi.org/10.1038/nm785

Mu, J., Awadalla, P., Duan, J., McGee, K.M., Keebler, J., Seydel, K., McVean, G.A.T., Su, X., 2007. Genome-wide variation and identification of vaccine targets in the *Plasmodium falciparum* genome. Nat. Genet. 39, 126–130. https://doi.org/10.1038/ng1924

Mu, J., Duan, J., Makova, K.D., Joy, D.A., Huynh, C.Q., Branch, O.H., Li, W.H., Su, X.Z., 2002. Chromosome-wide SNPs reveal an ancient origin for *Plasmodium falciparum*. Nature 418, 323–326. https://doi.org/10.1038/nature00836

Mu, J., Ferdig, M.T., Feng, X., Joy, D.A., Duan, J., Furuya, T., Subramanian, G., Aravind, L., Cooper, R.A., Wootton, J.C., Xiong, M., Su, X.Z., 2003. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol. Microbiol. 49, 977–989. https://doi.org/10.1046/j.1365-2958.2003.03627.x

Mu, J., Myers, R.A., Jiang, H., Liu, S., Ricklefs, S., Waisberg, M., Chotivanich, K., Wilairatana, P., Krudsood, S., White, N.J., Udomsangpetch, R., Cui, L., Ho, M., Ou, F., Li, H., Song, J., Li, G., Wang, X., Seila, S., Sokunthea, S., Socheat, D., Sturdevant, D.E., Porcella, S.F., Fairhurst, R.M., Wellems, T.E., Awadalla, P., Su, X., 2010. *Plasmodium falciparum* genome-wide scans for positive selection, recombination hot spots and resistance to antimalarial drugs. Nat. Genet. 42, 268–271. https://doi.org/10.1038/ng.528

Mukherjee, A., Bopp, S., Magistrado, P., Wong, W., Daniels, R., Demas, A., Schaffner, S., Amaratunga, C., Lim, P., Dhorda, M., Miotto, O., Woodrow, C., Ashley, E.A., Dondorp, A.M., White, N.J., Wirth, D., Fairhurst, R., Volkman, S.K., 2017. Artemisinin resistance without *pfkelch13* mutations in *Plasmodium falciparum* isolates from Cambodia. Malar. J. 16, 195. https://doi.org/10.1186/s12936-017-1845-5

Mukherjee, A., Gagnon, D., Wirth, D.F., Richard, D., 2018. Inactivation of *Plasmepsins 2 and 3* sensitizes *Plasmodium falciparum* to the antimalarial drug piperaquine. Antimicrob. Agents Chemother. 62. https://doi.org/10.1128/AAC.02309-17

Müller, I.B., Hyde, J.E., 2013. Folate metabolism in human malaria parasites–75 years on. Mol. Biochem. Parasitol. 188, 63–77. https://doi.org/10.1016/j.molbiopara.2013.02.008

Mungthin, M., Watanatanasup, E., Sitthichot, N., Suwandittakul, N., Khositnithikul, R., Ward, S.A., 2017. Influence of the *pfmdr1* gene on *In Vitro* sensitivities of piperaquine in Thai isolates of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 96, 624–629. https://doi.org/10.4269/ajtmh.16-0668

Murithi, J.M., Deni, I., Pasaje, C.F.A., Okombo, J., Bridgford, J.L., Gnädig, N.F., Edwards, R.L., Yeo, T., Mok, S., Burkhard, A.Y., Coburn-Flynn, O., Istvan, E.S., Sakata-Kato, T., Gomez-Lorenzo, M.G.,

Cowell, A.N., Wicht, K.J., Le Manach, C., Kalantarov, G.F., Dey, S., Duffey, M., Laleu, B., Lukens, A.K., Ottilie, S., Vanaerschot, M., Trakht, I.N., Gamo, F.-J., Wirth, D.F., Goldberg, D.E., Odom John, A.R., Chibale, K., Winzeler, E.A., Niles, J.C., Fidock, D.A., 2021. The *Plasmodium falciparum* ABC transporter ABCI3 confers parasite strain-dependent pleiotropic antimalarial drug resistance. Cell Chem. Biol. https://doi.org/10.1016/j.chembiol.2021.06.006

Mutabingwa, T., Nzila, A., Mberu, E., Nduati, E., Winstanley, P., Hills, E., Watkins, W., 2001. Chlorproguanil-dapsone for treatment of drug-resistant falciparum malaria in Tanzania. Lancet 358, 1218–1223. https://doi.org/10.1016/S0140-6736(01)06344-9

Nacer, A., Claes, A., Roberts, A., Scheidig-Benatar, C., Sakamoto, H., Ghorbal, M., Lopez-Rubio, J.J., Mattei, D., 2015. Discovery of a novel and conserved *Plasmodium falciparum* exported protein that is important for adhesion of PfEMP1 at the surface of infected erythrocytes. Cell. Microbiol. 17, 1205–1216. https://doi.org/10.1111/cmi.12430

Naing, C., Whittaker, M.A., Htet, N.H., Aye, S.N., Mak, J.W., 2019. Efficacy of antimalarial drugs for treatment of uncomplicated *falciparum* malaria in Asian region: A network meta-analysis. PLOS ONE 14, e0225882. https://doi.org/10.1371/journal.pone.0225882

Nájera, J.A., González-Silva, M., Alonso, P.L., 2011. Some lessons for the future from the global malaria eradication programme (1955–1969). PLoS Med. 8. https://doi.org/10.1371/journal.pmed.1000412

Nduati, E., Diriye, A., Ommeh, S., Mwai, L., Kiara, S., Masseno, V., Kokwaro, G., Nzila, A., 2008. Effect of folate derivatives on the activity of antifolate drugs used against malaria and cancer. Parasitol. Res. 102, 1227–1234. https://doi.org/10.1007/s00436-008-0897-4

Nessler, S., Friedrich, O., Bakouh, N., Fink, R.H.A., Sanchez, C.P., Planelles, G., Lanzer, M., 2004. Evidence for activation of endogenous transporters in *Xenopus laevis* oocytes expressing the *Plasmodium falciparum* chloroquine resistance transporter, PfCRT. J. Biol. Chem. 279, 39438–39446. https://doi.org/10.1074/jbc.M404671200

Newbold, C.I., Boyle, D.B., Smith, C.C., Brown, K.N., 1982. Stage specific protein and nucleic acid synthesis during the asexual cycle of the rodent malaria *Plasmodium chabaudi*. Mol. Biochem. Parasitol. 5, 33–44. https://doi.org/10.1016/0166-6851(82)90004-4

Ng, C.L., Siciliano, G., Lee, M.C.S., de Almeida, M.J., Corey, V.C., Bopp, S.E., Bertuccini, L., Wittlin, S., Kasdin, R.G., Le Bihan, A., Clozel, M., Winzeler, E.A., Alano, P., Fidock, D.A., 2016. CRISPR-Cas9-modified pfmdr1 protects *Plasmodium falciparum* asexual blood stages and gametocytes against a class of piperazine-containing compounds but potentiates artemisinin-based combination therapy partner drugs. Mol. Microbiol. 101, 381–393. https://doi.org/10.1111/mmi.13397

Ngotho, P., Soares, A.B., Hentzschel, F., Achcar, F., Bertuccini, L., Marti, M., 2019. Revisiting gametocyte biology in malaria parasites. FEMS Microbiol. Rev. 43, 401–414. https://doi.org/10.1093/femsre/fuz010

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300

Nguyen, M.H., Davis, T.M.E., Cox-Singh, J., Hewitt, S., Tran, Q.T., Tran, B.K., Nguyen, T.H., Vo, N.P., Doan, H.N., Le, D.C., 2003. Treatment of uncomplicated falciparum malaria in southern Vietnam: can chloroquine or sulfadoxine-pyrimethamine be reintroduced in combination with artesunate? Clin. Infect. Dis. 37, 1461–1466. https://doi.org/10.1086/379323

Nguyen-Dinh, P., Spencer, H.C., Chemangey-Masaba, S., Churchill, F.C., 1982. Susceptibility of *Plasmodium falciparum* to pyrimethamine and sulfadoxine/pyrimethamine in Kisumu, Kenya. Lancet 1, 823–825. https://doi.org/10.1016/s0140-6736(82)91873-6

Ngwa, C.J., Kiesow, M.J., Papst, O., Orchard, L.M., Filarsky, M., Rosinski, A.N., Voss, T.S., Llinás, M., Pradel, G., 2017. Transcriptional profiling defines histone acetylation as a regulator of gene expression during human-to-mosquito transmission of the malaria parasite *Plasmodium falciparum*. Front Cell Infect. Microbiol. 7, 320. https://doi.org/10.3389/fcimb.2017.00320

Niven, J.C., 1938. Sulphanilamide in the treatment of malaria. Trans. R. Soc. Trop. Med. Hyg. 32, 413–418. https://doi.org/doi.org/10.1016/S0035-9203(38)90055-0

Nkrumah, L.J., Muhle, R.A., Moura, P.A., Ghosh, P., Hatfull, G.F., Jacobs, W.R., Fidock, D.A., 2006. Efficient site-specific integration in *Plasmodium falciparum* chromosomes mediated by mycobacteriophage Bxb1 integrase. Nat. Methods 3, 615–621. https://doi.org/10.1038/nmeth904

Nobel Media AB 2021, 2021. The nobel prize in physiology or medicine 2015 [WWW Document]. NobelPrize.org. URL https://www.nobelprize.org/prizes/medicine/2015/advanced-information/ (accessed 3.5.21).

Noedl, H., 2005. Artemisinin resistance: how can we find it? Trends Parasitol. 21, 404–405. https://doi.org/10.1016/j.pt.2005.06.012

Noedl, H., Se, Y., Schaecher, K., Smith, B.L., Socheat, D., Fukuda, M.M., Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium, 2008. Evidence of artemisinin-resistant malaria in western Cambodia. N. Engl. J. Med. 359, 2619–2620. https://doi.org/10.1056/NEJMc0805011

Nogueira, F., Lopes, D., Alves, A.C., Estólio Do Rosário, V., 2008. *Plasmodium falciparum* multidrug resistance protein (MRP) gene expression under chloroquine and mefloquine challenge. J. Cell Anim. Biol. 2, 10–20.

Nogueira, P.A., Wunderlich, G., Pereira da Silva, L.H., 2001. Variant antigens of *Plasmodium falciparum* encoded by the var multigenic family are multifunctional macromolecules. Res. Microbiol. 152, 141–147. https://doi.org/10.1016/s0923-2508(01)01184-6

Nosten, F., van Vugt, M., Price, R., Luxemburger, C., Thway, K.L., Brockman, A., McGready, R., ter Kuile, F., Looareesuwan, S., White, N.J., 2000. Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. Lancet 356, 297–302. https://doi.org/10.1016/s0140-6736(00)02505-8

Nosten, F., White, N.J., 2007. Artemisinin-based combination treatment of falciparum malaria. Am. J. Trop. Med. Hyg. 77, 181–192.

Novartis, 2021. Novartis reaches milestone delivery of 1 billion courses of antimalarial treatment | Novartis [WWW Document]. URL https://novartis.gcs-web.com/Novartis-reaches-milestone-delivery-of-1-billion-courses-of-antimalarial-treatment (accessed 4.19.22).

Nzila, A., Ward, S.A., Marsh, K., Sims, P.F.G., Hyde, J.E., 2005. Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy. Trends Parasitol. 21, 292–298. https://doi.org/10.1016/j.pt.2005.04.002

Nzila, A.M., Mberu, E.K., Sulo, J., Dayo, H., Winstanley, P.A., Sibley, C.H., Watkins, W.M., 2000. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. Antimicrob. Agents Chemother. 44, 991–996. https://doi.org/10.1128/aac.44.4.991-996.2000

Nzila-Mounda, A., Mberu, E.K., Sibley, C.H., Plowe, C.V., Winstanley, P.A., Watkins, W.M., 1998. Kenyan *Plasmodium falciparum* Field Isolates: Correlation between Pyrimethamine and Chlorcycloguanil Activity *In Vitro* and Point Mutations in the Dihydrofolate Reductase Domain. Antimicrob. Agents Chemother. 42, 164–169. https://doi.org/10.1128/AAC.42.1.164

Ocan, M., Akena, D., Nsobya, S., Kamya, M.R., Senono, R., Kinengyere, A.A., Obuku, E., 2019. K13-propeller gene polymorphisms in *Plasmodium falciparum* parasite population in malaria affected

countries: a systematic review of prevalence and risk factors. Malar. J. 18, 60. https://doi.org/10.1186/s12936-019-2701-6

Okombo, J., Abdi, A.I., Kiara, S.M., Mwai, L., Pole, L., Sutherland, C.J., Nzila, A., Ochola-Oyier, L.I., 2013. Repeat polymorphisms in the low-complexity regions of *Plasmodium falciparum* ABC transporters and associations with *in vitro* antimalarial responses. Antimicrob. Agents Chemother. 57, 6196–6204. https://doi.org/10.1128/AAC.01465-13

Olliaro, P., 2001. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol. Ther. 89, 207–219. https://doi.org/10.1016/s0163-7258(00)00115-7

Olliaro, P.L., Taylor, W.R., 2004. Developing artemisinin based drug combinations for the treatment of drug resistant falciparum malaria: A review. J. Postgrad. Med. 50, 40–44.

O'Neill, P.M., Posner, G.H., 2004. A medicinal chemistry perspective on artemisinin and related endoperoxides. J. Med. Chem. 47, 2945–2964. https://doi.org/10.1021/jm030571c

Ord, R., Alexander, N., Dunyo, S., Hallett, R., Jawara, M., Targett, G., Drakeley, C.J., Sutherland, C.J., 2007. Seasonal carriage of *pfcrt* and *pfmdr1* alleles in Gambian *Plasmodium falciparum* imply reduced fitness of chloroquine-resistant parasites. J. Infect. Dis. 196, 1613–1619. https://doi.org/10.1086/522154

Ouattara, A., Kone, A., Adams, M., Fofana, B., Maiga, A.W., Hampton, S., Coulibaly, D., Thera, M.A., Diallo, N., Dara, A., Sagara, I., Gil, J.P., Bjorkman, A., Takala-Harrison, S., Doumbo, O.K., Plowe, C.V., Djimde, A.A., 2015. Polymorphisms in the K13-propeller gene in artemisinin-susceptible *Plasmodium falciparum* parasites from Bougoula-Hameau and Bandiagara, Mali. Am. J. Trop. Med. Hyg. 92, 1202–1206. https://doi.org/10.4269/ajtmh.14-0605

Pacheco, C., Moreno, J., Herrera, F., 2019. A high number of *pfmdr1* gene copies in *P. falciparum* from Venezuela. Parasitol. Res. 118, 3085–3089. https://doi.org/10.1007/s00436-019-06409-4

Packard, R.M., 2014. The origins of antimalarial-drug resistance. N. Engl. J. Med. 371, 397–399. https://doi.org/10.1056/NEJMp1403340

Paddon, C.J., Keasling, J.D., 2014. Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nat. Rev. Microbiol. 12, 355–367. https://doi.org/10.1038/nrmicro3240

Park, D.J., Lukens, A.K., Neafsey, D.E., Schaffner, S.F., Chang, H.-H., Valim, C., Ribacke, U., Tyne, D.V., Galinsky, K., Galligan, M., Becker, J.S., Ndiaye, D., Mboup, S., Wiegand, R.C., Hartl, D.L., Sabeti, P.C., Wirth, D.F., Volkman, S.K., 2012. Sequence-based association and selection scans identify drug resistance loci in the *Plasmodium falciparum* malaria parasite. Proc. Natl. Acad. Sci. 109, 13052–13057. https://doi.org/10.1073/pnas.1210585109

PATH, 2020. A Phase 2b randomized, open-label, controlled, single center study in *Plasmodium Falciparum*-infected and uninfected adults age 18-55 years old in Kenya to evaluate the efficacy of the delayed, fractional dose RTS,S/AS01E malaria vaccine in subjects treated with artemisinin combination therapy plus primaquine (Clinical trial registration No. NCT04661579). clinicaltrials.gov.

Paton, D.G., Childs, L.M., Itoe, M.A., Holmdahl, I.E., Buckee, C.O., Catteruccia, F., 2019. Exposing *Anopheles* mosquitoes to antimalarials blocks *Plasmodium* parasite transmission. Nature 567, 239–243. https://doi.org/10.1038/s41586-019-0973-1

Patzewitz, E.-M., Salcedo-Sora, J.E., Wong, E.H., Sethia, S., Stocks, P.A., Maughan, S.C., Murray, J.A.H., Krishna, S., Bray, P.G., Ward, S.A., Müller, S., 2013. Glutathione transport: A new role for PfCRT in chloroquine resistance. Antioxid. Redox Signal. 19, 683–695. https://doi.org/10.1089/ars.2012.4625

Petersen, I., Eastman, R., Lanzer, M., 2011. Drug-resistant malaria: molecular mechanisms and implications for public health. FEBS Lett. 585, 1551–1562. https://doi.org/10.1016/j.febslet.2011.04.042

Petersen, I., Gabryszewski, S.J., Johnston, G.L., Dhingra, S.K., Ecker, A., Lewis, R.E., de Almeida, M.J., Straimer, J., Henrich, P.P., Palatulan, E., Johnson, D.J., Coburn-Flynn, O., Sanchez, C., Lehane, A.M., Lanzer, M., Fidock, D.A., 2015. Balancing drug resistance and growth rates via compensatory mutations in the *Plasmodium falciparum* chloroquine resistance transporter. Mol. Microbiol. 97, 381–395. https://doi.org/10.1111/mmi.13035

Peterson, D.S., Milhous, W.K., Wellems, T.E., 1990. Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. 87, 3018–3022.

Peterson, D.S., Walliker, D., Wellems, T.E., 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc. Natl. Acad. Sci. 85, 9114–9118. https://doi.org/10.1073/pnas.85.23.9114

Phompradit, P., Muhamad, P., Chaijaroenkul, W., Na-Bangchang, K., 2014a. Genetic polymorphisms of candidate markers and in vitro susceptibility of *Plasmodium falciparum* isolates from Thai-Myanmar border in relation to clinical response to artesunate-mefloquine combination. Acta Trop. 139, 77–83. https://doi.org/10.1016/j.actatropica.2014.06.015

Phompradit, P., Muhamad, P., Wisedpanichkij, R., Chaijaroenkul, W., Na-Bangchang, K., 2014b. Four years' monitoring of in vitro sensitivity and candidate molecular markers of resistance of *Plasmodium falciparum* to artesunate-mefloquine combination in the Thai-Myanmar border. Malar. J. 13, 23. https://doi.org/10.1186/1475-2875-13-23

Phyo, A.P., Ashley, E.A., Anderson, T.J.C., Bozdech, Z., Carrara, V.I., Sriprawat, K., Nair, S., White, M.M., Dziekan, J., Ling, C., Proux, S., Konghahong, K., Jeeyapant, A., Woodrow, C.J., Imwong, M., McGready, R., Lwin, K.M., Day, N.P.J., White, N.J., Nosten, F., 2016. Declining efficacy of artemisinin combination therapy against *P. falciparum* malaria on the Thai-Myanmar border (2003-2013): The role of parasite genetic factors. Clin. Infect. Dis. 63, 784–791. https://doi.org/10.1093/cid/ciw388

Phyo, A.P., Nkhoma, S., Stepniewska, K., Ashley, E.A., Nair, S., McGready, R., Ler Moo, C., Al-Saai, S., Dondorp, A.M., Lwin, K.M., Singhasivanon, P., Day, N.P.J., White, N.J., Anderson, T.J.C., Nosten, F., 2012. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet 379, 1960–1966. https://doi.org/10.1016/S0140-6736(12)60484-X

Pickard, A.L., Wongsrichanalai, C., Purfield, A., Kamwendo, D., Emery, K., Zalewski, C., Kawamoto, F., Miller, R.S., Meshnick, S.R., 2003. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in *pfmdr1*. Antimicrob. Agents Chemother. 47, 2418–2423. https://doi.org/10.1128/AAC.47.8.2418-2423.2003

Plowe, C.V., Cortese, J.F., Djimde, A., Nwanyanwu, O.C., Watkins, W.M., Winstanley, P.A., Estrada-Franco, J.G., Mollinedo, R.E., Avila, J.C., Cespedes, J.L., Carter, D., Doumbo, O.K., 1997. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J. Infect. Dis. 176, 1590–1596. https://doi.org/10.1086/514159

Plucinski, M.M., Dimbu, P.R., Macaia, A.P., Ferreira, C.M., Samutondo, C., Quivinja, J., Afonso, M., Kiniffo, R., Mbounga, E., Kelley, J.S., Patel, D.S., He, Y., Talundzic, E., Garrett, D.O., Halsey, E.S., Udhayakumar, V., Ringwald, P., Fortes, F., 2017. Efficacy of artemether-lumefantrine, artesunate-amodiaquine, and dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* malaria in Angola, 2015. Malar. J. 16, 62. https://doi.org/10.1186/s12936-017-1712-4

Plucinski, M.M., Talundzic, E., Morton, L., Dimbu, P.R., Macaia, A.P., Fortes, F., Goldman, I., Lucchi, N., Stennies, G., MacArthur, J.R., Udhayakumar, V., 2015. Efficacy of artemether-lumefantrine and dihydroartemisinin-piperaquine for treatment of uncomplicated malaria in children in Zaire and

Uíge Provinces, angola. Antimicrob. Agents Chemother. 59, 437–443. https://doi.org/10.1128/AAC.04181-14

Powell, R.D., Brewer, G.J., Alving, A.S., 1963. Studies on a strain of chloroquine-resistant *Plasmodium falciparum* from Colombia, South America. Am. J. Trop. Med. Hyg. 12, 509–512. https://doi.org/10.4269/ajtmh.1963.12.509

Powell, R.D., Brewer, G.J., Alving, A.S., Millar, J.W., 1964. Studies on a strain of chloroquine-resistant *Plasmodium falciparum* from thailand. Bull. World Health Organ. 30, 29–44.

Price, R.N., Cassar, C., Brockman, A., Duraisingh, M., van Vugt, M., White, N.J., Nosten, F., Krishna, S., 1999. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob. Agents Chemother. 43, 2943–2949. https://doi.org/10.1128/AAC.43.12.2943

Price, R.N., Nosten, F., Luxemburger, C., van Vugt, M., Phaipun, L., Chongsuphajaisiddhi, T., White, N.J., 1997. Artesunate/mefloquine treatment of multi-drug resistant falciparum malaria. Trans. R. Soc. Trop. Med. Hyg. 91, 574–577. https://doi.org/10.1016/s0035-9203(97)90032-8

Price, R.N., Uhlemann, A.-C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N.J., Nosten, F., Krishna, S., 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 364, 438–447. https://doi.org/10.1016/S0140-6736(04)16767-6

Price, R.N., Uhlemann, A.-C., van Vugt, M., Brockman, A., Hutagalung, R., Nair, S., Nash, D., Singhasivanon, P., Anderson, T.J.C., Krishna, S., White, N.J., Nosten, F., 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. Clin. Infect. Dis. 42, 1570–1577. https://doi.org/10.1086/503423

Pulcini, S., Staines, H.M., Lee, A.H., Shafik, S.H., Bouyer, G., Moore, C.M., Daley, D.A., Hoke, M.J., Altenhofen, L.M., Painter, H.J., Mu, J., Ferguson, D.J.P., Llinás, M., Martin, R.E., Fidock, D.A., Cooper, R.A., Krishna, S., 2015. Mutations in the *Plasmodium falciparum chloroquine* resistance transporter, PfCRT, enlarge the parasite's food vacuole and alter drug sensitivities. Sci. Rep. 5, 14552. https://doi.org/10.1038/srep14552

Raj, D.K., Mu, J., Jiang, H., Kabat, J., Singh, S., Sullivan, M., Fay, M.P., McCutchan, T.F., Su, X.Z., 2009. Disruption of a *Plasmodium falciparum* multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione. J. Biol. Chem. 284, 7687–7696. https://doi.org/10.1074/jbc.M806944200

Rappa, G., Lorico, A., Flavell, R.A., Sartorelli, A.C., 1997. Evidence that the multidrug resistance protein (MRP) functions as a co- transporter of glutathione and natural product toxins. Cancer Res. 57, 5232–5237.

Rasmussen, S.A., Ceja, F.G., Conrad, M.D., Tumwebaze, P.K., Byaruhanga, O., Katairo, T., Nsobya, S.L., Rosenthal, P.J., Cooper, R.A., 2017. Changing antimalarial drug sensitivities in Uganda. Antimicrob. Agents Chemother. 61, e01516-17. https://doi.org/10.1128/AAC.01516-17

Rastelli, G., Sirawaraporn, W., Sompornpisut, P., Vilaivan, T., Kamchonwongpaisan, S., Quarrell, R., Lowe, G., Thebtaranonth, Y., Yuthavong, Y., 2000. Interaction of pyrimethamine, cycloguanil, WR99210 and their analogues with *Plasmodium falciparum* dihydrofolate reductase: structural basis of antifolate resistance. Bioorg. Med. Chem. 8, 1117–1128. https://doi.org/10.1016/s0968-0896(00)00022-5

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K., Cowman, A.F., 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403, 906–909. https://doi.org/10.1038/35002615

Rees, D.C., Johnson, E., Lewinson, O., 2009. ABC transporters: The power to change. Nat. Rev. Mol. Cell Biol. 10, 218–227. https://doi.org/10.1038/nrm2646

Reeves, D.S., Wilkinson, P.J., 1979. The pharmacokinetics of trimethoprim and trimethoprim/sulphonamide combinations, including penetration into body tissues. Infection 7 Suppl 4, S330-341. https://doi.org/10.1007/BF01639009

Reiling, S.J., Rohrbach, P., 2015. Monitoring PfMDR1 transport in *Plasmodium falciparum*. Malar. J. 14, 270. https://doi.org/10.1186/s12936-015-0791-3

Rempel, S., Gati, C., Nijland, M., Thangaratnarajah, C., Karyolaimos, A., de Gier, J.W., Guskov, A., Slotboom, D.J., 2020. A mycobacterial ABC transporter mediates the uptake of hydrophilic compounds. Nature 580, 409–412. https://doi.org/10.1038/s41586-020-2072-8

Renslo, A.R., 2013. Antimalarial drug discovery: from quinine to the dream of eradication. ACS Med. Chem. Lett. 4, 1126–1128. https://doi.org/10.1021/ml4004414

Ribacke, U., Mok, B.W., Wirta, V., Normark, J., Lundeberg, J., Kironde, F., Egwang, T.G., Nilsson, P., Wahlgren, M., 2007. Genome wide gene amplifications and deletions in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 155, 33–44. https://doi.org/10.1016/j.molbiopara.2007.05.005

Ribeiro, J.M., Garriga, M., Potchen, N., Crater, A.K., Gupta, A., Ito, D., Desai, S.A., 2018. Guide RNA selection for CRISPR-Cas9 transfections in *Plasmodium falciparum*. Int. J. Parasitol. 48, 825–832. https://doi.org/10.1016/j.ijpara.2018.03.009

Richards, S.N., Nash, M.N., Baker, E.S., Webster, M.W., Lehane, A.M., Shafik, S.H., Martin, R.E., 2016. Molecular mechanisms for drug hypersensitivity induced by the malaria parasite's chloroquine resistance transporter. PLoS Pathog. 12, e1005725. https://doi.org/10.1371/journal.ppat.1005725

Rijpma, S.R., van der Velden, M., Annoura, T., Matz, J.M., Kenthirapalan, S., Kooij, T.W.A., Matuschewski, K., van Gemert, G.-J., van de Vegte-Bolmer, M., Siebelink-Stoter, R., Graumans, W., Ramesar, J., Klop, O., Russel, F.G.M., Sauerwein, R.W., Janse, C.J., Franke-Fayard, B.M., Koenderink, J.B., 2016a. Vital and dispensable roles of *Plasmodium* multidrug resistance transporters during blood- and mosquito-stage development. Mol. Microbiol. 101, 78–91. https://doi.org/10.1111/mmi.13373

Rijpma, S.R., Van Der Velden, M., Bilos, A., Jansen, R.S., Mahakena, S., Russel, F.G.M., Sauerwein, R.W., Van De Wetering, K., Koenderink, J.B., 2016b. MRP1 mediates folate transport and antifolate sensitivity in *Plasmodium falciparum*. FEBS Lett. 590, 482–492. https://doi.org/10.1002/1873-3468.12079

Rijpma, S.R., van der Velden, M., González-Pons, M., Annoura, T., van Schaijk, B.C.L., van Gemert, G.J., van den Heuvel, J.J.M.W., Ramesar, J., Chevalley-Maurel, S., Ploemen, I.H., Khan, S.M., Franetich, J.F., Mazier, D., de Wilt, J.H.W., Serrano, A.E., Russel, F.G.M., Janse, C.J., Sauerwein, R.W., Koenderink, J.B., Franke-Fayard, B.M., 2016c. Multidrug ATP-binding cassette transporters are essential for hepatic development of *Plasmodium* sporozoites. Cell. Microbiol. 18, 369–383. https://doi.org/10.1111/cmi.12517

Robertson, G.I., Davey, D.G., Fairley, N.H., 1952. Cross-Resistance Between "Daraprim" And Proguanil. Brit. Med. J. 2, 1255–1256.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. https://doi.org/10.1093/bioinformatics/btp616

Rocamora, F., Zhu, L., Liong, K.Y., Dondorp, A., Miotto, O., Mok, S., Bozdech, Z., 2018. Oxidative stress and protein damage responses mediate artemisinin resistance in malaria parasites. PLoS Pathog. 14, e1006930. https://doi.org/10.1371/journal.ppat.1006930

Rohrbach, P., Friedrich, O., Hentschel, J., Plattner, H., Fink, R.H.A., Lanzer, M., 2005. Quantitative calcium measurements in subcellular compartments of *Plasmodium falciparum*-infected erythrocytes. J. Biol. Chem. 280, 27960–27969. https://doi.org/10.1074/jbc.M500777200

Rohrbach, P., Sanchez, C.P., Hayton, K., Friedrich, O., Patel, J., Sidhu, A.B.S., Ferdig, M.T., Fidock, D.A., Lanzer, M., 2006. Genetic linkage of *pfmdr1* with food vacuolar solute import in *Plasmodium falciparum*. EMBO J. 25, 3000–3011. https://doi.org/10.1038/sj.emboj.7601203

Rollo, I.M., 1955. Resistance of *Plasmodium falciparum* to pyrimethamine. Trans. R. Soc. Trop. Med. Hyg. 49, 94–95. https://doi.org/10.1016/0035-9203(55)90095-X

Rosenberg, E., Litus, I., Schwarzfuchs, N., Sinay, R., Schlesinger, P., Golenser, J., Baumeister, S., Lingelbach, K., Pollack, Y., 2006. *pfmdr2* confers heavy metal resistance to *Plasmodium falciparum*. J. Biol. Chem. 281, 27039–27045. https://doi.org/10.1074/jbc.M601686200

Ross, L.S., Dhingra, S.K., Mok, S., Yeo, T., Wicht, K.J., Kümpornsin, K., Takala-Harrison, S., Witkowski, B., Fairhurst, R.M., Ariey, F., Menard, D., Fidock, D.A., 2018. Emerging Southeast Asian PfCRT mutations confer *Plasmodium falciparum* resistance to the first-line antimalarial piperaquine. Nat. Commun. 9, 3314. https://doi.org/10.1038/s41467-018-05652-0

Roux, A.T., Maharaj, L., Oyegoke, O., Akoniyon, O.P., Adeleke, M.A., Maharaj, R., Okpeku, M., 2021. Chloroquine and sulfadoxine–pyrimethamine resistance in sub-Saharan Africa — a review. Front. Genet. 12, 668574. https://doi.org/10.3389/fgene.2021.668574

Rovira-Graells, N., Gupta, A.P., Planet, E., Crowley, V.M., Mok, S., De Pouplana, L.R., Preiser, P.R., Bozdech, Z., Cortés, A., 2012. Transcriptional variation in the malaria parasite *Plasmodium falciparum*. Genome Res. 22, 925–938. https://doi.org/10.1101/gr.129692.111

Sá, J.M., Kaslow, S.R., Krause, M.A., Melendez-Muniz, V.A., Salzman, R.E., Kite, W.A., Zhang, M., Barros, R.R.M., Mu, J., Han, P.K., Mershon, J.P., Figan, C.E., Caleon, R.L., Rahman, R.S., Gibson, T.J., Amaratunga, C., Nishiguchi, E.P., Breglio, K.F., Engels, T.M., Velmurugan, S., Ricklefs, S., Straimer, J., Gnädig, N.F., Deng, B., Liu, A., Diouf, A., Miura, K., Tullo, G.S., Eastman, R.T., Chakravarty, S., James, E.R., Udenze, K., Li, S., Sturdevant, D.E., Gwadz, R.W., Porcella, S.F., Long, C.A., Fidock, D.A., Thomas, M.L., Fay, M.P., Sim, B.K.L., Hoffman, S.L., Adams, J.H., Fairhurst, R.M., Su, X., Wellems, T.E., 2018. Artemisinin resistance phenotypes and K13 inheritance in a *Plasmodium falciparum* cross and Aotus model. Proc. Natl. Acad. Sci. 115, 12513–12518. https://doi.org/10.1073/pnas.1813386115

Sa, J.M., Twu, O., 2010. Protecting the malaria drug arsenal: halting the rise and spread of amodiaquine resistance by monitoring the PfCRT SVMNT type. Malar. J. 9, 374. https://doi.org/10.1186/1475-2875-9-374

Sá, J.M., Twu, O., Hayton, K., Reyes, S., Fay, M.P., Ringwald, P., Wellems, T.E., 2009. Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proc. Natl. Acad. Sci. 106, 18883–18889. https://doi.org/10.1073/pnas.0911317106

Safari, F., Zare, K., Negahdaripour, M., Barekati-Mowahed, M., Ghasemi, Y., 2019. CRISPR Cpf1 proteins: Structure, function and implications for genome editing. Cell Biosci. 9. https://doi.org/10.1186/s13578-019-0298-7

Saito, M., Mansoor, R., Kennon, K., Anvikar, A.R., Ashley, E.A., Chandramohan, D., Cohee, L.M., D'Alessandro, U., Genton, B., Gilder, M.E., Juma, E., Kalilani-Phiri, L., Kuepfer, I., Laufer, M.K., Lwin, K.M., Meshnick, S.R., Mosha, D., Mwapasa, V., Mwebaza, N., Nambozi, M., Ndiaye, J.-L.A., Nosten, F., Nyunt, M., Ogutu, B., Parikh, S., Paw, M.K., Phyo, A.P., Pimanpanarak, M., Piola, P., Rijken, M.J., Sriprawat, K., Tagbor, H.K., Tarning, J., Tinto, H., Valéa, I., Valecha, N., White, N.J., Wiladphaingern, J., Stepniewska, K., McGready, R., Guérin, P.J., 2020. Efficacy and tolerability of artemisinin-based and quinine-based treatments for uncomplicated *falciparum* malaria in pregnancy: a systematic review and individual patient data meta-analysis. Lancet Infect. Dis. 20, 943–952. https://doi.org/10.1016/S1473-3099(20)30064-5

Salcedo-Sora, J.E., Ochong, E., Beveridge, S., Johnson, D., Nzila, A., Biagini, G.A., Stocks, P.A., O'Neill, P.M., Krishna, S., Bray, P.G., Ward, S.A., 2011. The molecular basis of folate salvage in

Plasmodium falciparum: Characterization of two folate transporters. J. Biol. Chem. 286, 44659–44668. https://doi.org/10.1074/jbc.M111.286054

Sanchez, C.P., Rotmann, A., Stein, W.D., Lanzer, M., 2008. Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in *Plasmodium falciparum*. Mol. Microbiol. 70, 786–798. https://doi.org/10.1111/j.1365-2958.2008.06413.x

Saunders, D.L., Vanachayangkul, P., Lon, C., 2014. Dihydroartemisinin–piperaquine failure in Cambodia. N. Engl. J. Med. 371, 484–485. https://doi.org/10.1056/NEJMc1403007

Schellenberg, K.A., Coatney, G.R., 1961. The influence of antimalarial drugs on nucleic acid synthesis in *Plasmodium gallinaceum* and *Plasmodium berghei*. Biochem. Pharmacol. 6, 143–152. https://doi.org/10.1016/0006-2952(61)90158-7

Shafik, S.H., Cobbold, S.A., Barkat, K., Richards, S.N., Lancaster, N.S., Llinás, M., Hogg, S.J., Summers, R.L., McConville, M.J., Martin, R.E., 2020. The natural function of the malaria parasite's chloroquine resistance transporter. Nat. Commun. 11, 3922. https://doi.org/10.1038/s41467-020-17781-6

Shibeshi, W., Alemkere, G., Mulu, A., Engidawork, E., 2021. Efficacy and safety of artemisinin-based combination therapies for the treatment of uncomplicated malaria in pediatrics: a systematic review and meta-analysis. BMC Infect. Dis. 21, 326. https://doi.org/10.1186/s12879-021-06018-6

Shitan, N., Bazin, I., Dan, K., Obata, K., Kigawa, K., Ueda, K., Sato, F., Forestier, C., Yazaki, K., 2003. Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc. Natl. Acad. Sci. 100, 751–756. https://doi.org/10.1073/pnas.0134257100

Siddiqui, F.A., Cabrera, M., Wang, M., Brashear, A., Kemirembe, K., Wang, Z., Miao, J., Chookajorn, T., Yang, Z., Cao, Y., Dong, G., Rosenthal, P.J., Cui, L., 2018. *Plasmodium falciparum* falcipain-2a polymorphisms in Southeast Asia and their association with artemisinin resistance. J. Infect. Dis. 218, 434–442. https://doi.org/10.1093/infdis/jiy188

Siddiqui, G., Srivastava, A., Russell, A.S., Creek, D.J., 2017. Multi-omics based identification of specific biochemical changes associated with PfKelch13-mutant artemisinin-resistant *Plasmodium falciparum*. J. Infect. Dis. 215, 1435–1444. https://doi.org/10.1093/infdis/jix156

Sidhu, A.B.S., Uhlemann, A.-C., Valderramos, S.G., Valderramos, J.-C., Krishna, S., Fidock, D.A., 2006. Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J. Infect. Dis. 194, 528–535. https://doi.org/10.1086/507115

Sidhu, A.B.S., Valderramos, S.G., Fidock, D.A., 2005. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol. Microbiol. 57, 913–926. https://doi.org/10.1111/j.1365-2958.2005.04729.x

Sidhu, A.B.S., Verdier-Pinard, D., Fidock, D.A., 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science 298, 210–213. https://doi.org/10.1126/science.1074045

Simonsson, U.S.H., Jansson, B., Hai, T.N., Huong, D.X., Tybring, G., Ashton, M., 2003. Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9. Clin. Pharmacol. Ther. 74, 32–43. https://doi.org/10.1016/S0009-9236(03)00092-4

Sirawaraporn, W., Yuthavong, Y., 1984. Kinetic and molecular properties of dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant *Plasmodium chabaudi*. Mol. Biochem. Parasitol. 10, 355–367. https://doi.org/10.1016/0166-6851(84)90033-1

Sisowath, C., Ferreira, P.E., Bustamante, L.Y., Dahlström, S., Mårtensson, A., Björkman, A., Krishna, S., Gil, J.P., 2007. The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-

lumefantrine in Africa. Trop. Med. Int. Health 12, 736–742. https://doi.org/10.1111/j.1365-3156.2007.01843.x

Sisowath, C., Petersen, I., Veiga, M.I., Mårtensson, A., Premji, Z., Björkman, A., Fidock, D.A., Gil, J.P., 2009. *In Vivo* Selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with Artemether-Lumefantrine in Africa. J. Infect. Dis. 199, 750–757. https://doi.org/10.1086/596738

Sisowath, C., Strömberg, J., Mårtensson, A., Msellem, M., Obondo, C., Björkman, A., Gil, J.P., 2005. *In vivo* selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). J. Infect. Dis. 191, 1014–1017. https://doi.org/10.1086/427997

Skinner, T.S., Manning, L.S., Johnston, W.A., Davis, T.M.E., 1996. In vitro stage-specific sensitivity of *Plasmodium falciparum* to quinine and artemisinin drugs. Int. J. Parasitol. 26, 519–525. https://doi.org/10.1016/0020-7519(96)89380-5

Song, J., Socheat, D., Tan, B., Seila, S., Xu, Y., Ou, F., Sokunthea, S., Sophorn, L., Zhou, C., Deng, C., Wang, Q., Li, G., 2011. Randomized trials of artemisinin-piperaquine, dihydroartemisinin-piperaquine phosphate and artemether-lumefantrine for the treatment of multi-drug resistant *falciparum malaria* in Cambodia-Thailand border area. Malar. J. 10, 231. https://doi.org/10.1186/1475-2875-10-231

Spalding, M.D., Allary, M., Gallagher, J.R., Prigge, S.T., 2010. Validation of a modified method for Bxb1 mycobacteriophage integrase-mediated recombination in *Plasmodium falciparum* by localization of the H-protein of the glycine cleavage complex to the mitochondrion. Mol. Biochem. Parasitol. 172, 156–160. https://doi.org/10.1016/j.molbiopara.2010.04.005

Spencer, H.C., 1985. Drug-resistant malaria - changing patterns mean difficult decisions. Trans. R. Soc. Trop. Med. Hyg. 79, 748–758. https://doi.org/10.1016/0035-9203(85)90108-7

Spielmann, T., Gras, S., Sabitzki, R., Meissner, M., 2020. Endocytosis in *Plasmodium* and *Toxoplasma* Parasites. Trends Parasitol. 36, 520–532. https://doi.org/10.1016/j.pt.2020.03.010

Sridaran, S., McClintock, S.K., Syphard, L.M., Herman, K.M., Barnwell, J.W., Udhayakumar, V., 2010. Anti-folate drug resistance in Africa: meta-analysis of reported dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) mutant genotype frequencies in African *Plasmodium falciparum* parasite populations. Malar. J. 9, 247. https://doi.org/10.1186/1475-2875-9-247

Steketee, R.W., Campbell, C.C., 2010. Impact of national malaria control scale-up programmes in Africa: magnitude and attribution of effects. Malar. J. 9, 299. https://doi.org/10.1186/1475-2875-9-299

Straimer, J., Gnädig, N.F., Stokes, B.H., Ehrenberger, M., Crane, A.A., Fidock, D.A., 2017. *Plasmodium falciparum* K13 mutations differentially impact ozonide susceptibility and parasite fitness *In Vitro*. mBio 8. https://doi.org/10.1128/mBio.00172-17

Straimer, J., Gnädig, N.F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A.P., Dacheux, M., Khim, N., Zhang, L., Lam, S., Gregory, P.D., Urnov, F.D., Mercereau-Puijalon, O., Benoit-Vical, F., Fairhurst, R.M., Ménard, D., Fidock, D.A., 2015. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. Science 347, 428–431. https://doi.org/10.1126/science.1260867

Straimer, J., Lee, M.C.S., Lee, A.H., Zeitler, B., Williams, A.E., Pearl, J.R., Zhang, L., Rebar, E.J., Gregory, P.D., Llinás, M., Urnov, F.D., Fidock, D.A., 2012. Site-specific genome editing in *Plasmodium falciparum* using engineered zinc-finger nucleases. Nat. Methods. 9, 993–998. https://doi.org/10.1038/nmeth.2143

Striepen, B., Pruijssers, A.J.P., Huang, J., Li, C., Gubbels, M.J., Umejiego, N.N., Hedstrom, L., Kissinger, J.C., 2004. Gene transfer in the evolution of parasite nucleotide biosynthesis. Proc. Natl. Acad. Sci. 101, 3154–3159. https://doi.org/10.1073/pnas.0304686101

Sullivan, D.J., 2017. Quinolines block every step of malaria heme crystal growth. Proc. Natl. Acad. Sci. 114, 7483–7485. https://doi.org/10.1073/pnas.1708153114

Sulo, J., Chimpeni, P., Hatcher, J., Kublin, J.G., Plowe, C.V., Molyneux, M.E., Marsh, K., Taylor, T.E., Watkins, W.M., Winstanley, P.A., 2002. Chlorproguanil-dapsone versus sulfadoxine-pyrimethamine for sequential episodes of uncomplicated falciparum malaria in Kenya and Malawi: a randomised clinical trial. Lancet 360, 1136–1143. https://doi.org/10.1016/s0140-6736(02)11198-6

Summers, R.L., Dave, A., Dolstra, T.J., Bellanca, S., Marchetti, R.V., Nash, M.N., Richards, S.N., Goh, V., Schenk, R.L., Stein, W.D., Kirk, K., Sanchez, C.P., Lanzer, M., Martin, R.E., 2014. Diverse mutational pathways converge on saturable chloroquine transport via the malaria parasite's chloroquine resistance transporter. Proc. Natl. Acad. Sci. 111, E1759–E1767. https://doi.org/10.1073/pnas.1322965111

Takala-Harrison, S., Jacob, C.G., Arze, C., Cummings, M.P., Silva, J.C., Dondorp, A.M., Fukuda, M.M., Hien, T.T., Mayxay, M., Noedl, H., Nosten, F., Kyaw, M.P., Nhien, N.T.T., Imwong, M., Bethell, D., Se, Y., Lon, C., Tyner, S.D., Saunders, D.L., Ariey, F., Mercereau-Puijalon, O., Menard, D., Newton, P.N., Khanthavong, M., Hongvanthong, B., Starzengruber, P., Fuehrer, H.-P., Swoboda, P., Khan, W.A., Phyo, A.P., Nyunt, M.M., Nyunt, M.H., Brown, T.S., Adams, M., Pepin, C.S., Bailey, J., Tan, J.C., Ferdig, M.T., Clark, T.G., Miotto, O., MacInnis, B., Kwiatkowski, D.P., White, N.J., Ringwald, P., Plowe, C.V., 2015. Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. J. Infect. Dis. 211, 670–679. https://doi.org/10.1093/infdis/jiu491

Tan-Ariya, P., Brockelman, C.R., 1983. *Plasmodium falciparum*: variations in p-aminobenzoic acid requirements as related to sulfadoxine sensitivity. Exp. Parasitol. 55, 364–371. https://doi.org/10.1016/0014-4894(83)90033-4

Tangpukdee, N., Krudsood, S., Thanachartwet, W., Chalermrut, K., Pengruksa, C., Srivilairit, S., Silachamroon, U., Wilairatana, P., Phongtananant, S., Kano, S., Looareesuwan, S., 2005. An open randomized clinical trial of Artekin vs artesunate-mefloquine in the treatment of acute uncomplicated falciparum malaria. Southeast Asian J. Trop. Med. Public Health 36, 1085–1091.

Taylor, S.M., Parobek, C.M., DeConti, D.K., Kayentao, K., Coulibaly, S.O., Greenwood, B.M., Tagbor, H., Williams, J., Bojang, K., Njie, F., Desai, M., Kariuki, S., Gutman, J., Mathanga, D.P., Mårtensson, A., Ngasala, B., Conrad, M.D., Rosenthal, P.J., Tshefu, A.K., Moormann, A.M., Vulule, J.M., Doumbo, O.K., Ter Kuile, F.O., Meshnick, S.R., Bailey, J.A., Juliano, J.J., 2015. Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in Sub-Saharan Africa: a molecular epidemiologic study. J. Infect. Dis. 211, 680–688. https://doi.org/10.1093/infdis/jiu467

Taylor, W.R.J., White, N.J., 2004. Antimalarial drug toxicity: a review. Drug Saf. 27, 25–61. https://doi.org/10.2165/00002018-200427010-00003

Ter Beek, J., Guskov, A., Slotboom, D.J., 2014. Structural diversity of ABC transporters. J. Gen. Physiol. 143, 419–435. https://doi.org/10.1085/jgp.201411164

Terasaka, K., Blakeslee, J.J., Titapiwatanakun, B., Peer, W.A., Bandyopadhyay, A., Makam, S.N., Lee, O.R., Richards, E.L., Murphy, A.S., Sato, F., Yazaki, K., 2005. PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. Plant Cell 17, 2922–2939. https://doi.org/10.1105/tpc.105.035816

Thanh, N.V., Thuy-Nhien, N., Tuyen, N.T.K., Tong, N.T., Nha-Ca, N.T., Dong, L.T., Quang, H.H., Farrar, J., Thwaites, G., White, N.J., Wolbers, M., Hien, T.T., 2017. Rapid decline in the susceptibility of *Plasmodium falciparum* to dihydroartemisinin–piperaquine in the south of Vietnam. Malar. J. 16, 27. https://doi.org/10.1186/s12936-017-1680-8

Thera, M.A., Sehdev, P.S., Coulibaly, D., Traore, K., Garba, M.N., Cissoko, Y., Kone, A., Guindo, A., Dicko, A., Beavogui, A.H., Djimde, A.A., Lyke, K.E., Diallo, D.A., Doumbo, O.K., Plowe, C.V., 2005. Impact of trimethoprim-sulfamethoxazole prophylaxis on falciparum malaria infection and disease. J. Infect. Dis. 192, 1823–1829. https://doi.org/10.1086/498249

Thomas, C., Aller, S.G., Beis, K., Carpenter, E.P., Chang, G., Chen, L., Dassa, E., Dean, M., Duong Van Hoa, F., Ekiert, D., Ford, R., Gaudet, R., Gong, X., Holland, I.B., Huang, Y., Kahne, D.K., Kato, H., Koronakis, V., Koth, C.M., Lee, Y., Lewinson, O., Lill, R., Martinoia, E., Murakami, S., Pinkett, H.W., Poolman, B., Rosenbaum, D., Sarkadi, B., Schmitt, L., Schneider, E., Shi, Y., Shyng, S.L., Slotboom, D.J., Tajkhorshid, E., Tieleman, D.P., Ueda, K., Váradi, A., Wen, P.C., Yan, N., Zhang, P., Zheng, H., Zimmer, J., Tampé, R., 2020. Structural and functional diversity calls for a new classification of ABC transporters. FEBS Lett. 594, 3767–3775. https://doi.org/10.1002/1873-3468.13935

Thomas, K., Ying, W., 2008. Artemisinin — an innovative cornerstone for anti-malaria therapy, in: Petersen, F., Amstutz, R. (Eds.), Natural Compounds as Drugs: Volume II, Progress in Drug Research. Birkhäuser, Basel, pp. 383–422. https://doi.org/10.1007/978-3-7643-8595-8_9

Tilley, L., Straimer, J., Gnädig, N.F., Ralph, S.A., Fidock, D.A., 2016. Artemisinin action and resistance in *Plasmodium falciparum*. Trends Parasitol. 32, 682–696. https://doi.org/10.1016/j.pt.2016.05.010

Tivnan, A., Zakaria, Z., O'Leary, C., Kögel, D., Pokorny, J.L., Sarkaria, J.N., Prehn, J.H.M., 2015. Inhibition of multidrug resistance protein 1 (MRP1) improves chemotherapy drug response in primary and recurrent glioblastoma multiforme. Front. Neurosci. 9, 218. https://doi.org/10.3389/fnins.2015.00218

Toenhake, C.G., Fraschka, S.A.K., Vijayabaskar, M.S., Westhead, D.R., van Heeringen, S.J., Bártfai, R., 2018. Chromatin accessibility-based characterization of the gene regulatory network underlying *Plasmodium falciparum* blood-stage development. Cell Host Microbe 23, 557-569.e9. https://doi.org/10.1016/j.chom.2018.03.007

Tonkin-Hill, G.Q., Trianty, L., Noviyanti, R., Nguyen, H.H.T., Sebayang, B.F., Lampah, D.A., Marfurt, J., Cobbold, S.A., Rambhatla, J.S., McConville, M.J., Rogerson, S.J., Brown, G.V., Day, K.P., Price, R.N., Anstey, N.M., Papenfuss, A.T., Duffy, M.F., 2018. The *Plasmodium falciparum* transcriptome in severe malaria reveals altered expression of genes involved in important processes including surface antigen–encoding var genes. PLOS Biol. 16, e2004328. https://doi.org/10.1371/journal.pbio.2004328

Toyoda, T., Brobey, R.K., Sano, G., Horii, T., Tomioka, N., Itai, A., 1997. Lead discovery of inhibitors of the dihydrofolate reductase domain of *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase. Biochem. Biophys. Res. Commun. 235, 515–519. https://doi.org/10.1006/bbrc.1997.6814

Trager, W., 1958. Folinic acid and non-dialyzable materials in the nutrition of malaria parasites. J. Exp. Med. 108, 753–772. https://doi.org/10.1084/jem.108.5.753

Triglia, T., Cowman, A.F., 1999. The mechanism of resistance to sulfa drugs in *Plasmodium falciparum*. Drug. Resist. Updat. 2, 15–19. https://doi.org/10.1054/drup.1998.0060

Triglia, T., Cowman, A.F., 1994. Primary structure and expression of the dihydropteroate synthetase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. 91, 7149–7153. https://doi.org/10.1073/pnas.91.15.7149

Triglia, T., Menting, J.G.T., Wilson, C., Cowman, A.F., 1997. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. 94, 13944–13949. https://doi.org/10.1073/pnas.94.25.13944

Triglia, T., Wang, P., Sims, P.F., Hyde, J.E., Cowman, A.F., 1998. Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves the role of dihydropteroate synthase in

sulfadoxine-resistant malaria. EMBO J. 17, 3807–3815. https://doi.org/10.1093/emboj/17.14.3807

Tu, Y., 2017. From *Artemisia annua L.* to Artemisinins: The Discovery and Development of Artemisinins and Antimalarial Agents. Academic Press.

Tu, Y., 2016. Artemisinin-A gift from traditional chinese medicine to the world (Nobel Lecture). Angew. Chem. Int. Ed. Engl. 55, 10210–10226. https://doi.org/10.1002/anie.201601967

Tun, K.M., Imwong, M., Lwin, K.M., Win, A.A., Hlaing, T.M., Hlaing, T., Lin, K., Kyaw, M.P., Plewes, K., Faiz, M.A., Dhorda, M., Cheah, P.Y., Pukrittayakamee, S., Ashley, E.A., Anderson, T.J.C., Nair, S., McDew-White, M., Flegg, J.A., Grist, E.P.M., Guerin, P., Maude, R.J., Smithuis, F., Dondorp, A.M., Day, N.P.J., Nosten, F., White, N.J., Woodrow, C.J., 2015. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: A cross-sectional survey of the K13 molecular marker. Lancet Infect. Dis. 15, 415–421. https://doi.org/10.1016/S1473-3099(15)70032-0

United Nations, 2015. Transforming our world: the 2030 Agenda for Sustainable Development | Department of Economic and Social Affairs [WWW Document]. URL https://sdgs.un.org/2030agenda (accessed 8.19.21).

Ursing, J., Zakeri, S., Gil, J.P., Björkman, A., 2006. Quinoline resistance associated polymorphisms in the *pfcrt*, *pfmdr1* and *pfmrp* genes of *Plasmodium falciparum* in Iran. Acta Trop. 97, 352–356. https://doi.org/10.1016/j.actatropica.2006.01.002

US AID, 2018. Cambodia malaria operational plan FY2018. Washington, DC.

Uwimana, A., Legrand, E., Stokes, B.H., Ndikumana, J.-L.M., Warsame, M., Umulisa, N., Ngamije, D., Munyaneza, T., Mazarati, J.-B., Munguti, K., Campagne, P., Criscuolo, A., Ariey, F., Murindahabi, M., Ringwald, P., Fidock, D.A., Mbituyumuremyi, A., Menard, D., 2020. Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. Nat. Med. 26, 1602–1608. https://doi.org/10.1038/s41591-020-1005-2

Vadivelan, M., Dutta, T., 2014. Recent advances in the management of *Plasmodium knowlesi* infection. Trop. Parasitol. 4, 31–34. https://doi.org/10.4103/2229-5070.129158

Valderramos, S.G., Valderramos, J.-C., Musset, L., Purcell, L.A., Mercereau-Puijalon, O., Legrand, E., Fidock, D.A., 2010. Identification of a mutant PfCRT-mediated chloroquine tolerance phenotype in *Plasmodium falciparum*. PLoS Pathog. 6, e1000887. https://doi.org/10.1371/journal.ppat.1000887

van der Pluijm, R.W., Imwong, M., Chau, N.H., Hoa, N.T., Thuy-Nhien, N.T., Thanh, N.V., Jittamala, P., Hanboonkunupakarn, B., Chutasmit, K., Saelow, C., Runjarern, R., Kaewmok, W., Tripura, R., Peto, T.J., Yok, S., Suon, S., Sreng, S., Mao, S., Oun, S., Yen, S., Amaratunga, C., Lek, D., Huy, R., Dhorda, M., Chotivanich, K., Ashley, E.A., Mukaka, M., Waithira, N., Cheah, P.Y., Maude, R.J., Amato, R., Pearson, R.D., Gonçalves, S., Jacob, C.G., Hamilton, W.L., Fairhurst, R.M., Tarning, J., Winterberg, M., Kwiatkowski, D.P., Pukrittayakamee, S., Hien, T.T., Day, N.P., Miotto, O., White, N.J., Dondorp, A.M., 2019. Determinants of dihydroartemisinin-piperaquine treatment failure in *Plasmodium falciparum* malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. Lancet Infect. Dis. 19, 952–961. https://doi.org/10.1016/S1473-3099(19)30391-3

van der Velden, M., Rijpma, S.R., Russel, F.G.M., Sauerwein, R.W., Koenderink, J.B., 2015. PfMDR2 and PfMDR5 are dispensable for *Plasmodium falciparum* asexual parasite multiplication but change *in vitro* susceptibility to anti-malarial drugs. Malar. J. 14, 76. https://doi.org/10.1186/s12936-015-0581-y

van Dijk, M.R., Waters, A.P., Janse, C.J., 1995. Stable transfection of malaria parasite blood stages. Science 268, 1358–1362. https://doi.org/10.1126/science.7761856

Vaughan, A.M., Kappe, S.H.I., 2017. Malaria parasite liver infection and exoerythrocytic biology. Cold Spring Harb. Perspect. Med. 7. https://doi.org/10.1101/cshperspect.a025486

Veiga, M.I., Asimus, S., Ferreira, P.E., Martins, J.P., Cavaco, I., Ribeiro, V., Hai, T.N., Petzold, M.G., Björkman, A., Ashton, M., Gil, J.P., 2009. Pharmacogenomics of CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP3A4, CYP3A5 and MDR1 in vietnam. Eur. J. Clin. Pharmacol. 65, 355–363. https://doi.org/10.1007/s00228-008-0573-8

Veiga, M.I., Dhingra, S.K., Henrich, P.P., Straimer, J., Gnädig, N., Uhlemann, A.-C., Martin, R.E., Lehane, A.M., Fidock, D.A., 2016. Globally prevalent PfMDR1 mutations modulate *Plasmodium falciparum* susceptibility to artemisinin-based combination therapies. Nat. Commun. 7, 11553. https://doi.org/10.1038/ncomms11553

Veiga, M.I., Ferreira, P.E., Jörnhagen, L., Malmberg, M., Kone, A., Schmidt, B.A., Petzold, M., Björkman, A., Nosten, F., Gil, J.P., 2011. Novel polymorphisms in *Plasmodium falciparum* ABC transporter genes are associated with major ACT antimalarial drug resistance. PLoS One 6, e20212. https://doi.org/10.1371/journal.pone.0020212

Veiga, M.I., Ferreira, P.E., Malmberg, M., Jörnhagen, L., Björkman, A., Nosten, F., Gil, J.P., 2012. *pfmdr1* amplification is related to increased *Plasmodium falciparum in vitro* sensitivity to the bisquinoline piperaquine. Antimicrob. Agents Chemother. 56, 3615–3619. https://doi.org/10.1128/AAC.06350-11

Veiga, M.I., Ferreira, P.E., Schmidt, B.A., Ribacke, U., Björkman, A., Tichopad, A., Gil, J.P., 2010. Antimalarial exposure delays *Plasmodium falciparum* intra-erythrocytic cycle and drives drug transporter genes expression. PLoS One 5, e12408. https://doi.org/10.1371/journal.pone.0012408

Veiga, M.I., Osório, N.S., Ferreira, P.E., Franzén, O., Dahlstrom, S., Lum, J.K., Nosten, F., Gil, J.P., 2014. Complex polymorphisms in the *Plasmodium falciparum* multidrug resistance protein 2 gene and its contribution to antimalarial response. Antimicrob. Agents Chemother. 58, 7390–7397. https://doi.org/10.1128/AAC.03337-14

Venkatesan, M., Gadalla, N.B., Stepniewska, K., Dahal, P., Nsanzabana, C., Moriera, C., Price, R.N., Mårtensson, A., Rosenthal, P.J., Dorsey, G., Sutherland, C.J., Guérin, P., Davis, T.M.E., Ménard, D., Adam, I., Ademowo, G., Arze, C., Baliraine, F.N., Berens-Riha, N., Björkman, A., Borrmann, S., Checchi, F., Desai, M., Dhorda, M., Djimdé, A.A., El-Sayed, B.B., Eshetu, T., Eyase, F., Falade, C., Faucher, J.-F., Fröberg, G., Grivoyannis, A., Hamour, S., Houzé, S., Johnson, J., Kamugisha, E., Kariuki, S., Kiechel, J.-R., Kironde, F., Kofoed, P.-E., LeBras, J., Malmberg, M., Mwai, L., Ngasala, B., Nosten, F., Nsobya, S.L., Nzila, A., Oguike, M., Otienoburu, S.D., Ogutu, B., Ouédraogo, J.-B., Piola, P., Rombo, L., Schramm, B., Somé, A.F., Thwing, J., Ursing, J., Wong, R.P.M., Zeynudin, A., Zongo, I., Plowe, C.V., Sibley, C.H., Asaq Molecular Marker Study Group, null, 2014. Polymorphisms in *Plasmodium falciparum* chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for *P. falciparum* malaria after artemether-lumefantrine and artesunate-amodiaquine. Am. J. Trop. Med. Hyg. 91, 833–843. https://doi.org/10.4269/ajtmh.14-0031

Vezmar, M., Georges, E., 2000. Reversal of MRP-mediated doxorubicin resistance with quinoline-based drugs. Biochem. Pharmacol. 59, 1245–1252. https://doi.org/10.1016/s0006-2952(00)00270-7

Vezmar, M., Georges, E., 1998. Direct binding of chloroquine to the multidrug resistance protein (MRP): possible role for MRP in chloroquine drug transport and resistance in tumor cells. Biochem. Pharmacol. 56, 733–742. https://doi.org/10.1016/s0006-2952(98)00217-2

Vincke, I., Lips, M., 1952. Medical prophylaxis with daraprim in rural areas. An. Inst. Med. Trop. 9, 563–569.

Visser, B.J., Wieten, R.W., Kroon, D., Nagel, I.M., Bélard, S., van Vugt, M., Grobusch, M.P., 2014. Efficacy and safety of artemisinin combination therapy (ACT) for non-falciparum malaria: a systematic review. Malar. J. 13, 463. https://doi.org/10.1186/1475-2875-13-463

Volkman, S.K., Sabeti, P.C., DeCaprio, D., Neafsey, D.E., Schaffner, S.F., Milner, D.A., Daily, J.P., Sarr, O., Ndiaye, D., Ndir, O., Mboup, S., Duraisingh, M.T., Lukens, A., Derr, A., Stange-Thomann, N., Waggoner, S., Onofrio, R., Ziaugra, L., Mauceli, E., Gnerre, S., Jaffe, D.B., Zainoun, J., Wiegand, R.C., Birren, B.W., Hartl, D.L., Galagan, J.E., Lander, E.S., Wirth, D.F., 2007. A genome-wide map of diversity in *Plasmodium falciparum*. Nat. Genet. 39, 113–119. https://doi.org/10.1038/ng1930

Wagner, J.C., Platt, R.J., Goldfless, S.J., Zhang, F., Niles, J.C., 2014. Efficient CRISPR-Cas9-mediated genome editing in *Plasmodium falciparum*. Nat. Methods 11, 915–918. https://doi.org/10.1038/nmeth.3063

Walker, A.J., Lopez-Antunano, F.J., 1968. Response to drugs of South American strains of *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. 62, 654–667. https://doi.org/10.1016/0035-9203(68)90116-8

Waller, K.L., Muhle, R.A., Ursos, L.M., Horrocks, P., Verdier-Pinard, D., Sidhu, A.B.S., Fujioka, H., Roepe, P.D., Fidock, D.A., 2003. Chloroquine resistance modulated *in vitro* by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter. J. Biol. Chem. 278, 33593–33601. https://doi.org/10.1074/jbc.M302215200

Walliker, D., Carter, R., Sanderson, A., 1975. Genetic studies on *Plasmodium chabaudi*: recombination between enzyme markers. Parasitology 70, 19–24. https://doi.org/10.1017/s0031182000048824

Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R., Carter, R., 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science 236, 1661–1666. https://doi.org/10.1126/science.3299700

Wang, J., Huang, Y., Zhao, Y., Ye, R., Zhang, D., Pan, W., 2018. Introduction of F446I mutation in the K13 propeller gene leads to increased ring survival rates in *Plasmodium falciparum* isolates. Malar. J. 17, 248. https://doi.org/10.1186/s12936-018-2396-0

Wang, J., Zhang, C.-J., Chia, W.N., Loh, C.C.Y., Li, Z., Lee, Y.M., He, Y., Yuan, L.-X., Lim, T.K., Liu, M., Liew, C.X., Lee, Y.Q., Zhang, J., Lu, N., Lim, C.T., Hua, Z.-C., Liu, B., Shen, H.-M., Tan, K.S.W., Lin, Q., 2015. Haem-activated promiscuous targeting of artemisinin in *Plasmodium falciparum*. Nat. Commun. 6, 10111. https://doi.org/10.1038/ncomms10111

Wang, P., Brobey, R.K., Horii, T., Sims, P.F., Hyde, J.E., 1999. Utilization of exogenous folate in the human malaria parasite *Plasmodium falciparum* and its critical role in antifolate drug synergy. Mol. Microbiol. 32, 1254–1262. https://doi.org/10.1046/j.1365-2958.1999.01437.x

Wang, Ping, Read, M., Sims, P.F.G., Hyde, J.E., 1997. Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. Mol. Microbiol. 23, 979–986. https://doi.org/10.1046/j.1365-2958.1997.2821646.x

Wang, P., Sims, P.F., Hyde, J.E., 1997. A modified in vitro sulfadoxine susceptibility assay for *Plasmodium falciparum* suitable for investigating Fansidar resistance. Parasitology 115 (Pt 3), 223–230. https://doi.org/10.1017/s0031182097001431

Wang, P., Wang, Q., Aspinall, T.V., Sims, P.F.G., Hyde, J.E., 2004. Transfection studies to explore essential folate metabolism and antifolate drug synergy in the human malaria parasite *Plasmodium falciparum*. Mol. Microbiol. 51, 1425–1438. https://doi.org/10.1111/j.1365-2958.2003.03915.x

Wang, P., Wang, Q., Sims, P.F.G., Hyde, J.E., 2007. Characterisation of exogenous folate transport in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 154, 40–51. https://doi.org/10.1016/j.molbiopara.2007.04.002

Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A., Wei, G., Agre, P., Jacobs-Lorena, M., 2017. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. Science 357, 1399–1402. https://doi.org/10.1126/science.aan5478

Wang, Z., Hu, W., Zheng, H., 2020. Pathogenic siderophore ABC importer YbtPQ adopts a surprising fold of exporter. Sci. Adv. 6, eaay7997. https://doi.org/10.1126/sciadv.aay7997

Wang, Z., Parker, D., Meng, H., Wu, L., Li, J., Zhao, Z., Zhang, R., Fan, Q., Wang, H., Cui, L., Yang, Z., 2012. *In Vitro* Sensitivity of *Plasmodium falciparum* from China-Myanmar border area to major ACT drugs and polymorphisms in potential target genes. PLoS One 7. https://doi.org/10.1371/journal.pone.0030927

Wanke, D., Üner Kolukisaoglu, H., 2010. An update on the ABCC transporter family in plants: Many genes, many proteins, but how many functions? Plant Biol. 12, 15–25. https://doi.org/10.1111/j.1438-8677.2010.00380.x

Ward, A., Reyes, C.L., Yu, J., Roth, C.B., Chang, G., 2007. Flexibility in the ABC transporter MsbA: Alternating access with a twist. Proc. Natl. Acad. Sci. 104, 19005–19010. https://doi.org/10.1073/pnas.0709388104

Warhurst, D.C., Craig, J.C., Adagu, I.S., 2002. Lysosomes and drug resistance in malaria. Lancet 360, 1527–1529. https://doi.org/10.1016/S0140-6736(02)11577-7

Wellems, T.E., Plowe, C.V., 2001. Chloroquine-resistant malaria. J. Infect. Dis. 184, 770–776. https://doi.org/10.1086/322858

Wellems, T.E., Sá, J.M., Su, X.-Z., Connelly, S.V., Ellis, A.C., 2020. "Artemisinin resistance": Something new or old? Something of a misnomer? Trends Parasitol. 36, 735–744. https://doi.org/10.1016/j.pt.2020.05.013

West African Network for Clinical Trials of Antimalarial Drugs (WANECAM), 2018. Pyronaridine-artesunate or dihydroartemisinin-piperaquine versus current first-line therapies for repeated treatment of uncomplicated malaria: a randomised, multicentre, open-label, longitudinal, controlled, phase 3b/4 trial. Lancet 391, 1378–1390. https://doi.org/10.1016/S0140-6736(18)30291-5

White, N.J., 2013. Pharmacokinetic and pharmacodynamic considerations in antimalarial dose optimization. Antimicrob. Agents Chemother. 57, 5792–5807. https://doi.org/10.1128/AAC.00287-13

White, N.J., 2008. Qinghaosu (Artemisinin): The price of success. Science 320, 330–334. https://doi.org/10.1126/science.1155165

White, N.J., 1999. Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia 41, 301–308.

White, N.J., Pukrittayakamee, S., Hien, T.T., Faiz, M.A., Mokuolu, O.A., Dondorp, A.M., 2014. Malaria. Lancet 383, 723–735. https://doi.org/10.1016/S0140-6736(13)60024-0

White, N.J., van Vugt, M., Ezzet, F., 1999. Clinical pharmacokinetics and pharmacodynamics and pharmacodynamics of artemether-lumefantrine. Clin. Pharmacokinet. 37, 105–125. https://doi.org/10.2165/00003088-199937020-00002

WHO, 2018. Meeting report of the WHO Evidence Review Group on mass drug administration for malaria. Geneva, Switzerland.

Wieczorek, H., Putzenlechner, M., Zeiske, W., Klein, U., 1991. A vacuolar-type proton pump energizes K+/H+ antiport in an animal plasma membrane. J. Biol. Chem. 266, 15340–15347. https://doi.org/10.1016/S0021-9258(18)98621-7

Wilson, C.M., Serrano, A.E., Wasley, A., Bogenschutz, M.P., Shankar, A.H., Wirth, D.F., 1989. Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. Science 244, 1184–1186. https://doi.org/10.1126/science.2658061

Winstanley, P., Watkins, W., Muhia, D., Szwandt, S., Amukoye, E., Marsh, K., 1997. Chlorproguanil/dapsone for uncomplicated *Plasmodium falciparum* malaria in young children: pharmacokinetics and therapeutic range. Trans. R. Soc. Trop. Med. Hyg. 91, 322–327. https://doi.org/10.1016/s0035-9203(97)90093-6

Winstanley, P.A., Watkins, W.M., Newton, C.R., Nevill, C., Mberu, E., Warn, P.A., Waruiru, C.M., Mwangi, I.N., Warrell, D.A., Marsh, K., 1992. The disposition of oral and intramuscular pyrimethamine/sulphadoxine in Kenyan children with high parasitaemia but clinically non-severe falciparum malaria. Br. J. Clin. Pharmacol. 33, 143–148. https://doi.org/10.1111/j.1365-2125.1992.tb04016.x

Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S., Lim, P., Mao, S., Sopha, C., Sam, B., Anderson, J.M., Duong, S., Chuor, C.M., Taylor, W.R.J., Suon, S., Mercereau-Puijalon, O., Fairhurst, R.M., Menard, D., 2013. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: *in-vitro* and *ex-vivo* drug-response studies. Lancet Infect. Dis. 13, 1043–1049. https://doi.org/10.1016/S1473-3099(13)70252-4

Witkowski, B., Duru, V., Khim, N., Ross, L.S., Saintpierre, B., Beghain, J., Chy, S., Kim, S., Ke, S., Kloeung, N., Eam, R., Khean, C., Ken, M., Loch, K., Bouillon, A., Domergue, A., Ma, L., Bouchier, C., Leang, R., Huy, R., Nuel, G., Barale, J.-C., Legrand, E., Ringwald, P., Fidock, D.A., Mercereau-Puijalon, O., Ariey, F., Ménard, D., 2017. A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype-genotype association study. Lancet Infect. Dis. 17, 174–183. https://doi.org/10.1016/S1473-3099(16)30415-7

Wong, W., Bai, X.-C., Sleebs, B.E., Triglia, T., Brown, A., Thompson, J.K., Jackson, K.E., Hanssen, E., Marapana, D.S., Fernandez, I.S., Ralph, S.A., Cowman, A.F., Scheres, S.H.W., Baum, J., 2017. The antimalarial mefloquine targets the *Plasmodium falciparum* 80S ribosome to inhibit protein synthesis. Nat. Microbiol. 2, 17031. https://doi.org/10.1038/nmicrobiol.2017.31

Wongsrichanalai, C., Meshnick, S.R., 2008. Declining artesunate-mefloquine efficacy against Falciparum malaria on the Cambodia–Thailand border. Emerg. Infect. Dis. 14, 716–719. https://doi.org/10.3201/eid1405.071601

Woodrow, C.J., Haynes, R.K., Krishna, S., 2005. Artemisinins. Postgrad. Med. J. 81, 71–78. https://doi.org/10.1136/pgmj.2004.028399

World Health Organization, 2021. World malaria report 2021. Geneva, Switzerland.

World Health Organization, 2020. World malaria report 2020, WHO Regional Office for Africa. Geneva, Switzerland.

World Health Organization, 2015. Serum and red blood cell folate concentrations for assessing folate status in populations. Vitamin and Mineral Nutrition Information System 1–7.

Wu, Y., Sifri, C.D., Lei, H.H., Su, X.Z., Wellems, T.E., 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc. Natl. Acad. Sci. 92, 973–977. https://doi.org/10.1073/pnas.92.4.973

Wunderlich, J., Rohrbach, P., Dalton, J.P., 2012. The malaria digestive vacuole. Front. Biosci. 4 S, 1424–1448. https://doi.org/10.2741/s344

Wurtz, N., Fall, B., Pascual, A., Fall, M., Baret, E., Camara, C., Nakoulima, A., Diatta, B., Fall, K.B., Mbaye, P.S., Diémé, Y., Bercion, R., Wade, B., Pradines, B., 2014. Role of *Pfmdr1* in *In Vitro Plasmodium falciparum* Susceptibility to Chloroquine, Quinine, Monodesethylamodiaquine, Mefloquine, Lumefantrine, and Dihydroartemisinin. Antimicrob. Agents Chemother. 58, 7032–7040. https://doi.org/10.1128/AAC.03494-14

WWARN K13 Genotype-Phenotype Study Group, 2019. Association of mutations in the *Plasmodium falciparum* Kelch13 gene (Pf3D7_1343700) with parasite clearance rates after artemisinin-based treatments-a WWARN individual patient data meta-analysis. BMC Med. 17, 1. https://doi.org/10.1186/s12916-018-1207-3

Xie, S.C., Dogovski, C., Hanssen, E., Chiu, F., Yang, T., Crespo, M.P., Stafford, C., Batinovic, S., Teguh, S., Charman, S., Klonis, N., Tilley, L., 2016. Haemoglobin degradation underpins the sensitivity of early ring stage *Plasmodium falciparum* to artemisinins. J. Cell Sci. 129, 406–416. https://doi.org/10.1242/jcs.178830

Xie, S.C., Ralph, S.A., Tilley, L., 2020. K13, the cytostome, and artemisinin resistance. Trends Parasitol. 36, 533–544. https://doi.org/10.1016/j.pt.2020.03.006

Yamauchi, L.M., Coppi, A., Snounou, G., Sinnis, P., 2007. *Plasmodium* sporozoites trickle out of the injection site. Cell. Microbiol. 9, 1215–1222. https://doi.org/10.1111/j.1462-5822.2006.00861.x

Yang, C., Zhang, H., Zhou, R., Qian, D., Liu, Y., Zhao, Y., Li, S., Xu, B., 2017. Polymorphisms of *Plasmodium falciparum* k13-propeller gene among migrant workers returning to Henan Province, China from Africa. BMC Infect. Dis. 17, 560. https://doi.org/10.1186/s12879-017-2634-z

Yang, T., Yeoh, L.M., Tutor, M.V., Dixon, M.W., McMillan, P.J., Xie, S.C., Bridgford, J.L., Gillett, D.L., Duffy, M.F., Ralph, S.A., McConville, M.J., Tilley, L., Cobbold, S.A., 2019. Decreased K13 abundance reduces hemoglobin catabolism and proteotoxic stress, underpinning artemisinin resistance. Cell Reports 29, 2917-2928.e5. https://doi.org/10.1016/j.celrep.2019.10.095

Young, M.D., Contacos, P.G., Stitcher, J.E., Millar, J.W., 1963. Drug resistance in *Plasmodium falciparum* from Thailand. Am. J. Trop. Med. Hyg. 12, 305–314. https://doi.org/10.4269/ajtmh.1963.12.305

Yuan, W.-C., Lee, Y.-R., Lin, S.-Y., Chang, L.-Y., Tan, Y.P., Hung, C.-C., Kuo, J.-C., Liu, C.-H., Lin, M.-Y., Xu, M., Chen, Z.J., Chen, R.-H., 2014. K33-linked polyubiquitination of coronin 7 by Cul3-KLHL20 ubiquitin E3 ligase regulates protein trafficking. Mol. Cell. 54, 586–600. https://doi.org/10.1016/j.molcel.2014.03.035

Yuvaniyama, J., Chitnumsub, P., Kamchonwongpaisan, S., Vanichtanankul, J., Sirawaraporn, W., Taylor, P., Walkinshaw, M.D., Yuthavong, Y., 2003. Insights into antifolate resistance from malarial DHFR-TS structures. Nat. Struct. Biol. 10, 357–365. https://doi.org/10.1038/nsb921

Zeng, H., Chen, Z.S., Belinsky, M.G., Rea, P.A., Kruh, G.D., 2001. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: Effect of polyglutamylation on MTX transport. Cancer Res. 61, 7225–7232.

Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., Van Der Oost, J., Regev, A., Koonin, E.V., Zhang, F., 2015. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell 163, 759–771. https://doi.org/10.1016/j.cell.2015.09.038

Zhang, H., Howard, E.M., Roepe, P.D., 2002. Analysis of the antimalarial drug resistance protein Pfcrt expressed in yeast. J. Biol. Chem. 277, 49767–49775. https://doi.org/10.1074/jbc.M204005200

Zhang, M., Gallego-Delgado, J., Fernandez-Arias, C., Waters, N.C., Rodriguez, A., Tsuji, M., Wek, R.C., Nussenzweig, V., Sullivan, W.J., 2017. Inhibiting the *Plasmodium* eIF2α kinase PK4 prevents artemisinin-induced latency. Cell Host Microbe 22, 766-776.e4. https://doi.org/10.1016/j.chom.2017.11.005

Zhang, M., Wang, C., Otto, T.D., Oberstaller, J., Liao, X., Adapa, S.R., Udenze, K., Bronner, I.F., Cassandra, D., Mayho, M., Brown, J., Li, S., Swanson, J., Rayner, J.C., Jiang, R.H.Y., Adams, J.H., 2018. Uncovering the essential genome of the human malaria parasite *Plasmodium*

falciparum by saturation mutagenesis. Science 360, eaap7847. https://doi.org/10.1126/science.aap7847

Zurovac, D., Ndhlovu, M., Sipilanyambe, N., Chanda, P., Hamer, D.H., Simon, J.L., Snow, R.W., 2007. Paediatric malaria case-management with artemether-lumefantrine in Zambia: a repeat cross-sectional study. Malar. J. 6. https://doi.org/doi.org/10.1186/1475-2875-6-31