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**MOLECULAR BASIS FOR THE MECHANISMS  
OF ACTION AND RESISTANCE TO  
ARTEMISININ COMBINATION THERAPY IN  
*PLASMODIUM FALCIPARUM***

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## ABSTRACT

Malaria, caused by the *Plasmodium falciparum* parasite, remains a leading cause of death among children in Africa. To improve treatment efficacy and delay development and spread of antimalarial drug resistance artemisinin artemisinin-based combination therapy (ACT) is now globally recommended as first-line treatment of uncomplicated *P. falciparum* malaria as a cornerstone in modern malaria control.

The aim of this thesis is to improve the understanding of the molecular basis of potential evolution of *P. falciparum* resistance to ACT.

After the worldwide introduction of ACT several reports demonstrate that the multidrug resistance protein 1 (*pfmdr1*) and chloroquine resistance transporter (*pfcr1*) genes are under selective pressure. This thesis describes the *in vivo* selective process for *pfmdr1* haplotype coding for aminoacids 86N, 184F 1246D in reinfections after artemether-lumefantrine treatment. The selective window is within 35 days after treatment during the elimination phase of the partner drug.

PFMDR1 homologue model structures unveiled the functional interference of 86N, 184F and 1246D in antimalarial drug transport. This was further supported by *in vitro* susceptibility of *P. falciparum* *pfmdr1* transfectants clones to aminoquinolines indicating that PFMDR1 may act as a vacuolar importer.

Since the resistance mechanisms of *P. falciparum* to the major ACTs are largely unknown other candidate genes were analysed. Therefore the multidrug resistance-associated 1 (*pfmrp1*) gene diversity in *P. falciparum* and its potential contribution to decreased ACT sensitivity was studied. Some 21 nonsynonymous and 6 synonymous single nucleotide polymorphisms were identified. The polymorphism I876V appears to be significantly ( $P<0.05$ ) selected in reinfections after artemether-lumefantrine. The structural role of I876V polymorphism and impact for PFMRP1 transport was then studied in bacterial ABC transporter homologue, MsbA, and shown to be related to the nucleotide binding region of ABC transporters.

To investigate mechanism of action of artemisinins in *P. falciparum*, parasite's calcium homeostasis was studied using techniques of live single cell imaging and flow cytometry. Our work suggests that artemisinin triggers  $\text{Ca}^{2+}$  signalling- dependent cell death in *P. falciparum*. Parasite cell death was partially rescued (31%) by the  $\text{Ca}^{2+}$  chelator Bapta.

In conclusion, *P. falciparum* is adapting to the new ACTs. Complex mechanisms of *pfmdr1/pfcr1* are being selected by partner drugs and may represent entry points towards alarming evolution of tolerance and resistance to ACT.

**Key words:** *Plasmodium falciparum*; ACT; antimalarial resistance; drug selection; evolution; drug transporters

## LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numerals:

- I** - Sisowath C, **Ferreira PE**, Bustamante LY, Dahlström S, Mårtensson A, Björkman A, Krishna S, Gil JP. The role of pfmdr1 in Plasmodium falciparum tolerance to artemether-lumefantrine in Africa. Trop Med Int Health. 2007 Jun;12(6):736-42.
- II** - **Ferreira PE**, Veiga MI, Uhlen P, Kaneko A, Gil JP. PfMDR1: mechanisms of transport modulation by functional polymorphisms. Manuscript.
- III** - Dahlström S\*, **Ferreira PE\***, Veiga MI, Sedighi N, Wiklund L, Mårtensson A, Färnert A, Sisowath C, Osório L, Darban H, Andersson B, Kaneko A, Conseil G, Björkman A, Gil JP. Plasmodium falciparum multidrug resistance protein 1 and artemisinin-based combination therapy in Africa. J Infect Dis. 2009 Nov 1;200(9):1456-64.
- IV** - **Ferreira PE**, Westman M, Joseph B, Gil JP, Björkman A, Krishna S, Kaneko A, Uhlén P. Artemisinin Triggers Ca<sup>2+</sup> Signalling-Dependent Cell Death in *Plasmodium falciparum*. Manuscript

\*- Authors share first authorship.

### Publications not included in this thesis:

Veiga MI, **Ferreira PE**, Schmidt BA, Ribacke U, Bjorkman A, Tichopad A, Gil JP. Antimalarial Exposure Delays Plasmodium falciparum Intra-Erythrocytic Cycle and Drives Drug Transporter Genes Expression. Plos One 2010, 5 (8)

Veiga MI, Asimus S, **Ferreira PE**, Martins JP, Cavaco I, Ribeiro V, Hai TN, Petzold MG, Bjorkman A, Ashton M, Gil JP. Pharmacogenomics of CYP2A6 CYP2B6 CYP2C19 CYP2D6 CYP3A4 CYP3A5 and MDR1 in Vietnam. European Journal of Clinical Pharmacology 2009, 65 (4)

Dahlstrom S, Veiga MI, **Ferreira P**, Martensson A, Kaneko A, Andersson B, Bjorkman A, Gil JP. Diversity of the sarco/endoplasmic reticulum Ca2+-ATPase orthologue of Plasmodium falciparum (PfATP6). Infection, Genetics and Evolution 2008, 8 (3)

**Ferreira PE**, Cavaco I, Gil JP Pharmacogenetic tools for malaria and TB in the Developing World. Personalized Medicine 2008, 5 (6)

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Holmgren G, Gil JP, **Ferreira PM**, Veiga MI, Obonyo CO, Bjorkman A. Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pfcr1 76T and pfmdr1 86Y Infection, Genetics and Evolution 2006, 6 (4)

Veiga MI, **Ferreira PE**, Bjorkman A, Gil JP. Multiplex PCR-RFLP methods for pfcr1 pfmdr1 and pfdhfr mutations in Plasmodium falciparum. Molecular and Cellular Probes. 2006 20 (2)

## LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
AQ	Amodiaquine
AL	Artemether plus lumefantrine combination
ART	Artemisinin
ACT	Artemisinin-based combination therapy
AS-AQ	Artesunate plus amodiaquine combination
Ca <sup>2+</sup>	Calcium
CQ	Chloroquine
TCA	Citric acid cycle
CQR	Chloroquine resistant
CQS	Chloroquine sensitive
CytC	Cytochrome C
DEAQ	desethylamodiaquine
DDT	dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
FACS	Fluorescence Activated Cell Sorting
G6PD	Glucose-6-phosphate dehydrogenase
IRS	Indoor Residual Spraying
ITNs	Insecticide-treated bed nets
IPTp	Intermittent Preventive Treatment in pregnancy
LLINs	Long lasting insecticide nets
Lum	Lumefantrine
MQ	Mefloquine
<i>Msp-1</i>	Merozoite surface protein gene 1
<i>Msp-2</i>	Merozoite surface protein gene 2
<i>NBD</i>	Nucleotide Binding Domain
<i>PfATP6</i>	<i>P. falciparum</i> adenosine triphosphatase 6 protein
<i>Pfcr1</i>	<i>P. falciparum</i> chloroquine resistance transporter gene
<i>Pfdhfr</i>	<i>P. falciparum</i> dihydrofolate reductase gene
<i>Pfdhps</i>	<i>P. falciparum</i> dihydroptereroate synthetase gene
<i>Pfmdr1</i>	<i>P. falciparum</i> multidrug resistance gene 1
<i>Pfmrp1</i>	<i>P. falciparum</i> multidrug resistance protein 1 gene
PCR	Polymerase chain reaction
PHC	Primary health care
QN	Quinine
RBC	Red blood cell
SNP	Single nucleotide polymorphism
TM	Transmembrane
WHO	World Health Organization

# CONTENTS

1	Malaria prologue .....	1
2	Malaria Etiology .....	2
3	The malaria carousel .....	3
3.1	The Human HOST .....	4
3.2	The mosquito VECTOR .....	5
3.3	The parasites .....	7
4	The Plasmodium falciparum .....	8
4.1	The intraerythrocytic phase .....	8
4.2	Metabolism .....	9
4.2.1	Proteins .....	9
4.2.2	Carbohydrates .....	10
4.2.3	Nucleic Acids .....	10
4.2.4	Lipids .....	11
4.2.5	Calcium (Ca <sup>2+</sup> ) homeostasis .....	11
4.3	Malaria treatment and drug resistance .....	12
4.4	Antimalarial DRUGS .....	12
4.4.1	Quinolines .....	12
4.4.2	Quinine .....	12
4.4.3	Chloroquine .....	13
4.4.4	Amodiaquine and desethylamodiaquine .....	14
4.4.5	Antifolates .....	15
4.4.6	Arylaminoalcohols .....	16
4.4.7	Sesquiterpene lactones .....	16
5	Antimalarial drug resistance .....	17
5.1	Quinolines .....	18
5.2	Antifolates .....	18
5.3	Arylaminoalcohol .....	19
5.4	Artemisinins .....	19
6	Antimalarial resistance genes .....	21
6.1	PfCRT .....	21
6.2	PfMDR1 .....	22
6.3	PfMRP1 .....	24
7	Artemisinin combination therapies (ACTs) .....	24
7.1	Artemisinins in ACT .....	25
7.2	Partner drugs in ACT .....	25
7.3	Rationale for combination therapy .....	26
7.4	ACT impact .....	27
8	Aims of the thesis .....	28
8.1	Overall aim .....	28
8.2	Specific objectives: .....	28
9	Material and methods .....	28
9.1	Study sites .....	28
9.2	<i>In vivo</i> follow up trials .....	29
9.3	<i>In vitro</i> studies .....	29
9.3.1	Parasites cultures .....	29
9.3.2	Susceptibility testing .....	30

9.3.3	Blood sampling and storage .....	30
9.3.4	DNA extraction .....	30
9.3.5	<i>pfmsp1</i> and <i>pfmsp2</i> analysis .....	30
9.3.6	PCR- Restriction Fragment Length Polymorphism (RFLP) .....	31
9.3.7	Real-time PCR .....	31
9.3.8	Pyrosequencing .....	32
9.3.9	Sequencing .....	32
9.3.10	Statistical analysis of mixed infections .....	33
9.3.11	<i>In silico</i> .....	33
9.3.12	Ca <sup>2+</sup> records .....	33
9.3.13	Mitochondrial membrane potential measurements .....	34
9.3.14	Cytochrome C (CytC) detection .....	34
9.4	Ethical considerations .....	34
10	Results .....	35
10.1	Paper I .....	35
10.2	Paper II .....	36
10.3	Paper III .....	38
10.4	Paper IV .....	40
11	Discussion .....	43
12	Conclusions .....	47
12.1	Overall conclusion .....	47
12.2	Specific conclusions .....	47
13	Personal views and future perspectives .....	48
14	Acknowledgements .....	50
15	References .....	52

## **1 MALARIA PROLOGUE**

Malaria is generally recognized as a disease of poverty and is a major hindrance for economic development [1]. The optimal conditions for the propagation of malaria overlap geographically with countries, which have low gross domestic products. In spite of these circumstances (and despite having limited access to treatment and other control tools), which generate a situation of attrition for elimination attempts, poverty does not solely explain the failure of malaria control initiatives.

Persistent malarial infections have been associated with a breakdown in antimalarial and insecticidal efficacy [2]. As will be further discussed in this thesis, plans for malaria eradication and control have failed several times due to antimalarial drug resistance. We are now beginning a new era of optimism for malaria eradication, with the development of new and different drugs, as well as increased use of bednets and insecticides. However, we are still using the same old therapeutic strategies.

How can we assure the sustainable effectiveness of new malaria control tools? New and increased efforts in applicable, basic research are required to develop a rational use antimalarial for the control and cure of this disease. Insight into the complexity of malaria pathogenesis is vital to understand the disease and will provide a major step towards its control [3]. Those of us who work on pathogenesis must widen our approach and look for new strategies to reduce the prevalence of this disease. The inability of many countries to fund expensive campaigns and expensive antimalarial treatments require these new tools to be feasible, highly effective and affordable.

The goal of the work presented in this thesis is to contribute to our general knowledge and generate applicable data for rationalized drug policies aimed at obstructing anti-malarial drug resistance in the era of ACT.



## 2 MALARIA ETIOLOGY

Malaria is caused by obligate intracellular protozoan parasites of the genus *Plasmodium* and belonging to the Apicomplexa group. Several mammals, birds and reptiles have their own specific malaria parasite. The presence of specialized *Plasmodium spp* across different taxonomic classes reveals an ancient common ancestor and millions of years of parasite/host co-evolution [4].

Five different species of malaria parasites can infect, cause symptoms and eventually lead to death in humans. These parasites are: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* [5,6]. . Due to the process of speciation, through bounce and the adaptation of parasites to different hosts, some malaria species still remain capable of infecting their evolutionarily hosts. As an example of this, *Plasmodium knowlesi* causes malaria in long-tailed macaques (*Macaca fascicularis*), but it also can infect humans, *in vitro* [7] and naturally [8]. On the other hand, *Plasmodium falciparum* was recently detected in wild gorillas [9].

Work on the fascinating biological complexity of this disease and its public health impact has resulted in the recognition of five Nobel prizes thus far to the following researchers:

**Ronald Ross, 1902:** "For his work on malaria, by which he has shown how it enters the organism and thereby has laid the foundation for successful research on this disease and methods of combating it". Ronald Ross discovered the oocyst of a malaria parasite in the gut wall of a mosquito on August 20, 1897 in Secunderabad, India.

**Alphonse Laveran, 1907:** "In recognition of his work on the role played by protozoa in causing diseases". Laveran was the first to notice parasites in the blood of a patient suffering from malaria on November 6, 1880 at Constantine, Algeria.

**Julius Wagner-Jauregg, 1927:** "For his discovery of the therapeutic value of malaria inoculation in the treatment of dementia paralytica". A professor of psychiatry and neurology in Vienna (Austria), Wagner-Jauregg developed methods for treating general paresis (advanced stage of neurosyphilis) by inducing fever through deliberate infection of patients with malaria parasites. This method was used in the 1920s and 1930s. In the 1940s, the advent of penicillin and more modern methods of treatment made such "malaria therapy" obsolete.

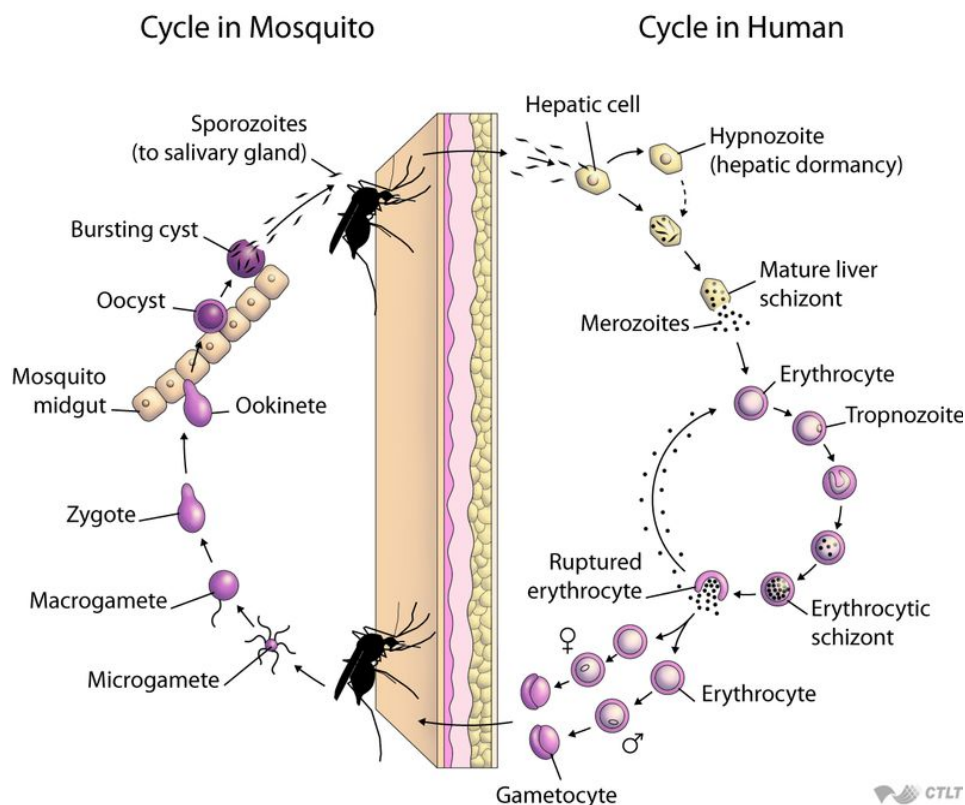
**Paul Hermann Müller, 1948:** "For his discovery of the high efficiency of DDT as a contact poison against several arthropods".

**Camillo Golgi, 1906:** Golgi shared the Nobel Prize with Santiago Ramón Cajal for their studies on the structure of the nervous system. Golgi made significant contributions to malaria research as well.

### 3 THE MALARIA CAROUSEL

As is true for all cases of malaria, the disease in humans consists of three major components: the **human** (host), the **mosquito** (vector) and the **parasite**. These constituents are essential for the propagation of malaria as a human disease. Malaria control and elimination tools intend to block this trilogy at the three different stage transitions [10]. Before entering into detail of each of the parts, a broad view of the full life cycle will be described (**Fig. 1**).

The starting point occurs when a *P. falciparum* infected female *Anopheles* mosquito feeds on a human. When this happens, sporozoites in the salivary glands are injected into the host along with the anti-coagulative saliva of the mosquito. Sporozoites enter the bloodstream and quickly reach the liver where they invade hepatocytes. The sporozoites remain in the liver for 1-2 weeks and undergo asexual replication (tissue schizogony) in which each sporozoite can give rise to tens of thousands of merozoites.



**Figure 1 - The malaria life cycle**, from Epidemiology of Infectious Diseases. Available at: <http://ocw.jhsph.edu>. Copyright © Johns Hopkins Bloomberg School of Public Health. Creative Commons BY-NC-SA.

When the hepatocytes rupture, merozoites are released into the blood stream and can readily invade erythrocytes. Once inside the erythrocyte, asexual replication begins and the parasite develops through a series of specific stages (erythrocytic schizogony). The parasite matures from a merozoite to an early trophozoite (ring stage), to the enlarged late trophozoite containing hemozoin pigmentation, and further to a schizont containing 16-18 merozoites. The infected erythrocyte is eventually lysed and merozoites are released into the bloodstream, where they can infect further erythrocytes to continue the asexual replication. The intra-erythrocytic cycle of *P. falciparum* takes approximately 48 hours. Erythrocytes infected at mature stages undergo sequestration, i.e. adhesion to endothelial cells in deep blood vessels, to avoid clearance by the spleen. Therefore, only the earlier parasite stages can normally be seen in peripheral blood and not schizonts. A small proportion of the merozoites in erythrocytes eventually differentiate to produce micro- (male) and macrogametocytes (female). In the *Anopheles* mosquito, it takes approximately 10-18 days for gametocytes to be ingested and form a zygote. Then, the zygote transforms into an ookinete that penetrates the wall of the midgut and develops into an oocyst which produces more sporozoites to initiate this millennial uninterrupted cycle.

### **3.1 THE HUMAN HOST**

Approximately half of the world's population is at risk of malaria infection, which is endemic in more than 100 countries [11]. Human and parasite evolution are inextricably linked and one piece of evidence in support of evolutionary theory.

Indeed, the reciprocal adaptation of parasite and host creates a magnificent, epic story in biology. The mortality and morbidity caused naturally by malaria [12] is thought to be the greatest selective pressure on the human genome in recent history [13]. Malaria parasites have modulated the human genome, and *vice-versa*, as will be discussed here.

A classic example of the effect of the malaria parasite on the human genome is sickle-cell disease [14]. In sickle-cell disease, a single nucleotide polymorphism (SNP) in the hemoglobin beta (*HBB*) gene, which encodes the beta-globin subunit of hemoglobin, promotes the polymerization of hemoglobin, deforming red blood cells (RBCs) into a "sickle" shape [15]. Homozygote carriers have an inevitable haemolysis and a severe, most part fatal, haemolytic anaemia. However, heterozygotes, which have both insoluble and normal hemoglobin, with low or insignificant levels of anemia, have a greatly reduced chance of serious malaria infection. Other mutations in the hemoglobin gene have also been identified and reported to behave identically [16,17]. Genetic advantages are reflected by the high

prevalence of this allele in populations where malaria is endemic, creating a case of heterozygote advantage.

Another well documented case of evolutionary selection is the Duffy antigen/chemokine receptor (DARC) gene, also known as Fy glycoprotein (FY) or CD234 (Cluster of Differentiation 234). This gene codes for a glycosylated protein that localizes to the membrane of the RBC. The Duffy antigen is used as a non-specific receptor by *Plasmodium vivax* and *Plasmodium knowlesi* to invade RBCs. Individuals who do not express Duffy antigen on the RBC are completely resistant to *P. vivax* infection. As for the sickle cell anemia, this genetic advantage has been evolutionary selected and is prevalent in almost all African populations, while being rare in other populations.

Other blood disorders, such as Thalassaemias [18] and glucose-6-phosphate dehydrogenase deficiency (G6PD) [19] have also been linked to protection against malaria. These disorders are well-documented processes of evolutionary adaptation of humans to naturally resist malaria [19,20]. In the present and future era of Genomics and high-throughput sequencing, it seems reasonable to expect many others to be unveiled.

Human genetic selection by malaria is driven by the disease's endemicity [21]. In nature, endemicity is established by the mosquito's transmissibility. However, malaria control activities can play a major role in malaria endemicity. These activities, which include early diagnosis and treatment with insecticide-treated bed nets (ITNs), indoor residual spraying (IRS) and intermittent preventive treatment in pregnancy (IPTp), create evolutionary unbalances [22].

### **3.2 THE MOSQUITO VECTOR**

About 80 mosquito species from the genus *Anopheles* transmit malaria to humans, of which approximately 40 are significant vectors. *Anopheles gambiae*, *A. arabiensis* and *A. funestus* transmit most instances of human malaria [23].

However, even within the same species, not all individuals can transmit malaria. Only the female mosquito possesses the capacity to transmit malaria. This capability is due the hematophagic (blood feeding) nature of the female, which is essential for egg production and reproduction. For malaria, humans are an intermediate host whereas mosquitoes constitute the definitive host where the sexual phase of the parasite occurs.

The period of development in the mosquito vector varies depending on the species and is controlled by ambient temperature. A lower limit of 15°C has been determined for

development in *P. vivax* and *P. malariae*. For *P. ovale*, 16°C, and for *P. falciparum*, a temperature of at least 18–21°C is necessary. The optimal ambient temperature for all malaria parasites has been shown to be 25°C, although, different time periods are required for each species to form infectious sporozoites [24]. In the case of *P. vivax*, it takes 9–10 days; for *P. falciparum*, 10–12 days; for *P. ovale*, 12–16 days; and for *P. malariae*, it takes 15–21 days until the process of sporogony is completed within the mosquito. With an ambient temperature of only 20°C, these processes can take significantly longer, for example 16–17 days (*P. vivax*), 22–28 days (*P. falciparum*), and 20–25 days (*P. malariae*) [25]. Alterations in parasite development time lengthen the span of malaria infection risk.

Mosquitoes are under selective, evolutionary pressure from both parasite and man. Similar to humans (chapter 3.1), mosquito populations are naturally affected by malaria parasites to favor the acquisition of infection resistance mechanisms [26].

Mosquitoes are the driving force responsible for malaria propagation and different characteristics and behavior of the overall mosquito population in a certain setting determines malaria endemicity [27]. Different species of mosquitoes are characterized by different feeding habits. Some are predominantly indoor biters (*A. gambiae* and *A. funestus*) or outdoors biters (*A. arabiensis*); others are mainly anthropophilic biters (*A. gambiae* and *A. funestus*) or zoophilic biters (*A. arabiensis*). This combined with factors related to their intrinsic biology, such as life expectancy, life cycle, and reproductive behaviours, make mosquitoes the prime conductors of transmission and determine malaria's endemicity [28]. In this sense, malaria can be stratified as: holo-endemic (>75%), where transmission occurs all year long; hyper-endemic (50-75%), which is intense, but with periods of no transmission during the dry season; meso-endemic (11-50%), with regular seasonal transmission; or hypo-endemic (<10%), in which malaria transmission is very intermittent.

The importance of the mosquito for malaria transmission in the principle is the reason why vector control is considered an essential tool in malaria control. In 1955, the WHO launched The Global Malaria Eradication Programme with an emphasis on vector control with dichlorodiphenyltrichloroethane (DDT) residual spraying. Development and use of insecticides had an enormous impact and the work was rewarded with a Nobel Prize. However, the massive use of insecticides led to the selection and propagation of genes conferring resistance among the mosquito population. This program was abandoned in 1969 with insecticide resistance being a major factor for its failure.

### 3.3 THE PARASITES

The present thesis is focused on the main killer among all human parasites, *Plasmodium falciparum*. The World Health Organization (WHO) estimates that in 2008, there were 243 million cases of malaria and 863.000 deaths, mostly among children under five years of age in sub-Saharan Africa due to *falciparum* malaria [11].

**Table 1 - *P. falciparum* selection process [22]**

	<b>Parasite adaptation</b>		<b>Constraints and trade-offs</b>
	<b>Parasite Variation</b>	<b>Selection</b>	
<b>Within genome within host</b>	<ul style="list-style-type: none"> <li>• Multigene families</li> </ul>	<ul style="list-style-type: none"> <li>• Invasion and adhesion receptors</li> </ul>	<ul style="list-style-type: none"> <li>• Diversity vs. function</li> <li>• Recombination</li> </ul>
<b>Between genome within host</b>	<ul style="list-style-type: none"> <li>• Antibody type</li> <li>• Ligands</li> <li>• Virulence</li> <li>• Drug resistance</li> <li>• Competitive ability</li> </ul>	<ul style="list-style-type: none"> <li>• Antibody</li> <li>• Host death</li> <li>• Drugs</li> <li>• RBCs</li> <li>• Receptors</li> <li>• General Immunity</li> </ul>	<ul style="list-style-type: none"> <li>• Diversity vs. Function</li> <li>• Mutation rate</li> </ul>
<b>Between host within population</b>	<ul style="list-style-type: none"> <li>• Antigenic repertoire</li> <li>• Drug resistance genes</li> <li>• Virulence</li> <li>• Transmission potential</li> </ul>	<ul style="list-style-type: none"> <li>• Antibody</li> <li>• Host death</li> <li>• Drugs</li> <li>• General Immunity</li> <li>• Co-infections</li> <li>• Host genetics</li> </ul>	<ul style="list-style-type: none"> <li>• Deletions vs. function</li> <li>• Virulence vs. transmissibility</li> <li>• Gametocytes vs. persistence</li> <li>• In-host ant. Diversity vs. future infection</li> <li>• Transmission bottlenecks</li> <li>• Recombination</li> </ul>
<b>Between populations</b>	<ul style="list-style-type: none"> <li>• Antigenic repertoire</li> <li>• Drug resistance frequencies</li> <li>• Virulence</li> <li>• Transmission potential</li> </ul>	<ul style="list-style-type: none"> <li>• Antibody cross-reactivity</li> <li>• Drugs</li> <li>• Proportion of immune host</li> <li>• Vector abundance</li> <li>• Host mortality</li> <li>• Co-infections</li> </ul>	<ul style="list-style-type: none"> <li>• Migration and gene flow vector</li> <li>• Abundance</li> </ul>

Evolution and modulation of the *P. Falciparum* genome occurs naturally through immunity, host death, mosquito availability and co-infection, and more recently through malaria control actions (drugs) (**Table 1**) [22].

A major hurdle to overcome for the eradication of malaria is the parasites high adaptation capacity to its highly heterogeneous natural environment. *P. falciparum*, undergoes 10 morphological transitions in five different host tissues, proliferates asexually within three of these, and must propagate sexually at each transfer between hosts. The parasite can infect most of its host population by reinvading people who have already mounted an immune response during previous or existing infections, and from each infection it can transmit for months and even years. Thus, this parasite has evolved the capacity to maximally exploit human beings for its own reproduction [29,30] .

A strategy to elucidate selection is by evaluating low sequence diversity (bottleneck) in a parasite gene and high linkage disequilibrium around the locus. These features indicate strong directional selection on a gene inside that region. This may have occurred, for instance, due to selective pressure by an antimalarial drug on a resistant parasite which could have led to a bottleneck in the worldwide parasite population [31].

## **4 THE PLASMODIUM FALCIPARUM**

### **4.1 THE INTRAERYTHROCYTIC PHASE**

Most antimalarial drugs used today are extremely effective and particularly potent against the *P. falciparum* intraerythrocytic phase. For this reason, more focus will be given to its cellular and molecular constituents.

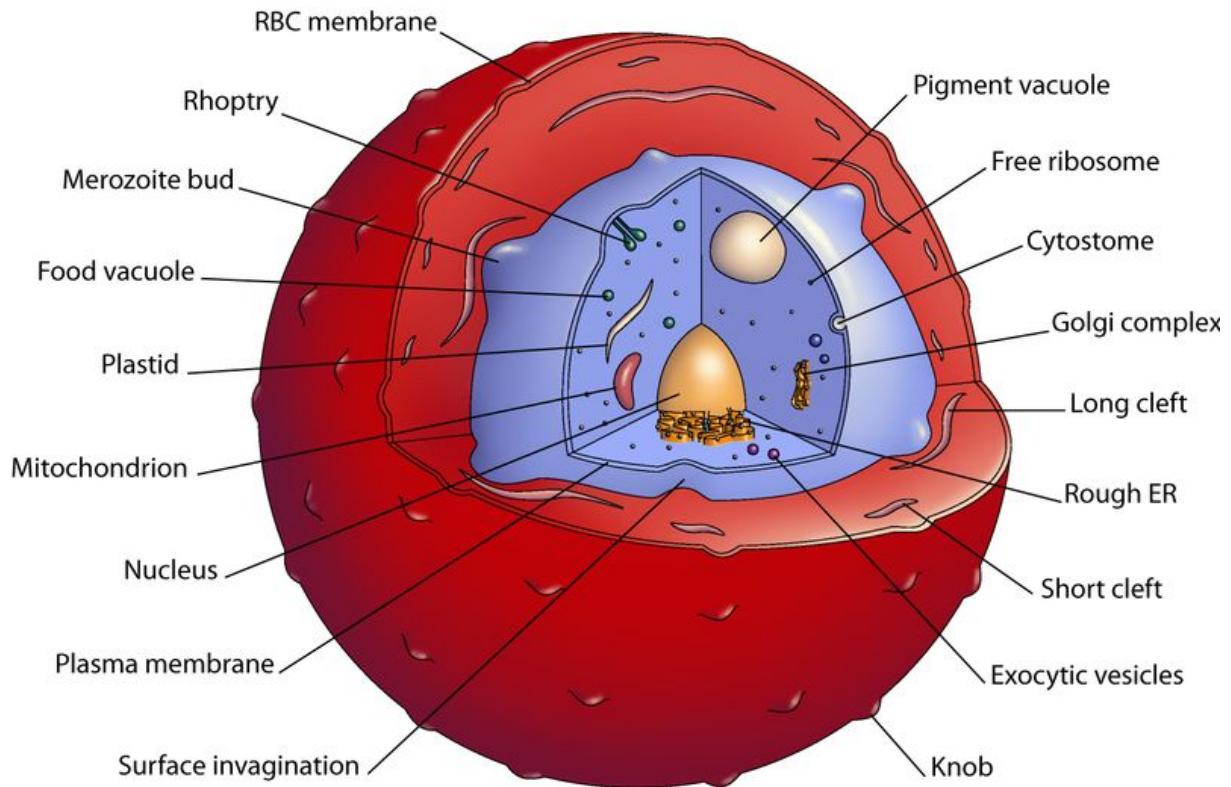
Classically, parasite morphology in the intraerythrocytic cycle is characterized in three main stages: the trophozoite stage, the ring and mature stages and the schizont stage. In this phase gametocytes are also produced.

After release from hepatocytes, the merozoites enter the bloodstream and subsequently infect RBCs. At this point, the merozoites, being approximately 1.5  $\mu\text{m}$  in length and 1  $\mu\text{m}$  in diameter, use the apicomplexan invasion organelle (apical complex, pellicle and surface coat) to recognize and enter the host erythrocyte. The parasite first binds to the erythrocyte in a random orientation. It then reorients itself such that the apical complex is in proximity to the erythrocyte membrane. A tight junction is then formed between the parasite and erythrocyte [32].

After invading the erythrocyte, the parasite loses its specific invasion organelles (the apical complex and surface coat) and de-differentiates into a round trophozoite located within a parasitophorous vacuole in the RBC's cytoplasm (**Fig. 2**). The young trophozoite (or "ring" stage) grows substantially after which it starts to replicate its DNA multiple times without cellular segmentation, which occurs prior to undergoing schizogonic division. Schizonts then undergo cellular segmentation and differentiation to form roughly 16-18 merozoite cells in the erythrocyte. The merozoites burst from the RBC and proceed to infect other erythrocytes quickly restarting (around 60 seconds) the cycle.

An important aspect of intraerythrocytic cycle is the fact that mature trophozoites and schizonts are sequestered in various human tissues and organs [33]. For this reason, in peripheral blood only early trophozoites can be detected and not mature stages. Sequestration

is caused by parasite-derived cell surface proteins being present on the RBC membrane which bind to receptors on human cells.



**Figure 2 - Red Blood cell Infection by *Plasmodium falciparum*,** from Malariology. Available at: <http://ocw.jhsph.edu>. Copyright © Johns Hopkins Bloomberg School of Public Health. Creative Commons BY-NC-SA. Adapted by CTLT from Parasitology Today, vol. 16, no. 10, 2000.

## 4.2 METABOLISM

Understand the basics of *P. falciparum* metabolism has been a principle goal toward looking for potential targets for drug development. For this matter, unique aspects of parasite metabolism are investigated in order to effect the parasite without effecting the human host. The following will give a brief overview regarding important topics of *P. falciparum* metabolism from the perspective of drug development.

### 4.2.1 Proteins

Within the RBC, parasite metabolism depends greatly on the digestion of haemoglobin. The protein is cleaved into peptides, and the haeme group is released and detoxified by biocrystallization in the form of hemozoin, creating the malaria pigment [34]. This process occurs in the digestive vacuole and is mainly driven by aspartic acid proteases, i.e.



Plasmepepsins [35]. Host uptake and haemoglobin degradation are the main sources of amino acids for the malaria parasites. This notion is further supported by the lack of amino acid biosynthesis machinery in the parasite. Genomic screening has revealed only the existence of enzymes capable of performing glycine-serine, cysteine-alanine, aspartate-asparagine, proline-ornithine, and glutamine-glutamate interconversions [29].

#### **4.2.2 Carbohydrates**

The earliest metabolic studies on malaria parasites examined their capacity for the uptake glucose [36]. Whether this uptake occurs by passive equilibration or through an active process is a matter of debate [37]. However, recently a hexose transporter (PfHT) was characterized in *P. falciparum* which demonstrated that both processes take place [38].

The citric acid (TCA) cycle and oxidative phosphorylation are generally carried out in the mitochondria of eukaryotes. These processes were generally assumed to be non-functional in the blood-stage parasite as evidenced by the acristae mitochondria. However, recently a functional electron transport chain and oxidative phosphorylation have been shown to exist in the blood-stage parasite [39]. What is known as TCA, in *P. Falciparum*, was shown to be branched in which the major carbon sources are the amino acids glutamate and glutamine and not pyruvate [40].

In addition, the antimalarial drug atovaquone has been shown to inhibit electron transport and to collapse the mitochondrial membrane potential in the malaria parasite [41].

#### **4.2.3 Nucleic Acids**

Deoxyribonucleic acid (DNA) is the blueprint for all known living organisms with the exception of some viruses. DNA consists of two long polymers of nucleotides, the purines (adenine and guanine) and the pyrimidines (cytosine and thymine). *P. falciparum* is unable to biosynthesize purines [42]. Instead, the parasite hijacks the infected RBC to transport and interconvert host purines. This process is mediated by the human equilibrative nucleoside transporter (hENT1) and the human facilitative nucleobase transporter (hFNT1). Hypoxanthine and adenine appear to enter erythrocytes mainly through the hFNT1 nucleobase transporter whereas adenosine enters predominantly through the hENT1 nucleoside transporter [43].

With respect to the synthesis of pyrimidines, the parasite can produce these nucleotides *de novo* using glutamine, bicarbonate, and aspartate.

#### 4.2.4 Lipids

The rapidly growing parasite requires large amounts of lipids to both its surface area and volume of internal membranes. This huge demand for lipids makes lipid metabolism an attractive intervention target for anti-malarial drugs. Several potential drugs targeting lipid metabolism have been identified. *Plasmodium* parasites share common characteristics in glycerophospholipid (GPL) metabolism with other eukaryotes, particularly with the lower eukaryote, yeast. *Plasmodium* parasites do have the capacity to synthesize fatty acid (FA), and have the genes encoding type II FA synthase (FAS), which are responsible for *de novo* fatty acid synthesis in the apicoplast, a plastid-like organelle unique in Apicomplexa parasites [44]. In *P. falciparum*, the parasite shows quite unique and amazingly diverse features in its lipid metabolism, some which share pathways with close similarities to the lower eukaryote, yeast which belongs to fungi, and some which share close similarities to bacteria and plants.

#### 4.2.5 Calcium ( $\text{Ca}^{2+}$ ) homeostasis

Movement of  $\text{Ca}^{2+}$  between different cellular organelles often produces a very adaptable cell signal that conveys information regulating numerous cellular processes [45,46,47]. These signals can be modulated by the concerted actions of  $\text{Ca}^{2+}$  transporters and  $\text{Ca}^{2+}$ -binding proteins to produce specific messages that trigger downstream molecular events. The parasite's ability to control its cytosolic  $\text{Ca}^{2+}$ -level in relation to the host's  $\text{Ca}^{2+}$ -level using various internal stores and cytosolic compartments is crucial to the parasites survival [48,49]. During the parasite's maturation, changes in  $\text{Ca}^{2+}$  levels between these stores and compartments fluctuate according to developmental steps though the ring-stage, the trophozoite stage and the schizont [49,50]. During early maturation, the compartment between the parasitophorous vacuole (PV) membrane and parasite membrane appears to contain relatively more  $\text{Ca}^{2+}$  than at later stages. The internal  $\text{Ca}^{2+}$  stores are thought to be the endoplasmic reticulum (ER), the digestive vacuole (DV) and so called acido-calciosomes, which are located in the parasite's cytosol. Although there is some conflicting evidence, the erythrocyte, parasite cytosol and DV are estimated to contain about 90 nM, 350 nM and 400 nM free  $\text{Ca}^{2+}$ , respectively [51]. The relatively high free  $\text{Ca}^{2+}$  levels of the parasite cytosol are thought to be confounded by superposition of extensive ER networks which are crucial for the high rate protein synthesis occurring during parasite maturation.

### 4.3 MALARIA TREATMENT AND DRUG RESISTANCE

The most important aspect to retain from this background is the fact that parasite survival happens due to adaption to constant selection and an ever-changing host environment [22]. The process of survival, metabolic adaption and establishment to a new environment, with a perspective of anti-malarial drug usage, is defined as resistance.

### 4.4 ANTIMALARIAL DRUGS

This chapter will give an introduction to the main antimalarial drugs with a focus on those used as partner drugs in ACT.

#### 4.4.1 Quinolines

Quinoline is a heterocyclic aromatic organic compound mainly used as a building block for other molecules. This backbone is the basic structure for many of the antimalarial drugs commercially available and in use nowadays (**Fig. 3**).

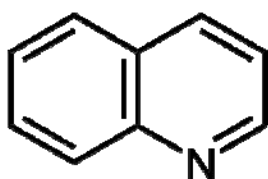


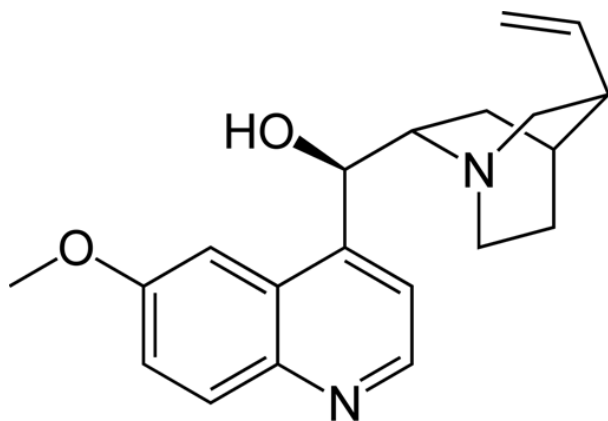
Figure 3 - Quinoline ring structure

#### 4.4.2 Quinine

Quinine (QN) (**Fig. 4**) became world famous by the hand of the Jesuit brother Agostino Salumbrino (1561–1642), an apothecary living in Peru who observed the Quechua Indians using the bark of the cinchona tree. The cinchona bark became known as Jesuit's bark and came to be one of the most valuable commodities shipped from Peru to Europe [52]. QN was first introduced to Europe around 1640 and was used to treat malaria in Italy where the disease was endemic to the swamps and marshes surrounding the city of Rome.

The name of the bark gave origin to the pathological condition caused by overdose of quinine, Cinchonism. Symptoms of mild cinchonism include flushed and sweaty skin, ringing of the ears (tinnitus), blurred vision, impaired hearing, confusion, reversible high-frequency hearing loss, headache, abdominal pain, rashes, drug-induced lichenoid reaction (lichenoid photosensitivity), vertigo, dizziness, dysphoria, nausea, vomiting and diarrhea. All adverse events are reversible and disappear once quinine is withdrawn [53,54].

As with other quinoline anti-malarial drugs, the therapeutic mechanism of quinine has not been fully resolved. The most widely accepted hypothesis of how quinine acts is based on the well-studied and closely related quinoline drug, chloroquine, which acts inside the digestive vacuole of the parasite, as explained in the next chapter 4.4.3.



**Figure 4 - Quinine molecule**

#### **4.4.3 Chloroquine**

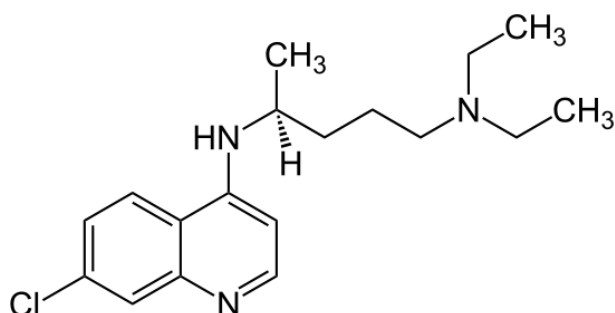
Chloroquine (CQ) (**Fig. 5**) has been, by far, the most used antimalarial medication. It was discovered in 1934 by Hans Andersag and co-workers at the Bayer laboratories and given the name "Resochin". Being a synthetic drug, it is cheap to produce and relatively well tolerated as compared with QN. This made CQ a very attractive antimalarial drug. During World War II, when malaria was a major concern for soldiers, the United States Government rapidly understood the benefits of CQ and promoted its testing, development and extensive use. Chloroquine gained special significance as part of the WHO Malaria Eradication Program begun in 1955 [55].

Besides malaria treatment, CQ is also used for treatment of other diseases including arthritis, viral infections and cancer [56].

Before the main mechanism of CQ action was identified, many congeners of the parent compound were synthesized in an attempt to improve its efficacy. Theories concerning CQ's mode of action included the DNA-binding theory, and theories involving inhibition of protein synthesis, inhibition of polyamine metabolism, inhibition of haemoglobin degradation and formation of a toxic haeme-chloroquine complex. It is now accepted that chloroquine disrupts the detoxification function of the malaria parasites. *Plasmodium* trophozoites take up large amounts of haemoglobin into their digestion vacuoles during their intraerythrocytic cycle and

release the toxic degradation by-product haematin. This process occurs by means of polymerization and binding of inert hemozoin crystals and presumably through an additional degradation process facilitated by glutathione. Chloroquine binds to haematin gamma-oxodimers and is deposited on the surface of the hemozoin crystals. This ultimately destroys the parasites [57].

CQ may cause side effects such as: dizziness, nausea, temporary hair loss, diarrhea, and worse psoriasis [53,54].



**Figure 5 - Chloroquine molecule**

#### **4.4.4 Amodiaquine and desethylamodiaquine**

Amodiaquine (AQ) (**Fig. 6**), which is structurally related to CQ, was developed in the late 1940s. AQ is not presently used in prophylaxis, owing to previous reports of the rare but serious toxic side effects (1:2000) agranulocytosis and hepatitis. From testing in animal models, AQ toxicity has been explained by its 4-hydroxyanilino moiety, which undergoes P-450 catalyzed oxidation to a reactive amodiaquine quinoneimine (AQQI), followed by the nucleophilic addition of glutathione. The formation of this conjugate *in vivo*, and its subsequent binding to cytosol macromolecules could affect the cellular function either directly or by immunological responses that initiate hypersensitivity reactions and cause myelotoxicity [53,54,58].

AQ is categorized as a prodrug, since it is readily metabolized in the liver into desethylamodiaquine (DEAQ) (**Fig. 6**), an oxidation step performed mainly by CYP2C8. AQ has a short half-life (4-12h) compared with its metabolite DEAQ (3-12 days). This aspect is of particular interest because, since DEAQ retains its antimalarial capacity longer and is responsible for post-treatment prophylaxis [59].

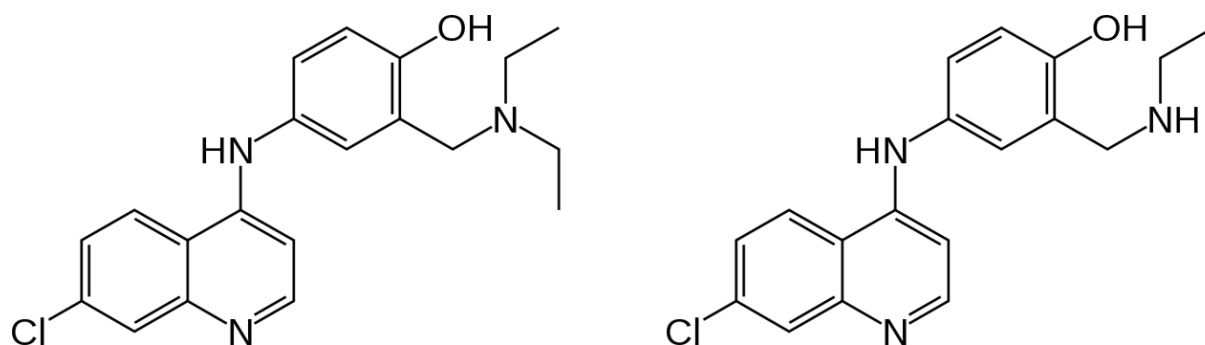


Figure 6 - Amodiaquine (left) and its metabolite Desethylamodiaquine (right).

#### 4.4.5 Antifolates

This class of drug interferes with the synthesis of folic acid and as a consequence with the synthesis of nucleotides required for DNA synthesis. Folate metabolism of malaria parasites provides two targets for current antimalarial therapy: dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). Sulfa drugs, sulfadoxine or dapsone, act as DHPS inhibitors while pyrimethamine or chlorcycloguanil are DHFR inhibitors [60] (**Fig. 7**).

In 1951, the first field trial of pyrimethamine monotherapy was carried out in African children [61]. The effectiveness of pyrimethamine as a prophylactic agent against *P. falciparum* was subsequently confirmed under controlled conditions [62]. During the 1950–1960s, pyrimethamine was mainly used as a causal prophylaxis of *P. falciparum* infection or for mass drug administration (MDA) due to the effectiveness of chloroquine in all endemic regions [63,64,65].

In 1959, sulfadoxine was found to potentiate the schizontocidal effect of pyrimethamine [66,67]. After that, faster schizontocidal activity and improved clinical response to *P. falciparum* infection were evident when sulfadoxine was used in combination with pyrimethamine [68]. In the late 1960s, an antifolate combination of sulfadoxine and pyrimethamine, SP, was first introduced in Thailand where the increased frequency of chloroquine-resistant *P. falciparum* infections reached an unacceptable level.

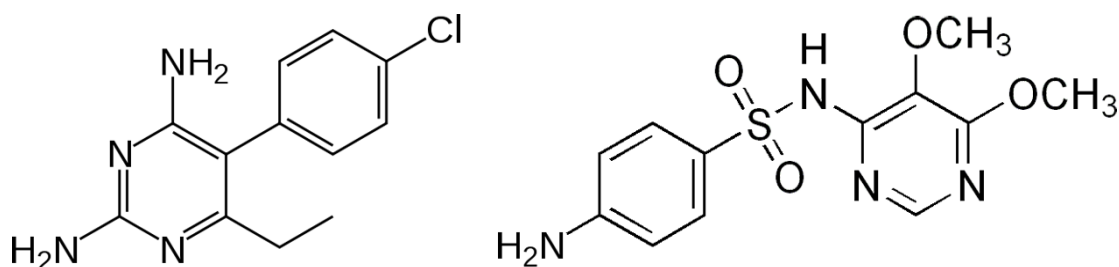


Figure 7 - Structure of pyrimethamine (left) and sulfadoxine (right)

#### 4.4.6 Arylaminoalcohols

The latest antimalarials to be adapted worldwide and used for the treatment of malaria are the arylaminoalcohols, such as mefloquine (MQ) and lumefantrine (Lum) (**Fig. 8**).

MQ emerged from the US Army's enormous drug discovery programme at the time of the Vietnam War between 1963 and 1976 when over a quarter of a million potential antimalarial compounds were screened [69]. The earliest reported trials of MQ were carried out on US prisoners [70]. MQ first became generally available for European travellers in 1985 [71] and is still broadly used mostly in South-east Asia. The side effects associated with MQ include adverse neuropsychiatric symptoms [53,54].

MQ is absorbed with a half-life of 1 to 4 hours and reaches peak concentration before 24 hours and has a terminal elimination half-life of 2 to 3 weeks in patients with malaria [69].

Lum is slowly and erratically absorbed, requiring 18 hours to complete maximal absorption. Low and variable bioavailability is the major factor contributing to interindividual inconsistency in pharmacokinetics. Food intake has a significant effect on the bioavailability of Lum, which is increased by a factor of 16 when the drug is taken with a high fat meal compared with that in fasting individuals. Because of variable bioavailability, the terminal elimination half-life may also vary from 30h to 107h depending on the population [72].

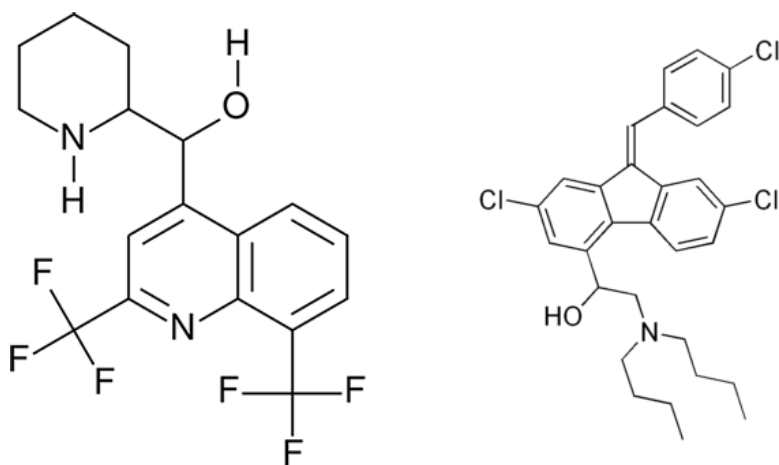


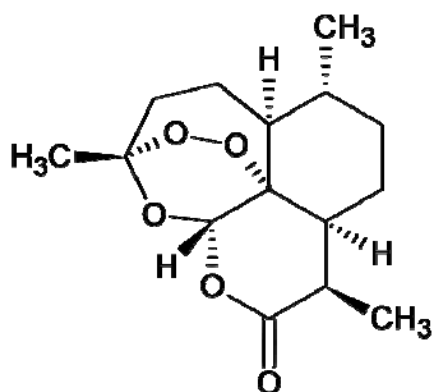
Figure 8 - Structures of mefloquine (left) and lumefantrine (right)

#### 4.4.7 Sesquiterpene lactones

Artemisinin (ART) (**Fig. 9**), isolated from the plant *Artemisia annua*, is an herb described in traditional Chinese medicine which makes it one of the oldest antimalarial used by man. Also known as qinghaosu, ART and its derivatives are a group of drugs that possess the most rapid action of all current drugs against *falciparum* malaria. The pharmacokinetics of artemisinins

have unique features, characterized by a very short half-life and auto-inducible elimination properties [73]. Its antimalarial activity resides in the endoperoxide bridge structure.

Artemisinin is generally well tolerated at the doses used to treat malaria. The side effects from the artemisinin class of medications are similar to the symptoms of malaria itself: nausea, vomiting, anorexia, and dizziness. Mild blood abnormalities have also been noted [53].



**Figure 9 - Artemisinin structure**

Use of artemisinins as a monotherapy is well known to have poor efficacy. In addition, the WHO has recommended cessation of monotherapy (press release 20.1.2006) in an attempt to limit emergence of artemisinin resistance. Recrudescences following monotherapy tend to occur rather late, and studies following patients up to day 42 detect a higher proportion of recrudescences than those stopping at 28 days. Estimates of recrudescence rates after short course artemisinin monotherapy at 28 days follow-up are 20–40% in Africa [73,74,75] and about 20% in Southeast Asia [76,77,78]

Duration of therapy has been reported to be of critical importance in efficacy of artemisinin based monotherapies, with extensions of 7 days improving cure rates significantly [79,80,81]. Artemisinin derivatives are presently only used in combination therapy as will be further discussed.

## **5 ANTIMALARIAL DRUG RESISTANCE**

Eradication efforts based on the use of CQ faltered in the 1960s due to the development of drug-resistant parasites. Nowadays, resistance to all main antimalarials used has been reported.



Recent genetic and genomic advances have paved the way for discoveries into the origins and spread of antimalarial drug resistance and the underlying molecular mechanisms.

## 5.1 QUINOLINES

Awareness and comprehension regarding the public health impact of antimalarial resistance came following the emergence of CQ resistant parasites. In 1969, the WHO declared that the major world effort to eradicate malaria, The Global Malaria Eradication Programme, had failed in part due to CQ resistance.

The first studies in antimalarial resistance were conducted on the quinolines. It took more than 30 years to go from clinical recognition of CQ resistance to the identification of the molecular cause, the chloroquine resistance transporter gene (*pfcr*) [82]. A specific mutation of lysine to threonine (K76T) was shown to confer *in vivo* [83] and *in vitro* [84] CQ resistance to the extent that it became an established biomarker.

Recent studies, analyzing a large number of geographically diverse *pfcr* alleles and microsatellite genotypes from parasite isolates, have identified at least three additional independent foci of resistance [31,85]. Origins of CQ resistance (CQR) have so far been discovered in the Thai–Cambodian border region (eventually spreading westward into Africa), Papua New Guinea, the Philippines, Colombia and Peru [86].

*Pfcr* was discovered in the search for mechanisms of CQ resistance, however, broader studies revealed that this transporter can also modulate drug susceptibility and tolerance to chemically unrelated antimalarials [84,87,88].

## 5.2 ANTIFOLATES

Sulfadoxine-pyrimethamine (SP) resistant parasites rapidly spread from Southeast Asia and South America in the 1970s–1980s and to Africa in the last two decades [89]. Unlike CQ, where resistance is due to a mutated transporter protein, SP resistance is driven by mutations of the drug target. Mutations in the DHFR and DHPS determine resistance to SP in *P. falciparum* [90,91,92].

Five mutations in *pfdhfr* and five in *pfdhps* are the main determinants for SP resistance. For *pfdhfr*, an amino acid change at position 108 from serine to asparagine (S108N) represents the initial mutation. This necessary mutation raises the parasite's resistance to a certain level, however, additional mutations are required to enhance resistance further. Additional mutation(s) at positions 50, 51, 59, and 164 synergistically increase the levels of resistance [93,94,95,96].

For *pfdhps*, an amino acid change at position 437 (A437G) represents the initial mutation for sulfadoxine resistance [92,97]. And additional mutation(s) at positions 436, 540, 581, and 613 elevate levels of sulfadoxine resistance.

The origin of resistance to SP seems to be more complex than that observed for CQ, with different foci discovered even within the same continent [89].

### 5.3 ARYLAMINOALCOHOL

The mode of action of arylaminoalcohols in *P. falciparum* is not currently known. However, genetic determinants of resistance in *P. falciparum* correlate with mutations in *pfcr* and the multidrug resistance gene 1 (*pfmdr1*).

This association was demonstrated by classical genetics, selection studies and reverse genetics. It was noted that parasite lines 3D7 and HB3, the parents of a genetic cross, differ in sensitivity to the hydrophobic arylaminoalcohol (mefloquine, halofantrine and lumefantrine) [98]. A particularity of this cross is that the parental lines have a *pfcr* wild type background.

*P. falciparum* reverse genetic analysis has confirmed these observations [99,100]. Importantly, in these studies mutations introduced into the chloroquine sensitive line were unable to confer chloroquine resistance. Nevertheless, introduction of *pfmdr1* wild-type polymorphisms in a chloroquine resistance line, resulted in the reduction of chloroquine resistance, suggesting that *pfmdr1*, although important in conferring higher levels of chloroquine resistance, is not sufficient to confer resistance [101].

There are two ways in which *pfmdr1* gene polymorphisms may lead to drug-resistance, through gene amplification and/or through mutation [102,103]. Amplification of *pfmdr1* was found to occur through multiple and independent events, suggesting it has arisen in several independent places [104].

*In vivo*, *pfmdr1* copy number variation (CNV) determines parasite resistance to MQ [105] and Lum [106].

### 5.4 ARTEMISININS

Artemisinin's (ART) structure is completely different from all other antimalarial described here (not based in the quinoline backbone) and therefore is expected to have a different mechanism of action. The first insight into ART's mode of action involved the establishment of the structural endoperoxide bridge [107]. Since peroxides are a known source of reactive oxygen species such as hydroxyl radicals and superoxide [108]. This observation suggests that free radicals might somehow be involved in the mechanism of action. Artemisinin interacts

with intraparasitic haeme, suggesting that intraparasitic haeme or iron might function to activate artemisinin inside the parasite to generate toxic free radicals [109]. When ART or its derivatives are incubated with haeme or iron, they decompose in a fashion that suggests the generation of free radical intermediates [110]. Despite these findings, artemisinin treatment of living intraerythrocytic *P. falciparum* caused no change in hemozoin, suggesting that haeme metabolism might not be the major intracellular target [111].

Six malaria specific proteins were detected as targets of ART. These are not highly abundant proteins, suggesting that ART reacts selectively [112].

The first protein to be suggested as a target for artemisinins is the malarial translationally controlled tumour protein (TCTP), a protein that binds haeme [113,114]. It is likely that the reaction between artemisinin and TCTP occurs because of an association between TCTP and haeme, since both are localized to the food vacuole. TCTP is involved in calcium binding and microtubule stabilization [115]. Despite that increased expression of TCTP correlates with artemisinin resistance, no genetic alterations have been described.

Membrane containing structures such as the plasma membrane, endoplasmic reticulum, nuclear envelope, food vacuolar membrane and mitochondria appear to be most sensitive to the action of artemisinin derivatives [116,117,118].

Structural similarities of ART to thapsigargin, which is also a sesquiterpene lactone, lead to the identification of another candidate target related to the endoplasmic reticulum, the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) encoded by the *pfATPase 6* gene in *P. falciparum* [119]. The function of the SERCA pump is to transport  $\text{Ca}^{2+}$  from the cytosol to the lumen of the endoplasmic reticulum [120]. Thapsigargin is a well described specific inhibitor of the mammalian SERCA pump [121]. Inhibition of the SERCA pump by thapsigargin is characterized by a transient  $\text{Ca}^{2+}$  increase in the cytosol.

ART and thapsigargin share the same binding pocket in the SERCA protein as demonstrated by competition assays [119]. Additional structural analysis on the binding pocket of different *Plasmodium spp.*, using the rabbit SERCA pump crystal structure, further supports SERCA as a drug target in malaria parasites [122].

The S769N mutation in *pfATPase 6*, noted exclusively in French Guiana, was reported to be associated with decreased sensitivity to artemisinins. *In vitro* studies of mutant *pfATPase 6* also showed an alteration of the half maximal effective concentration ( $\text{EC}_{50}$ ) to artemisinins [123].

In rodent malaria, the *ubp-1* gene encoding the *P. falciparum* orthologue of the deubiquitinating enzyme was also associated with artemisinins resistance [124].

Despite the fact that clinical failures to artemisinin has been reported [125,126], so far only *pfprt* and *pfmdr1* have correlated with artemisinin sensitivity *in vitro*. Contrary to resistance to CQ, the allele K76 correlates with artemisinin resistance. For *pfmdr1*, increase in the gene copy number variation promotes *P. falciparum* resistance to artemisinins. SNP in *pfmdr1* associated with arylaminoalcohols are associated also with artemisinin resistance [87,98,99,127].

## 6 ANTIMALARIAL RESISTANCE GENES

So far, the two main molecular players of antimalarial resistance in *P. falciparum* are the chloroquine resistance transporter gene (*pfprt*) and the multidrug resistant gene 1 (*pfmdr1*). In the next subchapter, the genetic and physiological characteristics of these two genes will be described.

### 6.1 PfCRT

A major breakthrough in the search for the genetic basis of CQR in *P. falciparum* was the identification of *Plasmodium falciparum* chloroquine resistance transporter gene (*pfprt*), which encodes a putative transporter or channel protein [82]. PfCRT is a 48 kDa protein containing 424 aminoacids, 10 predicted transmembrane-spanning domains and is localized to the DV membrane in erythrocytic stage parasites [82,87]. Fifteen polymorphic amino acid residues in PfCRT are associated with CQR in field isolates. These vary significantly depending on the geographic location and selection history, while CQ sensitive (CQS) strains maintain an invariable wild-type allele (**Table 2**) [31,85,128,129]

A K76T mutation appears to be necessary for the resistance phenotype, and is the most reliable molecular marker of resistance among the various *pfprt* mutations [83,130].

Mutant *pfprt* was associated with low CQ accumulation in the *P. falciparum*, however, only recently was conclusive evidence found for a role of PfCRT as an antimalarial vacuolar efflux transporter [131]. The endogenous role of PfCRT in the malaria parasite has yet to be revealed despite the wealth of epidemiological and *in vitro* drug response data demonstrating the critical role of mutations in *pfprt* which lead to CQR. PfCRT homologues in plants seem to play a role in glutathione and redox stress [132]. An understanding of the natural role of PfCRT in normally functioning *P. falciparum* parasite will provide a clearer picture of how drug resistance works in the malaria parasite.

It is now becoming clear that *pfcr1* is a major determinant of resistance not only for CQ but most probably also for other antimalarials including quinolines and arylaminoalcohols [88].

## 6.2 PfMDR1

In cancer cells, drug resistance frequently arises from the presence of an over-expressed P-glycoprotein. This molecule is an ABC transporter capable of actively expelling a wide range of structurally and functionally diverse chemotherapeutic agents in a verapamil-sensitive manner [133]. Inspired by the apparent phenotypic similarities between multi-drug resistance in cancer cells and CQ resistance in *P. falciparum*, it was once thought that a *P. falciparum* homologue of the mammalian P-glycoprotein, later termed PfMDR1 or Pgh-1, could be a major candidate molecule for conferring resistance to CQ [103]. Although this hypothesis was not completely accurate, it is now accepted that PfMDR1 can contribute to CQ resistance dependent on the genetic background of the parasite strain [99,100].

Mammalian P-glycoproteins localize within the plasma membrane and their nucleotide binding domains face the cytoplasm which aids in the export of drugs out of the cell (Higgins, 2007). However, PfMDR1 is present on the parasite's digestive vacuolar membrane [134] and that the topology of the protein leaves its ATP-binding domain facing the cytoplasm [135]. The molecular basis of CQR raised a lot of uncertainty regarding the exact role of PfMDR1 in drug resistance.

It is now becoming evident that vectorial transport by PfMDR1 is, therefore, inwardly directed, into and not out of the digestive vacuole [136,137]. This new insight into the cellular physiology of PfMDR1 caused a rethinking of its contribution to the molecular mechanism of antimalarial resistance.

**Table 2 - Geographic distribution of *pfprt* mutations.** Mutant aminoacids are shown highlighted with grey background. Adapted from [138]

Origin	Clones	pfprt aminoacid positions																			
		72	74	75	76	77	97	144	148	152	160	163	194	220	271	275	326	333	352	356	371
CQS																					
Honduras	HB3	C	M	N	K	I	H	A	L	T	L	S	I	A	Q	P	N	T	Q	I	R
Sudan	106/1	C	I	E	K	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
Labclones	106/1-IR	R	I	E	I	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
	106/1-IK	C	I	E	I	I	H	A	L	T	L	S	I	S	E	P	S	T	K	I	I
	K1AM	C	I	E	T	I	H	A	L	T	L	R	I	S	E	P	S	T	Q	V	I
	K1Hf	C	I	E	T	I	H	A	L	A	L	R	I	S	E	L	S	T	Q	I	I
CQR																					
Mali	S35CQ	C	I	E	T		H	A	L	T	L	S	I	S	E	P	N	T	Q	I	
SouthAfrica	RB8	C	I	E	T	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	
Labclones	106/1-N	C	I	E	N	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	
	106/1-I	C	I	E	I	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	
Thailand	Dd2	C	I	E	T	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	T	
Thailand	TM93-C1088	C	I	E	T		L	A			L			S	E		S			T	
Cambodia	783	C	I	E	T		H	A	L				I	S	E		N	T		T	
Cambodia	738	C	I	D	T		H	A	I				T	S	E		N	S		I	
Cambodia	734	C	I	D	T		H	F	I				T	S	E		N	S		I	
Cambodia	176	C	I	E	T	T															
Cambodia	108	C	I	D	T	I															
Cambodia	36	C	T	N	T	I															
Morong	PH1	C	M	N	T		H	T			Y			A	Q		D			I	
Morong	PH2	S	M	N	T		H	T			Y			A	Q		D			I	
Lombok	Field isolate	C	M	N	N																
Tamika	2300	C	I	K	T																
Armopa	CQ076	S	I	E	T																
Solomon	PNG4	S	M	N	T	I	H	A	L	T	L	S	I	A	Q	P	D	T	Q	L	
Ecuador	Ecu1110	C	M	N	T	I	H	A	L	T	L	S	I	S	Q	P	D	T	Q	L	
Colombia	Jav	C	M	E	T	I	Q	A	L	T	L	S	I	S	Q	P	N	T	Q	I	
Brazil	7G8	S	M	N	T	I	H	A	L	T	L	S	I	S	Q	P	D	T	Q	L	
Guyana	Field isolate	S	M	I	T																
Guyana	Field isolate	R	M	N	T																

Polymorphisms at amino acids 86, 184, 1034, 1042, and 1246 of PfMDR1 alter *in vitro* susceptibility to antimalarial drugs, including quinine, halofantrine, mefloquine and artemisinin [98,99,100,139]. In particular, the N1042D substitution seems to play a prominent role in quinine resistance [100], while the N86Y substitution has been implicated in contributing to resistance to lumefantrine and high levels of CQ [140,141]. Amplification of *pfmdr1* is associated with *in vitro* resistance to quinine, mefloquine, and halofantrine [105,142,143,144].

A single amino acid change, substituting the wild-type asparagine at position 86 for the aromatic amino acid tyrosine, completely alters the substrate specificity from quinine and CQ to halofantrine transporting capability [145].

PfMDR1 itself may also be a target of antimalarial drugs, including quinine and mefloquine, as demonstrated in competition studies which suggest that some of the drugs that interact with PfMDR1 may function as both substrates and inhibitors [137,145]. An analogous finding has been reported for human P-glycoprotein [146] where it was shown that the common drug binding site can accommodate several substrates of the same or different type, and at the same time [147].

### **6.3 PfMRP1**

The first ABC protein shown to confer resistance to multiple natural product drugs used in the treatment of cancer was the 170 kDa P-glycoprotein, originally described in 1976 [148]. Cases of multidrug resistance in the absence of P-glycoprotein overexpression together with studies that failed to detect P-glycoprotein in a variety of human tumors, suggested the existence of other multidrug resistance-conferring proteins [149,150].

Shortly thereafter, transfection experiments provided unequivocal evidence that overexpression of a second ABC protein could cause multidrug resistance in mammalian cells [151]. This new class was defined as the multidrug resistance-associated proteins (MRPs). MRP proteins have just recently begun to be studied and early reports indicate a correlation, both *in vivo* and *in vitro*, with antimalarial resistance [152,153]

## **7 ARTEMISININ COMBINATION THERAPIES (ACTs)**

The need to suppress antimalarial resistance and increase the efficacy of malaria chemotherapy led to the development and global implementation of artemisinin combination therapies (ACTs) as the principle strategy for the treatment of malaria in endemic countries.

ACTs are intended to improve the efficiency of individual components and provide some protection for individual components against the development of higher levels of resistance [154].

In the beginning of this century the WHO started to financially support and strongly recommend switching protocols to include artemisinin-based combination therapy (ACT) claiming that these drug combinations are more effective, allow for shorter treatment courses, and protect against drug resistance.

## **7.1 ARTEMISININS IN ACT**

ART derivatives have been used in ACT in place of ART itself. This decision is based on two characteristics: derivatives can have a higher hydrophilic capacity and/or can be more potent than ART itself. Most used ACTs have in its formulation derivatives of ART like artesunate, dihydroartemisinin or artemether. After administration and unlike artemisinin, artesunate and artemether convert to dihydroartemisinin [73]. Artemisinin compounds have several advantages over other antimalarial drugs for use in combinations as follows [155]:

- They are very active at reducing parasite numbers; more than the other antimalarial, approximately  $10^4$  per asexual cycle.
- They reduce considerably gametocyte carriage and thus transmissibility.
- Resistance has not yet spread
- These drugs are very rapidly eliminated and thus provide no opportunity for parasites to be exposed to sub-therapeutic concentrations if the dosage is correct.
- They have operational advantages: they produce a rapid clinical response, which encourages acceptance, and they have an excellent safety and side-effect profile that encourages compliance.

## **7.2 PARTNER DRUGS IN ACT**

The most common antimalarials used in combination therapy are: artemether-lumefantrine (AL), artesunate-amodiaquine (AS-AQ), artesunate-mefloquine (ASMQ), artesunate-sulfadoxine-pyrimethamine (AS-SP) and dihydroartemisinin-piperaquine (DHA-PQ). Lately, most countries have been adopted AL or AS-AQ as first-line therapy.

In the last decade, a large number of efficacy clinical trials were performed with ACTs to define baseline characteristics of the treatments. In general, ACT performed well; being in most cases above the 90% efficacy required by WHO for an ACT to be introduced as a first line treatment [11].

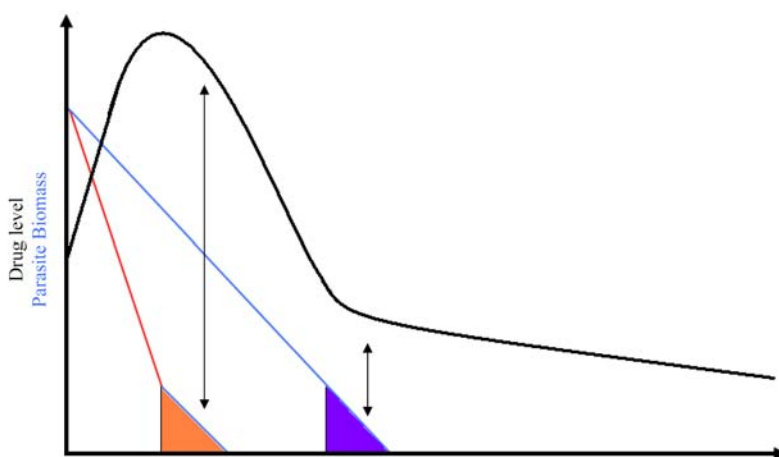


The most important conclusion regarding this initial work is that partner drugs efficacy by itself is an important determinant factor for ACT performance. This idea is supported by clinical trials where partner drugs have resulted in decreased efficacy, as in the cases of SP and AQ.

### 7.3 RATIONALE FOR COMBINATION THERAPY

The rationale for using drugs in combination is well established for the treatment of tuberculosis, infection by the human immunodeficiency virus (HIV), and cancer. The probability of a parasite arising that is resistant simultaneously to two drugs with unrelated modes of action is the product of the per parasite mutation frequencies multiplied by the total number of parasites exposed to drugs. Therefore, if the probability of a parasite being resistant to drug A is one in  $10^9$  and to drug B is one in  $10^9$  then the probability that a parasite will be simultaneously resistant to both is one in  $10^{18}$ , representing a billion-fold reduction in probability. As such, ACT fundamentals towards resistance were based in the probability that resistance events to ACT parts are based in independent events [156] which can be mathematically described as  $P_{ACT-R} = P_{ART-R} * P_{Partner\ drug\ resistance}$ . The implication of this assumption will be taken in consideration and discussed in this thesis.

**Figure 10** represents the difference in total parasite biomass reduction to a long half life antimalarial in combination with artemisinin (salmon line) or by itself (blue line). In ACT, it is expected that the fast reduction in parasite biomass, in a patient with malaria, promotes the exposure of more tolerant parasites to higher levels of the partner drug (salmon triangle) than if artemisinin were not used (purple triangle) [156].



**Figure 10 - Scheme of parasite biomass reduction in ACT (salmon) and non-ACT (blue) treatments.** The blue line refers to biomass reduction by partner drug in monotherapy. Salmon line describes reduction of biomass in combination with artemisinin. The triangles show the fraction of parasite biomass remaining after antimalarial exposure and expected to be more resistant.

## **7.4 ACT IMPACT**

So far, the introduction of ACT as first line treatment has being very successful. Countries where ACT and other malaria controls activities have been introduced have seen malaria endemicity reduced [157]. There is again a new wave of optimism as seen in the middle of the last century after initiation of The Global Malaria Eradication Programme. From lessons taken at that time, we should be well aware and be able to develop a sustainable system enabling us to fully control and eventually eliminate malaria.

## 8 AIMS OF THE THESIS

### 8.1 OVERALL AIM

To improve the understanding in the molecular basis of *P. falciparum* resistance to ACT.

### 8.2 SPECIFIC OBJECTIVES:

**Paper 1-** To determine the importance of *pfmdr1* natural polymorphisms, as markers in *P. falciparum* associated with clinical lumefantrine tolerance/resistance.

**Paper 2-** To study the structural impact of natural polymorphisms in PfMDR1.

**Paper 3-** To explore polymorphisms in the *pfmrp1* gene as new molecular markers associated with ACT *in vivo* resistance/tolerance.

**Paper 4-** To study the impact of dihydroartemisinin in *P. falciparum* calcium homeostasis.

## 9 MATERIAL AND METHODS

### 9.1 STUDY SITES

Zanzibar is located outside the coast of mainland Tanzania and consists of two large islands, Unguja and Pemba, and numerous small ones. Paper I and III includes a clinical drug efficacy trial conducted in two sites; Kivunge Hospital on Unguja and Micheweni Hospital on Pemba. Both hospitals are located in densely populated rural areas. The trial was performed in October 2002 to February 2003, when chloroquine and SP was still supplied to the study sites by the government. Antimalarial drugs were available in the private sector with exception to ACTs. Later in 2003 Zanzibar became one of the first regions in Africa to implement ACT, with artesunate-amodiaquine as first line treatment and artemether-lumefantrine as second line treatment for uncomplicated *P. falciparum* malaria.

Paper III includes a clinical trial conducted in Fukayosi Primary Health Care Centre in April to July 2004. Fukayosi is a village located in a relatively scarcely populated rural area in Bagamoyo district, on mainland Tanzania. At the time of the study SP was the first line

treatment for uncomplicated *P. falciparum* malaria and amodiaquine the second line treatment in Tanzania and ACTs were not available in the governmental health care. In 2006 Tanzania adopted artemether-lumefantrine as first line treatment.

## **9.2 *In vivo* FOLLOW UP TRIALS**

The two clinical trials performed in Zanzibar and mainland Tanzania respectively, included children with microscopically confirmed uncomplicated *P. falciparum* malaria. All enrolled children had a parasitaemia of 2000-200 000 asexual parasites/ $\mu$ l blood and an axillary temperature of 37.5°C or a history of fever the last 24 hours. Children with severe malaria were not included in the trial. Follow-up visits after initiation of treatment were conducted on days 1, 2, 3, 7, 14, 21, 28, 35, 42 and on any day of recurrent illness occurring during the 42-day follow-up period. On every visit blood smear was collected for the assessment of parasitaemia through microscopy. Parasites were counted against 200 white blood cells and parasitaemia was calculated with the assumption that one  $\mu$ l of peripheral blood contains 8000 white blood cells. Finger prick blood was collected on filter paper for molecular analysis.

In Zanzibar, the clinical trial was performed between October 2002 and February 2003 and included 200 children aged 12 to 59 months, with a body weight of at least 9 kg. The children were treated under supervision with a fixed combination of 20 mg/120mg artemether-lumefantrine (Coartem®) twice a day for three consecutive days. Children between  $\geq 9$ kg and  $<15$  kg were treated with one tablet, while children between  $\geq 15$  and  $<25$  kg were treated with two tablets.

## **9.3 *In vitro* STUDIES**

### **9.3.1 Parasites cultures**

*P. falciparum in vitro* culturing and experiments were used for Paper II and Paper IV. The different clones were acquired from Malaria Research and Reference Reagent Resource Center (MR4). Parasites were cultured in 4 ml or 12 ml flasks with Rh0<sup>+</sup> erythrocytes (washed three times) to 5 % hematocrite with culture media (Invitrogen, RPMI-1640 with phenol red) supplemented with 10 % human serum. Cultures were maintained in continuous cultures at 37°C. Parasite densities were monitored by microscopic assessment by Giemsa-stained smears.

### 9.3.2 Susceptibility testing

For drug susceptibility test and growth experiments, cell growth was calculated by relative quantification of *P. falciparum* histidine rich protein 2 pfHRP2 [158], analysed in a Multiskan EX (Thermo Electron Corporation) ELISA reader.

### 9.3.3 Blood sampling and storage

All blood samples were collected on 3MM filter paper for parasite genotyping. After the blood had dried, the filter papers were stored in individual sealable plastic bags in room temperature.

### 9.3.4 DNA extraction

Samples were extracted using the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems). The extraction was performed according to the manufacturer's recommendations with some modifications adjusted for filter paper: the filter paper with dried blood were cut in pieces and put in distilled water. The lysing mixture was incubated for 1 hour at 58°C and the lysed samples were incubated at 4°C overnight before performing the extraction. The samples were stored at -20°C for long term storage or in 4°C for shorter storage time.

### 9.3.5 *pfmsp1* and *pfmsp2* analysis

For categorisation of recurrent infections as recrudescences or reinfections, the *pfmsp2* was analysed as described [159]. *Pfmsp2* is a single copy gene that is highly polymorphic both in sequence and size. First the outer conserved region of polymorphic repetitive block 3 was amplified followed by two separate nested reactions. One pair of oligonucleotide primers specific for the FC27 allelic type was used in one of the nested reactions while one pair specific for the IC/3D7 type was used in the other. The polymerase chain reaction (PCR) products were separated by gel electrophoresis and visualized by UV transillumination, after staining with ethidium bromide. Samples with at least one matching band from samples from day 0 and day of recurrent infection respectively were interpreted as recrudescences; all other samples were interpreted as reinfections.

However, studies showed that two markers should be used for the discrimination between recrudescences and reinfections [160]. For that reason additional analysis of *pfmsp1* diversity was made. Similar to the analysis of *pfmsp2*, the outer conserved region of the polymorphic repetitive block 2 was amplified, followed by 3 separate nest amplifications. The nest amplifications were specific for the allelic type of MAD20, K1 and RO33.

### 9.3.6 PCR- Restriction Fragment Length Polymorphism (RFLP)

PCR-RFLP is, equipment wise, a simple way to analyse SNPs and is therefore a suitable method to use in the field.

All analyses were based on nested PCR, except for the analysis of *PfATP6* where a seminest was used. The nested PCR products were incubated over night at the conditions described by the restriction endonucleases manufacturer, New England Biolabs (NEB) or Fermentas. The restriction enzymes that were used are listed in **Table 3**. After incubation, restriction fragments were analysed on 2%–2.5% agarose gels with ethidium bromide and were visualized by UV transillumination in a BioRad GelDoc 2000.

**Table 3** Restriction enzymes used for RFLP

SNPs cleaved allele marked in <b>bold</b>	Restriction enzymes (manufacturer)
<i>Pfprt</i> <b>K76T</b>	<i>ApoI</i> (NEB)
<i>Pfprt</i> <b>S163R</b>	<i>HinfI</i> (NEB)
<i>Pfmdr1</i> <b>N86Y</b>	<i>ApoI</i> (NEB)
<i>Pfmdr1</i> <b>Y184F</b>	<i>Tsp509I</i> (NEB)/ <i>TasI</i> (Fermentas)
<i>Pfmdr1</i> <b>S1034C</b>	<i>DdeI</i> (NEB)
<i>Pfmdr1</i> <b>N1042D</b>	<i>AseI</i> (NEB)/ <i>VspI</i> (Fermentas)
<i>Pfmdr1</i> <b>D1246Y</b>	<i>EcoRV</i> (NEB)
<i>PfATP6</i> <b>S769N</b>	<i>RsaI</i> (NEB)

### 9.3.7 Real-time PCR

Assessment of *pfmdr1* copy number, was performed with TaqMan<sup>®</sup> probe based real-time PCR as described in [105] with minor modifications. The analysis was performed using an ABI PRISM 7000 Sequence Detection System. The machines, TaqMan<sup>®</sup> buffer and probes were from Applied Biosystems.

Briefly, this technology is based on oligonucleotide probes with a reporter dye covalently ligated at the 5'-end and a quencher dye at the 3'-end. The proximity between them suppresses the emission of fluorescence by the reporter dye. During PCR, the primers and probes specifically hybridise to their complementary DNA sequence and as the DNA polymerase extends the primers, the hybridised probe is cleaved. This separates the reporter dye and the quencher dye, resulting in increased fluorescence from the reported dye. For analysis of *pfmdr1* copy number, TAMRA<sup>®</sup> probes were used. The *pfmdr1* probe was labelled

with FAM<sup>®</sup> and the probe for the endogenous control, *β-tubulin*, was labelled with VIC<sup>®</sup>. The *P. falciparum* clone 3D7 was used as a one copy calibrator for *pfmdr1* and Dd2 was used as a multi-copy control. All samples were run in triplicate. The comparative  $\Delta\Delta C_t$  (cycle threshold) method was used.  $C_t$  is the cycle number where the fluorescent crosses a set threshold. The amount of target (*pfmdr1*) is:  $2^{-\Delta\Delta C_t}$ . Where  $\Delta C_t = C_{t_{pfmdr1}} - C_{t_{\beta-tubulin}}$  and  $\Delta\Delta C_t = \Delta C_{t_{sample}} - \Delta C_{t_{3D7}}$ . Results of triple replicate samples were excluded if: (1) more than one replicate exhibited a  $C_t > 35$ ; (2) the triple replicate samples had  $C_t$  SD  $> 0.5$  and the  $C_t$  difference between the two remaining replicates was  $> 0.7$  after the removal of any outlier. The parasites were considered to have an amplified *pfmdr1* gene if copy number was  $> 1.5$ .

### 9.3.8 Pyrosequencing

Pyrosequencing is a method that can be used for SNP analysis, where short fragments of DNA are sequenced directly from a PCR product. One of the PCR primers used in the PCR reaction is biotinylated, which enables the purification of specific PCR products since Streptavidin Sepharose Beads binds to biotin and single stranded PCR products can then be separated in a vacuum based system. A sequencing primer is further hybridized to the PCR product and incubated with reagents containing enzymes and substrates. One deoxynucleotide triphosphate (dNTP) is added to the reaction at a time and is incorporated into the DNA strand if it is complementary to the base in the template strand. The incorporation results in a release of pyrophosphate (PPi) that is converted to ATP, which drives conversion of the substrate luciferin to oxyluciferin that generates light. The light is proportional to the number of nucleotides incorporated and is visualised as a peak in a pyrogram. ([www.pyrosequencing.com](http://www.pyrosequencing.com)) Consequently, e.g. the incorporation of three consecutive dTTPs into the DNA strand results in a peak that is three times higher than the incorporation of one dTTP.

### 9.3.9 Sequencing

Sequencing was used to evaluate the accuracy of PCR-RFLP method and in prospective studies as Paper III to identified new mutation in the candidate genes. The method used was dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission. Sequencing was performed at Center for Genomics and Bioinformatics, Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden or by MacroGen, Inc, Seoul, South Korea.

### 9.3.10 Statistical analysis of mixed infections

There is no consensus on how to consider mixed genotypes derived from mixed infections i.e. where both mutant and wild type allele are present. In view of that, the calculations with mixed infections have been handled slightly different for the clinical trials. In Paper I, detected mixed infections were calculated to contribute equally to each of the allele.

In Paper III, we choose to remove the mixed infections from the association analyses of the particular genotype that was mixed. The rationale can be explained having as example of *pfmrp1* I876V analysis. Selection of I876 is based on the principle that parasite populations carrying the I876 allele survive, while all parasites carrying 876V tend to be eliminated. Elimination of parasites carrying 876V will occur independently if only these parasites are infecting (pure) or if there are also parasites with I876 in the infection (mixed). Mixed infections with I876 and 876V should consequently become pure I876 after selection. Thus we suggest that *in vivo*, the selected genotype should be analysed against the mixed infections together with the non-selected genotype.

Proportions were compared using Yates's corrected  $\chi^2$  testing (Microstat<sup>®</sup> software, release 4; Ecosoft) and confidence intervals were calculated with the Confidence Interval Analysis (CIA) program (version 1.1). Fisher's exact test was determined with GraphPad QuickCalcs software; GraphPad Software. Statistical significance was defined as  $P \leq 0.05$ .

### 9.3.11 *In silico*

*P. falciparum* genome sequence became available in 2002. Thousands of genes are specific for *P. falciparum*, although several still homologous to well studied proteins in prokaryotes and/or eukaryotes organisms. Crystallography data is available ([www.pdb.org](http://www.pdb.org)) from some of these homologous to *P. falciparum* proteins and enables the structural studies by computational homology. In Paper II and Paper III these strategy was used to unveil the importance of PfMDR1 and PfMRP1 polymorphisms based in the bacterial homologous ABC transporter MsbA. For this purposes programs as Modeller which performs comparative protein structure modelling by satisfaction of spatial restraints, WinCoot and Yasara were used.

### 9.3.12 $\text{Ca}^{2+}$ records

To perform  $\text{Ca}^{2+}$  measurements in *P. falciparum* the  $\text{Ca}^{2+}$  indicator Fluo-3 was used. Fluo-3 was developed by Tsien (Nobel laureate) and colleagues for use with visible-light excitation sources in flow cytometry and confocal laser-scanning microscopy. Since being introduced



in 1989, Fluo-3 imaging has revealed the spatial dynamics of many elementary processes in calcium signalling. The most important properties of fluo-3 in these applications are an absorption spectrum compatible with excitation at 488 nm by argon-ion laser sources, and a very large fluorescence intensity increase in response to  $\text{Ca}^{2+}$  binding. Unlike the ultraviolet light-excited indicators fura-2 and indo-1, there is no accompanying spectral shift. The fluorescence intensity increase on  $\text{Ca}^{2+}$  binding is typically >100-fold.

### **9.3.13 Mitochondrial membrane potential measurements**

Mitochondrial transmembrane potential was assessed by uptake of TMRE (25 nM; Invitrogen), a lipophilic, cationic fluorescent dye that is only taken up by mitochondria having an intact electrochemical gradient. Infected RBCs were double labelled with 5  $\mu\text{M}$  Hoechst 33342 and incubated for 30 min with TMRE and fluorescence analysed in a LSR-II flow cytometer (BD Biosciences) before and after challenging parasites with 72  $\mu\text{M}$  DHA. Acquired fluorescence-activated cell sorting (FACS) data was analysed in FlowJo software v.5.7.2.

### **9.3.14 Cytochrome C (CytC) detection**

Anti- CytC antibody (BD Pharmingen) was used to study CytC release from mitochondria by flow cytometry and microscopy. Flow cytometry of the parasites was performed on a LSR II (Becton-Dickinson) equipped with a 488nm argon laser and a 358 nm ultraviolet light source. Parasite slides were mounted using the Prolong Antifade Kit (Molecular Probes) and scanned in a Zeiss LSM510 and Olympus Fluorview F1000 confocal microscope equipped with C-Apochromat 60X/1.2 water immersion objectives (Zeiss and Olympus).

## **9.4 ETHICAL CONSIDERATIONS**

Ethical approval for Paper I and Paper III was obtained from ethical committees in the endemic countries and from Karolinska Institutet, Sweden. All samples were obtained upon informed consent of the patients or their guardians.

## 10 RESULTS

### 10.1 PAPER I

The aim of this study was to explore the selection of other known mutations in *pfmdr1* and *pfcr1* following artemether-lumefantrine treatment. In addition, PfATP6 S769N, suggested to be associated with decreased susceptibility to artemether (Jambou 2005) was also investigated.

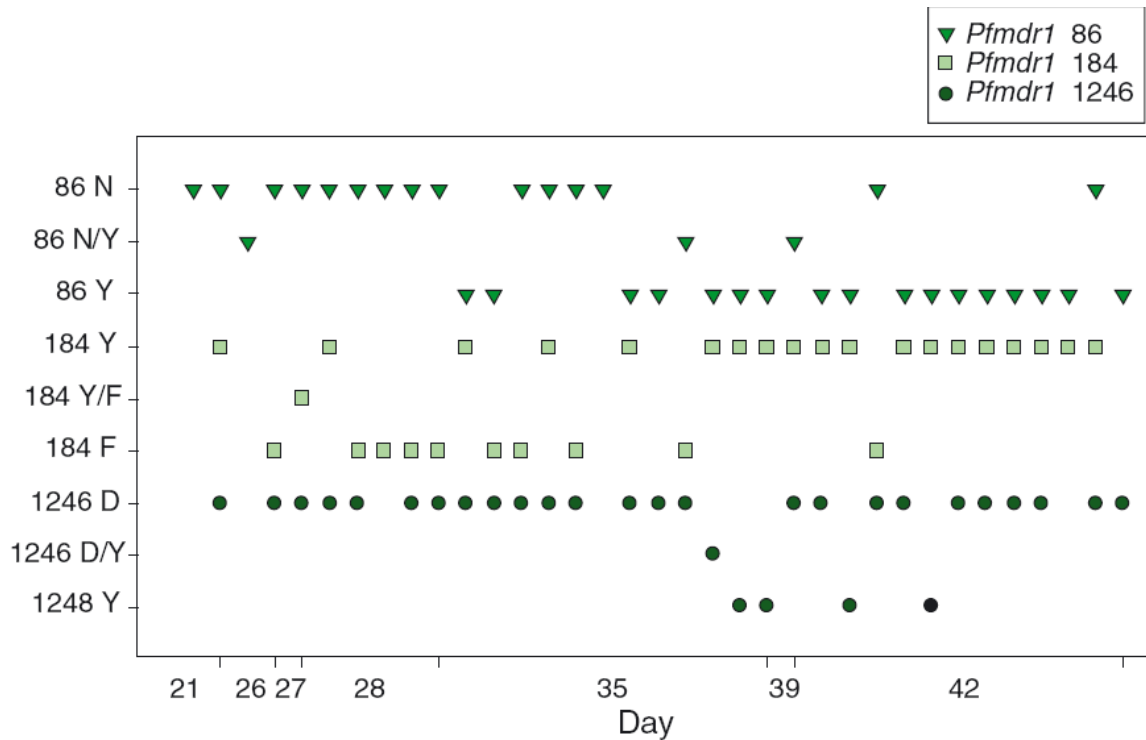
Recurrent infections classified as recrudescences by *pfmsp2* genotyping, were subjected to additional *pfmsp1* genotyping. Only 2 out of 11 remained recrudescences, while 7 were reclassified as reinfections and 2 were PCR negative for *pfmsp1*.

The PfMDR1 N86, 184F and D1246 alleles were observed to be selected after artemether lumefantrine treatment. When calculating mixed infections to contribute equally to each allele, the selection of PfMDR1 mutation comparing D<sub>0</sub> with reinfections was as follow: PfMDR1 N86 increase from 23.4% to 47.4% (P = 0.004). PfMDR1 184F showed a statistically significant increase from 16.6% at D<sub>0</sub> to 35.5% among reinfections (P = 0.027). Likewise, the PfMDR1 D1246 allele increased from 66.8% to 83.9% (P = 0.086).

An association between the N86/184F haplotype and reinfections was seen. The statistically significant selection of this haplotype (P = 0.001) was followed by a similar decrease in the prevalence of 86Y/Y184 (P = 0.009), while the other two haplotypes (86Y/I84F and N86/YI84) were not affected. The D1246 in combination with N86 was also significantly selected in reinfections (P = 0.001). This selection was not at the cost of any specific allele.

The two recrudescences analysed had N86/Y184/D1246 and 86Y/Y184/1246Y respectively. No *pfmdr1* gene amplification was detected. No polymorphism was found for PfMDR1 S1034C, 1042D, D1246Y, PfCRT SI63R and PfATP6 S769N.

The selection of mutations in PfMDR1 was seen to occur in time fashion. This observation lead was to describe the selective window for PfMDR1 N86, 184F and D1246 of being within the 35 days after artemether-lumefantrine treatment (**Fig. 11**).



**Figure 11 Selection of PfMDR1 after artemether-lumefantrine treatment.** Distribution of PfMDR1 N86Y, Y184F and D1246Y SNPs of the 28 *pfmsp2* adjusted *P. falciparum* reinfections during the 42-day follow-up period. Infections with PfMDR1 86N alleles are being selected particularly in the early reinfections, while in the later reinfections on day 42 the 86Y and 86N frequencies have returned towards the baseline frequency. A similar finding was seen for PfMDR1 Y184F.

## 10.2 PAPER II

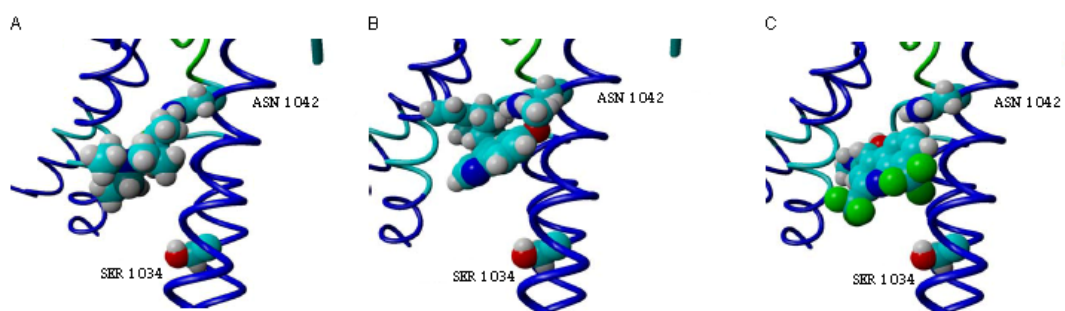
For this paper was considered the importance of polymorphism in *pfmdr1* (position 86, 184, 1034, 1042 and 1246) selected by lumefantrine in Paper I and analysed its importance in the transporter structure and for antimalarial resistance in general.

PfMDR1 is constituted by 12 transmembranes (TMs). Our results show that transmembrane (TM) 11 (harbor polymorphisms position 1034 and 1042) are close to TM1 in the open conformation of the transporter. On the top of TM1 locates residue 86 (polymorphism). The structural clustering of mutations in residues 86, 1034 and 1042 in this conformation suggests their role in the docking of antimalarial. To better understand the role of 1034 and 1042 we hypothesize TM11 as being part of a drug binding pocket. Our *in silico* docking results show that MQ, QN, CQ docked in the proposed binding site, preferentially interacting with residue 1042. The energies of docking for best pose were estimated to be in Kcal/mol: -6.89 for CQ (**Fig. 12A**), -7.86 for QN (**Fig. 12B**) and -5.69 for MQ (**Fig. 12C**).

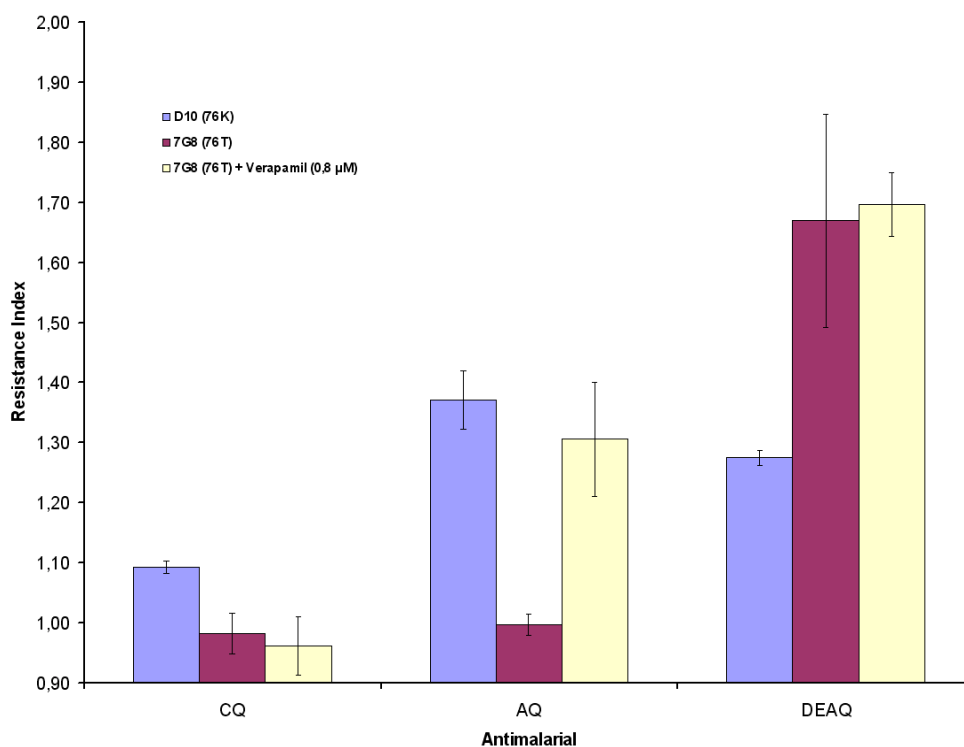
Residue 1246 happens to be located in the nucleotide-binding domain (NBD) 2. It is part of a cleft that interacts with the loop formed by TM4 and TM5. This particularity relates to residue 184 in TM3. The TM3 is located in the middle of TMD1 surrounded by TM1-TM2 in one

side and TM4-TM6 in the other. TM4 and TM5 come close to TM3 in the nucleotide bound conformation.

Our results support the hypothesis of PfMDR1 being a vacuolar importer of antimalarial. To acquire further evidence we evaluate the index of resistance for different aminoquinolines known to target the digestive vacuole, as CQ, AQ and DEAQ. The correspondent Log *D* (pH 7.2) values for these drug are CQ: 0.045 < DEAQ: 1.183 < AQ: 2.60. The contribution of PfMDR1 mutations 1034, 1042 and 1246 for index of resistance was higher for more hydrophobic (AQ and DEAQ) (**Fig. 13**) supporting the hypothesis that PfMDR1 is an importer which requires active transport for the vacuole. The contribution of PfMDR1 was also shown to be dependent on PfCRT background.



**Figure 12 - Docking of antimalarial in TM11.** Residues 1042 and 1034 are show in TM11 being a serine and aspartic acid respectively together with CQ (A), QN (B) and MQ (C) docks. The energies of docking for best pose were estimated to be -6.89 Kcal/mol for CQ, -7.8 Kcal/mol for QN and -5.69 Kcal/mol for MQ.



**Figure 13 - Resistance Index for 1034/1042/1246 *pfmdr1* haplotype.** Index was determined as the EC<sub>50</sub> ratio of isogenic transfectants CDY over SND for the different antimalarial.

### 10.3 PAPER III

Paper III is the first comprehensive study of *pfmrp1* diversity. The *pfmrp1* open reading frame (ORF) was sequenced in 103 *P. falciparum* infections originating from most malaria endemic regions and in 47 fresh *P. falciparum* isolates from Mae Sot, Thailand and 30 from Gulu, Uganda. *pfmrp1* was observed to harbor significant biodiversity with 23 non-synonymous SNPs, 8 synonymous SNPs and one insert were identified in the gene. The distribution of the SNPs showed distinct geographic patterns (**Table 4**). In Africa the most common SNPs were I876V and K1466R, with no other SNP seen in more than two samples. K1466R was only found in Africa and Papua New Guinea (PNG). In South America no SNPs in *pfmrp1* were observed.

The majority of SNPs were identified in samples with origin in Asia and Oceania. There the most common SNPs were H191Y and S437A, which were linked and mutated in almost all samples. Distinct haplotypes of *pfmrp1* SNPs in aminoacid positions 785, 876, 1007 and 1390 were observed. Either they were all wild-type, mutated in positions 876 and 1390 or mutated in positions 785, 876 and 1007 with or without mutation in position 572.

I876V was the most spread SNP worldwide, present in all parasite populations.

PfMRP1 I876V and K1466R, were analyzed in *P. falciparum* samples from malaria-infected patients, in two clinical drug efficacy trials in Tanzania (n=106) and Zanzibar (n=408), treated with artemether-lumefantrine vs. SP or artemether-lumefantrine vs. artesunate-amodiaquine, respectively.

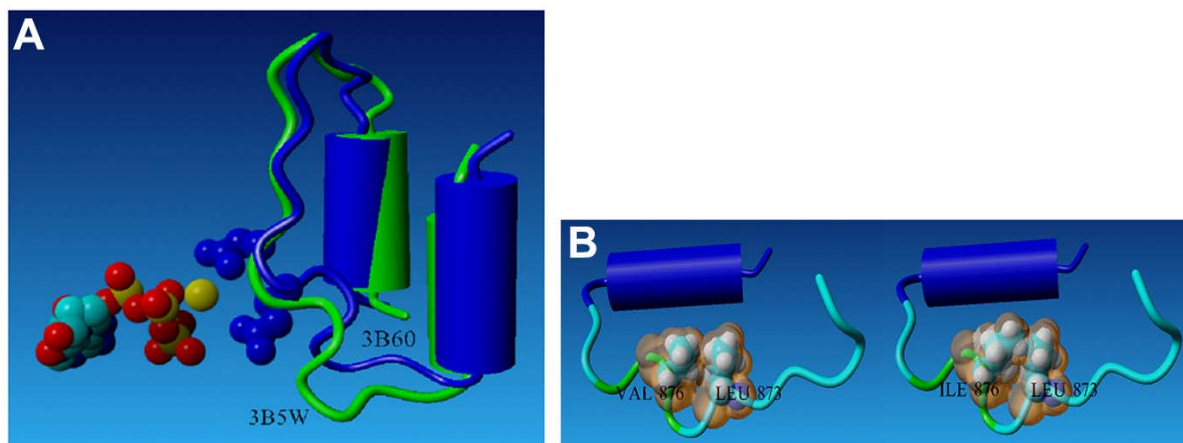
There were no statistically significant changes in frequency of K1466R after artemether-lumefantrine or artesunate-amodiaquine treatment. We observed a statistically significant positive selection of the pure I876 allele in the recurrent infections (92.1%) of the artemether-lumefantrine arm compared to the baseline (76.9%) ( $P=0.038$ ) in Zanzibar. In Tanzania the same tendency was detected after artemether-lumefantrine treatment, although it was not statistically significant. Pooling the two independent studies supplied robust evidence for a strong selection of the pure I876 in recurrent infections (89.5%) compared to the baseline (75.9%) ( $P=0.007$ ).

Residue 876 is located in nucleotide-binding domain (NBD) 1, immediately downstream of the Walker B motif between the LSGGQ motif and the H loop, an important region for ATP binding and hydrolysis. We then analysed the importance of this region analysing the dynamics of MsbA protein crystals trapped in different conformation (**Fig. 14A**). Our results show that this region is highly mobile required for nucleotide bound-unbound in the transporter.

*In silico* analysis suggested that variation in residue 876 (**Fig. 14B**) may influence ATP docking and consequently the catalytic cycle and potentially the overall protein function.

**Table 4 – Geographic genetic diversity of *pfmrp1***

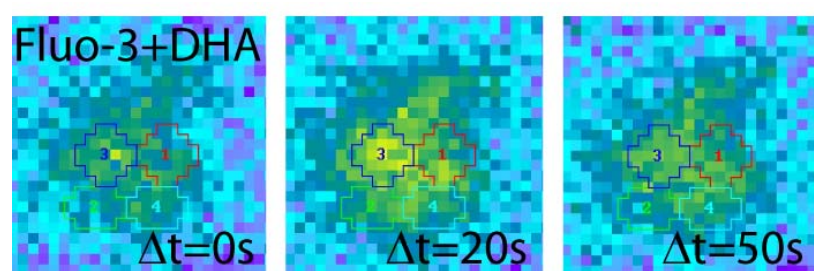
Origin		N	PfMRP1 polymorphisms											
			37	191	202	325	437	572	785	876	1007	1390	1431	1466
Africa														
Africa	19	P	H	K	N	S	F	H	I	T	F	K	K	
Benin	1	P	H	K	N	S	F	H	I	T	F	K	R	
Gambia	1	P	H	K	N	S	F	H	V	T	I	K	K	
Ghana	1	P	H	K	N	S	F	H	I	T	F	K	R	
Guinea Conakry	1	P	H	K	N	S	F	H	I	T	F	K	R	
Kenya	1	P	H	K	N	S	F	H	V	T	F	K	R	
Malawi	1	P	H	K	N	S	F	H	V	T	F	K	R	
Uganda	9	P	H	K	N	S	F	H	I	T	F	K	K	
	9	P	H	K	N	S	F	H	V	T	F	K	R	
	9	P	H	K	N	S	F	H	I	T	F	K	R	
	5	P	H	K	N	S	F	H	V	T	F	K	K	
Middle East														
Iran	2	P	Y	K	N	A	F	H	I	T	F	K	K	
	1	P	Y	K	N	A	F	H	V	T	F	K	K	
	1	P	Y	K	N	A	F	H	V	M	F	K	K	
Yemen	1	P	H	K	N	S	F	H	I	T	F	K	K	
South East Asia														
Cambodia	3	P	Y	K	N	A	F	N	V	M	F	K	K	
	2	P	Y	K	N	A	L	N	V	M	F	K	K	
	2	P	H	K	N	S	F	H	I	T	F	K	K	
	1	P	Y	K	N	A	F	H	V	T	I	K	K	
	1	P	Y	K	S	A	F	H	I	T	F	K	K	
Thailand	14	P	Y	K	N	A	L	N	V	M	F	K	K	
	8	P	Y	K	N	A	F	H	V	T	I	K	K	
	7	P	Y	K	S	A	F	H	I	T	F	K	K	
	6	P	Y	K	N	A	F	N	V	M	F	K	K	
	2	P	Y	K	N	A	F	H	I	T	F	K	K	
	2	P	H	K	N	S	F	H	I	T	F	K	K	
	1	P	Y	K	S	A	L	N	V	M	F	K	K	
	1	P	Y	K	N	A	F	N	V	T	I	K	K	
	1	P	Y	K	S	A	L	N	V	T	F	K	K	
	1	P	Y	K	S	A	F	H	V	T	I	K	K	
	1	P	Y	K	S	A	F	H	I	T	F	K	K	
Oceania														
Papau New Guinea	3	S	Y	E	N	A	F	H	V	T	I	K	K	
	1	P	Y	K	N	A	F	H	V	T	F	K	R	
	1	P	H	K	N	S	F	H	I	T	F	K	R	
Vanuatu	6	P	Y	K	N	A	F	H	V	T	I	I	K	
	1	P	Y	K	N	A	F	H	V	T	I	K	K	
South America														
Colombia	13	P	H	K	N	S	F	H	I	T	F	K	K	
Surinam	1	P	H	K	N	S	F	H	I	T	F	K	K	



**Figure 14 - Mobility of NBD in ABC transporters.** (A) MsbA structural alterations at NBD of open (3B5W) and close (3B60) conformations. Localization of ATP molecule is shown linked to the structures by a Mg<sup>2+</sup> bridge (yellow) (B) Visualization of V876I mutation model.

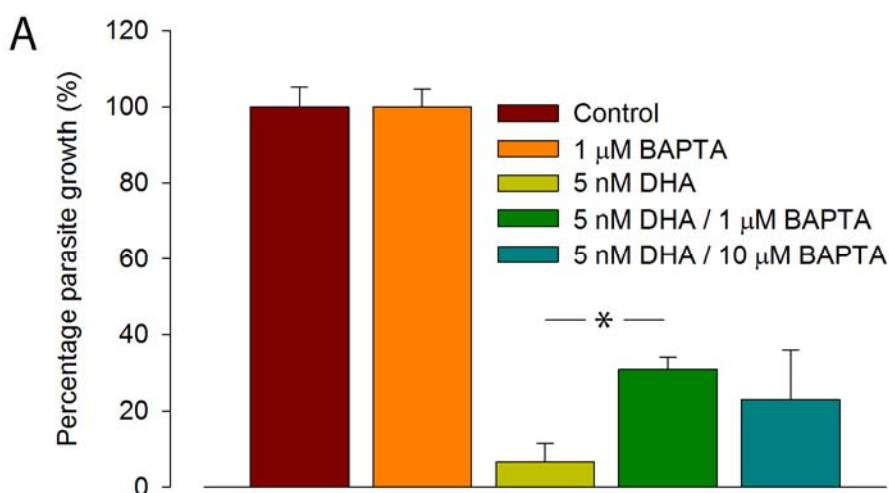
#### 10.4 PAPER IV

In Paper IV we evaluate the impact of dihydroartemisinin (DHA) in *P. falciparum*. When parasites were treated with DHA a similar transient Ca<sup>2+</sup> increase was observed as for thapsigargin (TG) (Fig. 15).



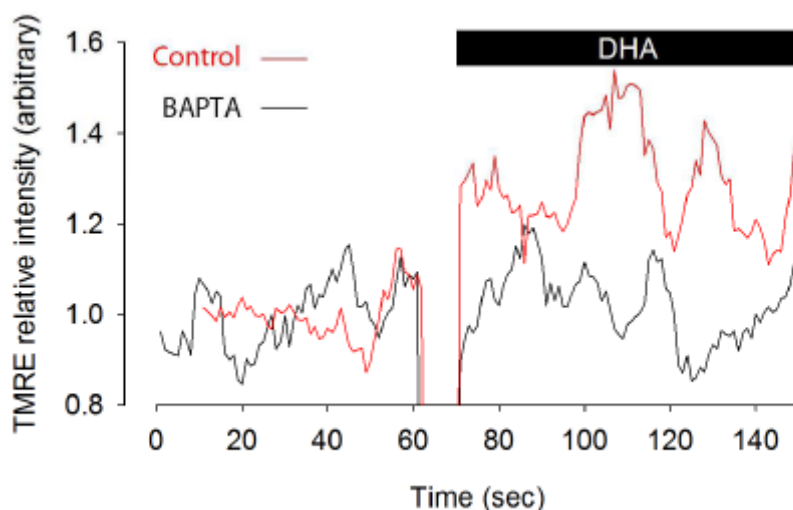
**Figure 15 - Induction of a Ca<sup>2+</sup> transient by DHA**

ART inhibits cell growth of *P. falciparum*. To examine if this effect caused by ART was dependent on cytosolic Ca<sup>2+</sup> signalling we next treated the cells with the Ca<sup>2+</sup> chelator BAPTA/AM and measured parasite growth using an ELISA assay. Although we block intracellular Ca<sup>2+</sup> with BAPTA *P. falciparum* parasites were able to grow to a certain extent (Fig. 16).



**Figure 16 – DHA induced cell death rescued by Bapta.**

In order to further elucidate the  $\text{Ca}^{2+}$  signalling pathway evoked by ART we monitored mitochondria membrane potential after challenge parasites to DHA 72  $\mu$ M. FACS data analysis revealed that DHA was able to increase the TMRE signal 26 %. The DHA-induced TMRE increase was blocked when pretreating the cells with BAPTA. The time lapse FACS recordings showed that hyperpolarisation of mitochondrial membrane by DHA 72  $\mu$ M was rapid and suppressed by BAPTA (**Fig. 17**).

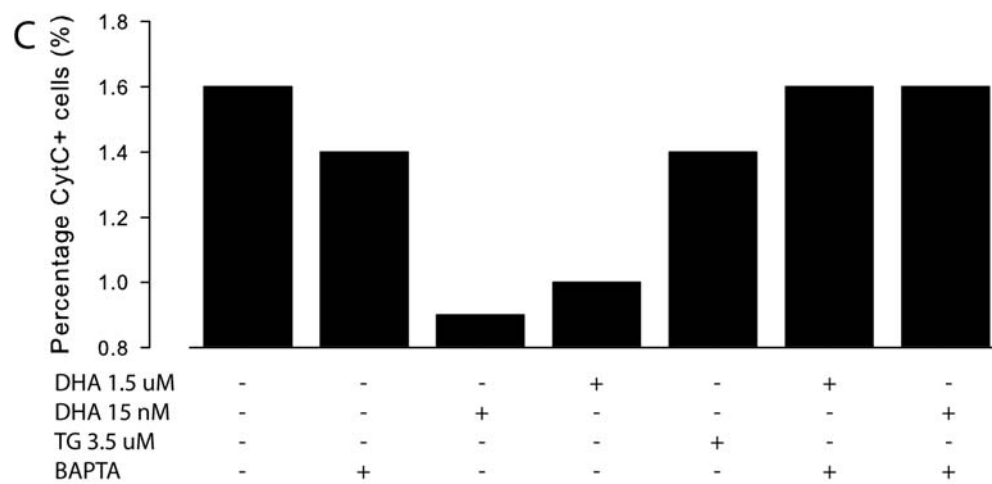


**Figure 17 – Mitochondrial membrane potential disruption by DHA**

To evaluate the mitochondrial reaction to  $\text{Ca}^{2+}$  signal induced by DHA we tracked CytCin *P. falciparum*. Approximately 10 % - 30 % of all DAPI stained parasites were staining positive for CytC. Treating the cells with DHA showed that CytC was released into the



cytosol of the cell and DNA fragmentation was apparent following DAPI staining of DHA treated cells. The CytC release effect was abolished when cells were pre-treated with BAPTA (Fig. 18).



**Figure 18 - CytC release induced by DHA in *P. falciparum*.**

## 11 DISCUSSION

With the enactment of ACT was hypothesized to delay the spread of drug resistant malaria parasites. We have now evidence that this is not the case for the African continent. Artesunate+sulfadoxine-pyrimethamine (AS-SP) treatment is an adequate regime to evaluate the capacity of ACT to reduce drug resistance spread since the mechanism of resistance for SP is well defined and associated with the targets, DHFR and DHPS. AS-SP was introduced in Mozambique in 2004 due to its high efficacy and its adequate clinical and parasitological response of 98% at 42 days, estimated between 2003 to 2004 [161]. After 4 years following its introduction, malaria prevalence decreased, however, dhfr/dhps “quintuple” mutations (related with high SP resistance) increased from 11.0% in 2004 to 75.0% by 2008 ( $P < 0.0001$ ) [162]. Based on the case of AS-SP and artemether-lumefantrine (Paper I) in this thesis, it is apparent that ACT does not prevent the spread of partner drug resistance markers. The determinant factor detected in Paper I driving *pfmdr1* haplotype selection is the time to reinfection. We described a selective window of 35 days, during which lumefantrine is in its terminal elimination phase reaching sub-therapeutical levels. The selection of *pfmdr1* markers related to lumefantrine resistance fit exactly within these 35 days. As opposed to Southeast Asia, where transmission and consequent reinfection rate is low, in Africa, reinfections after treatment are common suggesting that a fraction of the parasite population is exposed to sub-therapeutical dosages of antimalarials, promoting antimalarial resistance and spread.

We showed in Paper III that this ACT selection is not confined to *pfmdr1*. Other genes, including *pfmrp1*, are also under such pressure.

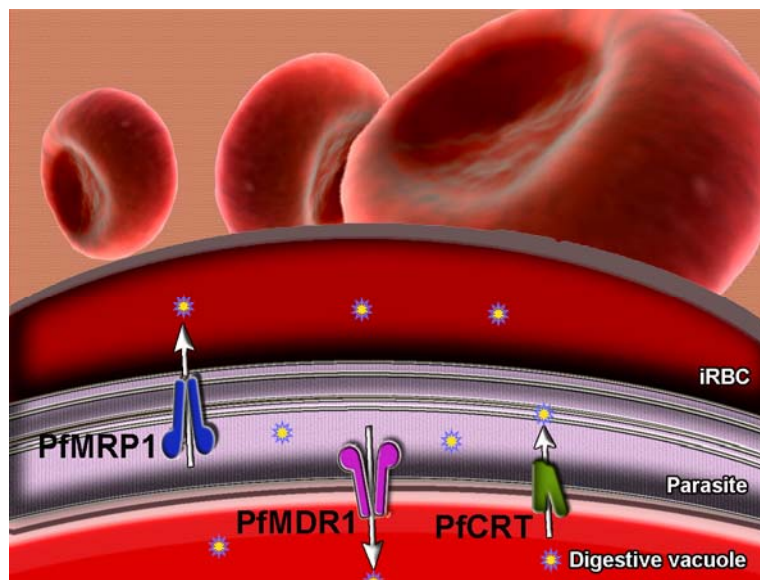
A more worrying scenario is that unlike SP, lumefantrine seems to have cross resistance with artemisinins, at least to a certain extent. This fact brings us back to the basics. An assumption for ACT deployment is the fact that the probability of ACT resistance was estimated to occur based upon independent resistance mutations to artemisinins and partner drugs. However, the involvement of PfMDR1 to lumefantrine and artemisinin seems to be cross related [140,141,144]. In these terms, it is reasonable to conclude that in some cases ACT resistance might not be based on independent resistance events but rather on linked events. Thus, the probability for a parasite developing resistance to a particular ACT should be assumed to be the probability of this parasite's resistance to artemisinin in the context of resistance to its partner drug. Described mathematically as  $P_{ACT-R} = P_{ART-R} * (P_{Partner\ drug\ resistance} | P_{ART-R})$ , this important assumption is of major implication in the context of drug policy establishment. Choice of the partner drug should then be considered in this perspective. Reducing the

probability of associated resistance (partner drug and artemisinins) may aid in slowing down the probability of resistance to ACT.

This fact is further supported by work from our laboratory in AS-AQ selection. Our data shows that following AS-AQ treatment, the opposed haplotype of *pfmdr1* occurs [163]. In which background (PfMDR1/PfMRP1/PfCRT) ART resistance is more prompt to happen we do not know, although it is reasonable to assume that there might exist a preferable genetic background promoting resistance development.

It is certain that unveiling the mode of action of antimalarial drugs is of extreme importance to improve and rationalize drug policies. In the context of PfMDR1, in Paper II, we tried to uncover more information concerning the structure and function of this transporter. Our data support the notion of PfMDR1 as a vacuolar importer. Its contribution seems to be dependent on the PfCRT, which acts as a vacuolar efflux pump. The vacuolar accumulation of antimalarials is of major importance especially for aminoquinolines. The dynamics of antimalarial influx/efflux determine the net accumulation of drug in the vacuole and is a topic for future study.

A relevant physiological feature of PfMRP1 is that it does not localize to the digestive vacuole, instead it is present on the plasma membrane. The three transporters (PfMDR1/PfMRP1/PfCRT) studied here enclose a broad spectrum of substrates enabling us to propose a general mechanism for antimalarial flux in *P. falciparum* (**Fig. 19**).



**Figure 19 - Physiologic model of antimalarial transport in *P. falciparum*.**

PfMRP1 was never found to be a determinant of antimalarial resistance which might be due to the fact that *P. falciparum* is an intracellular parasite. As opposed to other ABC resistant

pumps in other organisms, PfMRP1 does not pump to the extracellular milieu; instead, it expels its substrates to the cytosol of the hosting RBC. Increased oxidative stress on RBCs by antimalarials may also affect the integrity of the host cell, something which is vital for parasite's survival. This hypothesis is further supported by the fact that PfMDR1 pumps drugs into the digestive vacuole, protecting the hosting RBC. A balance between vacuolar accumulation and RBC accumulation may be required for overall *P. falciparum* antimalarial resistance.

This perspective of parasite and host as one entity brings forward once more the complexity of malaria. Survival of malaria parasites to xenobiotics is a result of concerted complex events leading to antimalarial resistance.

Artemisinins have a complex mechanism of action. In this thesis, we describe additional details regarding their impact on *P. falciparum* calcium homeostasis and downstream effects. It is known that Artemisinin resistance in *Toxoplasma gondii* is related to calcium homeostasis [164]. All proposed target proteins for artemisinins resistance in *P. falciparum* directly or indirectly involve  $\text{Ca}^{2+}$ . TCTP is a  $\text{Ca}^{2+}$  binding protein and SERCA is a  $\text{Ca}^{2+}$  pump. In Paper IV, we evaluate the effect of artemisinin on overall intracellular calcium. Artemisinins induce a cytosolic  $\text{Ca}^{2+}$  increase in *P. falciparum*-infected erythrocytes. We analysed the downstream effects of DHA-induced  $\text{Ca}^{2+}$  signalling and demonstrate that damages at the mitochondrial level triggers a cell death pathway. These observed effects were abolished by chelating intracellular  $\text{Ca}^{2+}$  with BAPTA. Subsequently, parasite viability was rescued.

Calcium is an almost universal intracellular messenger that controls a vast number of cellular processes from fertilization to cell death. Cells create large calcium concentration gradients ( $\sim 10000$  to 1) between the extracellular fluid, cytoplasm, and internal calcium stores by means of calcium-pumps located in the plasma membrane and in the membranes of internal calcium stores. These gradients provide ideal conditions for the use of calcium as cellular currency that supports the propagation of intracellular calcium waves. The concerted actions of calcium transporters located in the plasma membrane and in the membranes surrounding internal stores, including the endoplasmic and sarcoplasmic reticulum, the mitochondria, and the nucleus, can generate controlled calcium oscillations for cellular regulation and homeostasis. Deregulation of these processes can often lead to cell death.

In the case of artemisinin, calcium deregulation does cause parasite death. Convincing genetic factors for artemisinin resistance have not been reported yet. We showed that

increased resistance to DHA could be achieved chemically by chelating  $\text{Ca}^{2+}$ . We hypothesize that parasites may biologically mimic this event at critical points of  $\text{Ca}^{2+}$  homeostasis control resulting in increased resistance towards artemisinins.  $\text{Ca}^{2+}$  related targets in *P. falciparum* (SERCA and TCTP) were reported, however, the  $\text{Ca}^{2+}$  dependent mechanisms of resistance might occur up or downstream of these targets.

The recently reported *in vivo* and *in vitro* resistance to artemisinins has been related to general biological features: hyper-parasitaemia [165] and dormancy state [166]. These features of cell proliferation are well known to be controlled by intracellular  $\text{Ca}^{2+}$  signals whilst increased cell proliferation, like tumour cells, correlates with altered intracellular  $\text{Ca}^{2+}$  regulation [167]. In the case of *P. falciparum*, information on the regulation of cell proliferation by  $\text{Ca}^{2+}$  signalling in the erythrocytic cycle is scarce. The role of  $\text{Ca}^{2+}$  within artemisinin's mechanism of action and antimalarial resistance, in general, potentiates the interest and constitutes a great area of scientific interest for the near future.

## 12 CONCLUSIONS

### 12.1 OVERALL CONCLUSION

The antimalarial drug resistance proteins PfMDR1, PfCRT and PfMRP1 are under selective pressure by ACT partner drugs. For artemisinins, intracellular  $\text{Ca}^{2+}$  in *P. falciparum* plays a role in its mode of action.

### 12.2 SPECIFIC CONCLUSIONS

**Paper 1-** *pfmdr1* haplotype coding for 86N, 184Y, 1246D is selected for in re-infections after artemether-lumefantrine treatment.

**Paper 2-** Different haplotypes in *pfmdr1* correlates with two distinct functional modulation mechanisms at the protein level, one allosteric and another directly related to the drug binding pocket.

**Paper 3-** The I876V polymorphism in PfMRP1 is selected for in re-infections after artemether- lumefantrine treatment. We examine the importance of this residue for ATP hydrolysis at the transporter nucleotide binding domain.

**Paper 4-** Dihydroartemisinin disrupts calcium homeostasis in *P. falciparum* and induces cell death.

### 13 PERSONAL VIEWS AND FUTURE PERSPECTIVES

Enormous efforts were needed to convince international organizations to start a new era of malaria elimination and eradication after the failure of the World Malaria Eradication Program in the middle of last century. Now we have the opportunity to apply more than one century of research in malaria to jeopardized common goal. Due to its extreme complexity, to eliminate or even control malaria requires effective concerted interventions within the host, the mosquito and the parasite. To fully achieve these requirements, a deep understanding of the fundamentals of malaria is mandatory.

The studies presented in this thesis were focused on the parasite response to ACT with the intention of contributing to our basic knowledge of antimalarial selection and resistance. In brief, we described how the parasite population has been modulated by partner drugs due to the use of ACTs and further how alterations of intracellular  $\text{Ca}^{2+}$  regulation could contribute to resistance to artemisinins.

Unfortunately, there is more and more evidence that the durability of ACT efficacy might not be what was previously thought. The Efficacy of artemisinins seem to be degrading in Southeast Asia and resistance has been reported to the most used partner drugs. In a more cautious perspective, is wise to take into consideration that the parasite population is adapting to ACT and resistance may well happen sooner than previously expected. The question now is: what can we do to slow down this process and promote ACT sustainability? The Malaria Research Group at Karolinska Institute has made significant contributions in this field, reporting on the opposed selection of *pfmdr1* and *pfcr1*, which are also oppositely related regarding susceptibility, for the partner drugs amodiaquine and lumefantrine.

In Africa, transmission is a major driving force for the mechanism of selection of resistant haplotypes to ACT partner drugs. The identification of these two factors, parasite genetics and transmission, redefines the concept of time to re-infection of different resistant haplotypes. This new concept promotes the generation of new understanding and vision of antimalarial resistance and selection. In the future, time to re-infection may be an important tool to access populational parasite drug susceptibility in a longitudinal perspective. Evaluation of the importance of the terminal elimination phase of different partner drugs and risk of resistance selection might give us more information regarding the correct choice of partner drugs and long term planning of ACT deployment.

Artemisinin efficacy has been looked upon as the mainstay for ACT resistance. Our very little knowledge regarding the mechanism of artemisinin makes it difficult to define what

artemisinin resistance actually is. If we reflect on the definition of resistance: “Diminished or failed response of an organism, disease or tissue to the intended effectiveness of a chemical or drug”, (From the MeSH<sup>®</sup> online dictionary (Medical Subject Headings) ([http://www.nlm.nih.gov/cgi/mesh/2010/MB\\_cgi](http://www.nlm.nih.gov/cgi/mesh/2010/MB_cgi))) we may say that indeed resistance to artemisinin has always been present, as recognized by the WHO, and not recommended as a monotherapy because of its low effectiveness.

ACT effectiveness should then be looked at as a whole, focusing on all aspects, artemisinin and partner drug pharmacologic characteristics, the parasite’s background and transmission.

In a more broad research perspective, antimalarial resistance is one case in the scientific challenge of translational research where distinct areas of scientific knowledge converge in a multidisciplinary fashion to create a truly translational science.



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