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# *PLASMODIUM FALCIPARUM* DRUG TRANSPORTER GENES IN EMERGING MALARIA MULTIDRUG RESISTANCE

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The front cover is a light microscopy image of a Giemsa stained slide with *Plasmodium falciparum* infected red blood cells.



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# ABSTRACT

Malaria is caused by an intracellular protozoan parasite of the genus *Plasmodium.*
The use of chemotherapy, the foremost tool available for the control of the disease, has been challenged in the last decades by the development and spread of drug resistance among malaria parasites. A clear understanding behind the mechanisms of parasite resistance is required for the improvement of treatment efficacy, policy assessment and in the development of new drugs.

A common strategy used by parasites in achieving resistance involves decreasing drug accumulation inside the cell. This is typically accomplished by increasing the availability of transporter proteins that mediate the efflux of the active compound.

The goal of this thesis was to better understand the involvement of drug transporter genes in the molecular mechanisms underlying drug susceptibility in *Plasmodium falciparum* malaria. The approaches involved clinical drug trials, clinical isolates and extensive studies of laboratory *P. falciparum* parasites.

The contribution of *pfmrp1* polymorphisms in *in vivo* parasite drug response was studied in *P. falciparum* infected patients from drug efficacy clinical trials. After Sulphadoxine-Pyrimethamine treatment, recrudescent infections selected for parasites that had a lysine at amino acid position 1466 in *pfmrp1,* thus providing the first indication that this transporter gene may have a role in *P. falciparum* antifolate drug responses *in vivo*.

We examined the effect of the ACT partner drug, mefloquine, on the intra-erythrocytic cell cycle of *P. falciparum* laboratory parasites having different *in vitro* drug susceptibilities, while in parallel investigating the expression of four pivotal drug transporter genes: *pfcrt, pfmdr1, pfmrp1* and *pfmrp2*. This study revealed a delay in the cell cycle of the parasite after drug pressure, accompanied by gene induction of the transporter genes studied.

The genetic background of the drug transporter genes *pfcrt, pfmdr1, pfmrp1* and *pfmrp2* were further studied at length in field isolates collected at the Thai-Myanmar border, a historically known epicenter of resistance. The isolates were characterized *in vitro* for their sensitivity against a broad range of ACT relevant antimalarials. Correlation analyses revealed novel candidate markers for multidrug resistance against structurally unrelated antimalarial drugs used extensively in ACT regimens worldwide.

In conclusion, these studies reinforce the concept of malaria drug resistance as a multi-factorial and complex phenomenon that may involve not only the parasite's handling of the incoming drug, but also concomitant responses of its basic physiology.

# LIST OF PUBLICATIONS

I. Dahlström S[§], **Veiga MI[§],** Mårtensson A, Björkman A, Gil JP. Polymorphism in PfMRP1 (*Plasmodium falciparum* multidrug resistance protein 1) amino acid 1466 associated with resistance to sulfadoxine-pyrimethamine treatment. *Antimicrob Agents Chemother. 2009; 53(6):2553-6.* (§-shared authorship)

II. **Veiga MI**, Ferreira PE, Schmidt BA, Ribacke U, Björkman A, Tichopad A, Gil JP. Antimalarial exposure delays *Plasmodium falciparum* intra-erythrocytic cycle and drives drug transporter genes expression. *PLoS One. 2010; 5(8).*

III. **Veiga MI**, Ferreira PE, Jörnhagen L, Malmberg M, Kone A, Schmidt BA, Petzold M, Bjorkman A, Nosten F, Gil JP. Novel polymorphisms in *Plasmodium falciparum* ABC transporter genes associated to major ACT antimalarial drugs. *Submitted.*

IV. **Veiga MI**, Franzen O, Ferreira PE, Dahlstrom S, Lum JK, Nosten F, Gil JP. Complex polymorphism in the *Plasmodium falciparum* Multidrug Resistance Protein 2 gene (*pfmrp2*). *Manuscript.*

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# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-Binding Cassette |
| ACT | Artemisinin-based Combination Therapy |
| AL | Artemether + Lumefantrine combination |
| AQ | Amodiaquine |
| ART | Artemisinin |
| AS-AQ | Artesunate + Amodiaquine combination |
| AS-MQ | Artesunate + Mefloquine combination |
| AS-SP | Artesunate + Sulphadoxine + Pyrimethamine combination |
| ATP | Adenosine Triphosphate |
| BLAST | Basic Local Alignment Search Tool |
| CNV | Copy Number Variation |
| CQ | Chloroquine |
| $C_t$ | Cycle threshold |
| DEAQ | Desethylamodiaquine |
| DHA | Dihydroartemisinin |
| DHA-PQ | Dihydroartemisinin + Piperaquine combination |
| DHFR | Dihydrofolate Reductase |
| DHPS | Dihydropteroate Synthase |
| DNA | Deoxyribonucleic Acid |
| FV | Food Vacuole |
| G6PD | Glucose-6-Phosphate Dehydrogenase |
| GSH | Reduced Glutathione |
| GSSG | Oxidized Glutathione |
| HF | Halofantrine |
| HIV | Human Immunodeficiency Virus |
| HRP | Histidine Rich Protein |
| IC | Inhibitory Concentration |
| IDC | Intra-erythrocytic Developmental Cycle |
| IPT | Intermittent Preventive Treatment |
| LUM | Lumefantrine |
| MDR | Multi Drug Resistance |
| MQ | Mefloquine |

| | |
|---|---|
| MSD | Membrane Spanning Domain |
| MSP | Merozoite Surface Protein |
| NBD | Nucleotide Binding Domain |
| ORF | Open Reading Frame |
| PCR | Polymerase Chain Reaction |
| PfCRT | *P. falciparum* Chloroquine Resistance Transporter |
| PfMDR | *P. falciparum* Multidrug Resistance |
| PfMRP | *P. falciparum* Multidrug Resistance-associated Protein |
| PQ | Piperaquine |
| QN | Quinine |
| RBC | Red Blood Cell |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic Acid |
| SERCA | Sarco/Endoplasmatic Reticulum Ca2+-ATPase |
| SNP | Single Nucleotyde Polymorphism |
| SP | Sulphadoxine-Pyrimethamine |
| TCTP | Translationally Controlled Tumor Protein |
| TM | Transmembrane |
| TMD | Transmembrane Domain |
| WHO | World Health Organization |

*"THIS IS MALARIA. A scourge to mankind... a deadly plague that scatters misery and suffering among its victims. Deadlier than guns, more powerful than bombs, this mysterious killer has been responsible for the decline of nations, the lost of wars, the failure of explorations.... From earliest times right up to today, malaria has altered the march of history: Alexander the Great died from it on the eve of his greatest conquests.... It prevented the notorious Cesare Borgia from placing all of Italy under his power.... Poets, such as Dante and Lord Byron, statesmen like Garibaldi and James I, the famous Lord Nelson – all were plagued by this disease which had no respect for fame or position.... And in World War II, it proved almost as great an enemy as all of the Axis powers."*

**LEON J. WARSHAW (1917-2001)**

*Malaria, the Biography of a Killer.*
New York and Toronto; Rinehart and Company. 1949

# 1 BACKGROUND

## 1.1 MALARIA FACTS

Malaria is a disease caused by a parasite spread from person to person through the bites of infected mosquitoes. Malaria generally occurs in tropical and subtropical areas, leaving approximately 40% of the world at risk for the disease. Most lethality from malaria infection is focused in the African continent, especially among children under five. The most recent WHO World Malaria Report registers a 2009 global impact of approximately 225 million new malaria clinical reported infections, associated with 781,000 deaths (WHO, 2010).

Malaria is commonly associated with poverty, and represents a major burden to economic and social development, costing an estimated sum of greater than US$ 6 billion for the year 2010 (Sachs & Malaney, 2002, WHO, 2010).

Early diagnosis and prompt treatment are two basic elements in easing the impact of malaria. While progress in these areas has been remarkable, emerging insecticide resistant vectors, population movements, environmental disturbances, disintegrative health services and wide spread antimalarial drug resistance have constrained this mission.

## 1.2 MALARIA INFECTION AND TRANSMISSION

The parasite causing malaria is an intracellular protozoan belonging to the genus *Plasmodium*, which is comprised of approximately one hundred species (Levine, 1988) able to infect numerous vertebrate hosts including reptiles, birds and mammals. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* are the five species that can infect, cause symptoms and eventually death in humans (Cox-Singh *et al.*, 2008, Levine, 1988). *P. falciparum* is responsible for the most severe cases of the disease and mortality, and is the focus of this thesis.

Malaria infection is the result of complex interplay between the host, the parasite and the mosquito vector. The dynamics between these three elements is an important factor affecting malaria transmission. Many mosquitoes can transmit malaria, but within the

species able to do so, only females possess the capacity due to their hematophagic (blood feeding) nature. Mosquitoes are a driving force responsible for malaria propagation and different characteristics and behaviours of the mosquito population in certain settings often determines malaria endemicity (Elliott, 1972).

## 1.3 MALARIA CONTROL

In the last few years, there has been a substantial increase in international funding and political commitment toward malaria control and elimination. This has allowed a recent expansion of access to valuable public health tools such as long-lasting insecticide treated bed nets and indoor residual spraying, as well as early access to diagnosis and effective antimalarial drugs including artemisinin combination therapy (ACT). Consequently, the toll of the disease has decreased in a number of countries throughout the world, with notable decreases in the morbidity and mortality due to malaria (WHO, 2010).

Nevertheless, the feasibility of malaria eradication is a complex challenge, as several of these central strategies are under threat (Mendis *et al.*, 2009). Malaria vectors in several countries display a degree of resistance to pyrethroids (Chandre *et al.*, 1999), while – and most importantly – recent reports from South East Asia document decreased susceptibility to the latest medications (Noedl *et al.*, 2010, Wongsrichanalai & Meshnick, 2008, Price *et al.*, 2004, Price *et al.*, 2006). Furthermore, humans that become infected differ in their susceptibility to malaria as a result of genetic factors and/or acquired immunity (Mendis et al., 2009). Research and development to develop tools of greater potency and effectiveness are needed, especially those which impact transmission, as well as replacement drugs for antimalarials that are rendered ineffective by increased parasite resistance.

## *1.4 PLASMODIUM FALCIPARUM*

### 1.4.1 The life-cycle

As general features of the life-cycle, two factors are of primary importance in the living history of the parasite: (i) the alternation within two hosts (vertebrate and invertebrate)

in which three steps occur, two intracellular in the vertebrate and the third extracellular in the *Anopheles* mosquito with asexual and sexual growth and division in the vertebrate and invertebrate host respectively; and (ii) chronobiology; the time table of events in all stages is highly significant for microscopic clinical diagnosis, for taxonomic differentiation of subspecies and as a survival mechanism for the parasite (Bray & Garnham, 1982).

Figure 1 illustrates in detail the complete life cycle of *P. falciparum*. One may assume an arbitrary starting point in the cycle for description purposes: when a female *Anopheles* mosquito penetrates the human skin to obtain a blood meal. Through this action the vector injects saliva mixed with an anticoagulant. If the mosquito is infected with *P. falciparum*, it will simultaneously inject elongated sporozoites into the bloodstream of the host. The sporozoites travel to the liver where they enter the hepatocytes. In this intracellular environment, they rapidly divide asexually, generating the next lifecycle stage form, the merozoites. Following the rupture of the hepatocyte, merozoites are released into the bloodstream, where they invade erythrocytes and develop through the early trophozoite ("ring") and mature trophozoite stages. After these, the parasites undergo a series of asexual divisions to produce a large segmented schizont filled with mononucleated merozoites. The erythrocyte then ruptures, releasing the merozoites and initiating the well-known peak of fever and chills characteristic of malaria. A small proportion of merozoites do not divide, instead developing into sexual forms: the male and female gametocytes. These develop zygotes only inside the mosquito gut, after a mosquito blood meal. Within the gut, the male and female gametes fuse and the resultant diploid zygotes undergo meiosis and further development within the mosquito intestinal wall, ultimately differentiating into oocysts. After repeated mitotic divisions, these oocysts produce a large number of sporozoites. The sporozoites actively migrate to the salivary glands of the mosquito, ready to be injected by the mosquito into the bloodstream of a human, thus re-starting the life-cycle of the parasite again.

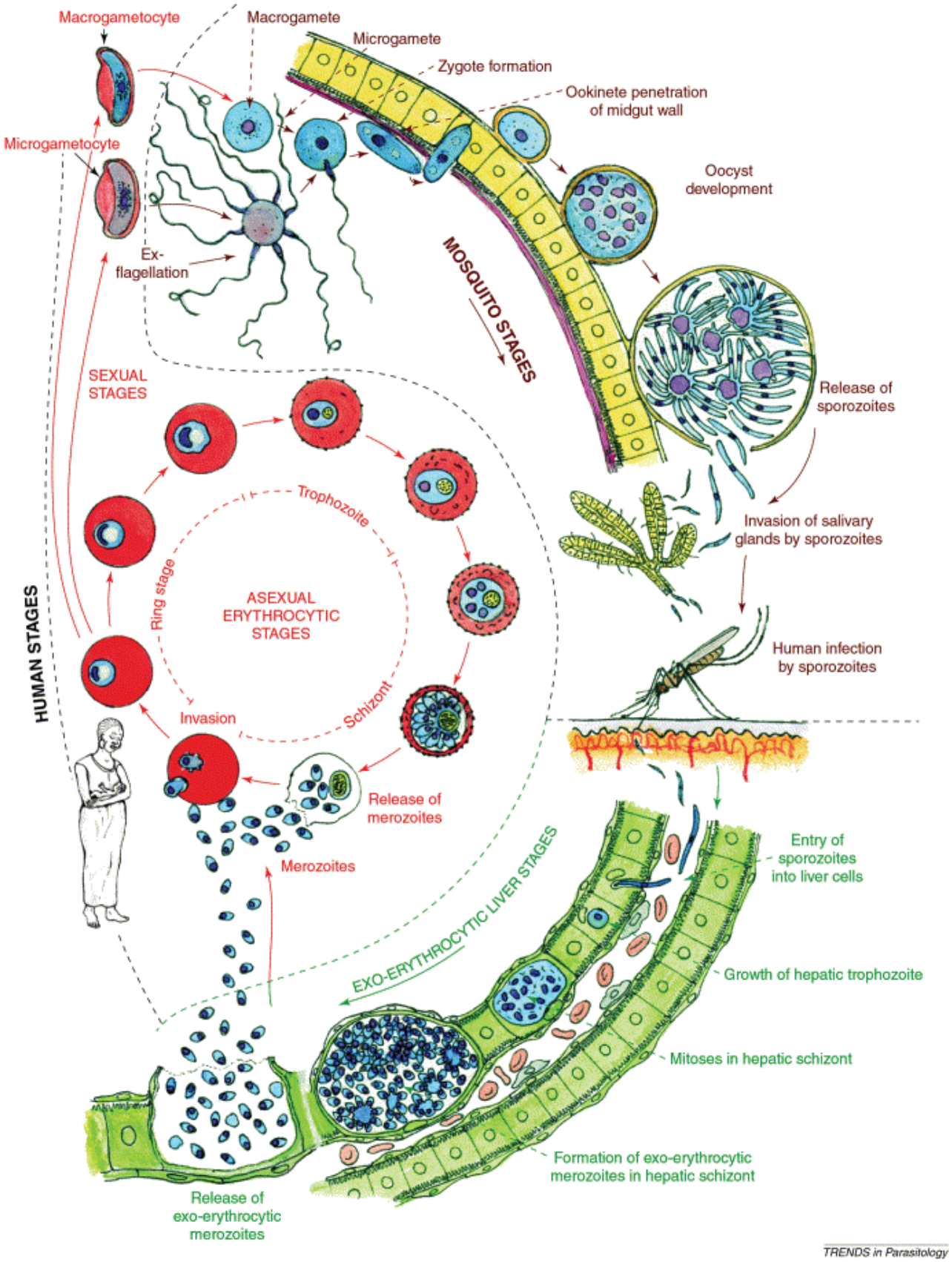



Figure 1 - The life-cycle of *Plasmodium falciparum*. The main phases in the liver and in the red blood cells (asexual and sexual erythrocytic stages) of the human host, and in the gut and in the salivary glands of the mosquito host are depicted. Reprinted from Trends in Parasitology (Bannister & Mitchell, 2003), with permission from Elsevier.

#### *1.4.1.1 Replication within the red blood cell*

The life-cycle stage receiving the most attention is the intra-erythrocytic developmental cycle (IDC), because it is responsible for the immediately visible pathology of the disease in humans and is the main target for most available antimalarial drugs. It is also the most amenable to study, as it is comparatively easy to obtain biological material from blood stage cultures. Nevertheless, the cell cycle division in the erythrocytic cycle are poorly understood (Arnot *et al.*, 2011). The IDC has an impact progressively amplified by repeated 48 hour cycles of invasion (*P. falciparum*), intracellular growth, multiplication and re-invasion. Due to the focus of this thesis, a brief description of these different morphological and replication stages will be given.

The discrete phases of DNA replication and cell division which constitute the life-cycles of malarial parasites do not appear to follow the general eukaryotic mitotic model (Arnot & Gull, 1998). "A cell-cycle can be defined as the period between the formation of a cell by the division of its mother cell and the time it itself divides to form two daughters, during each period there is a 2-fold increase in DNA content followed by equal division of the nucleos" (Mitchison, 1971). A clear correlation between the G1, S, G2 and M phases of the typical eukaryotic cell cycle has yet to be established in *Plasmodium*. Attempts to solve such questions using biochemical approaches are hampered by the difficulty in obtaining sufficient highly synchronized populations and the apparent asynchrony in nuclear divisions in a given schizont (Arnot & Gull, 1998, Waters & Janse, 2004). It is generally accepted that merozoites and rings are in G1, and S phase is initiated in trophozoites, around 18 hours post invasion (Figure 2).

The malariological terms used to describe *P. falciparum* IDC stages are based mainly in their structural features, observable by optical microscopy:

(i) Ring and early trophozoites stage associated with a single interphase nucleus. This stage is characterized under Giemsa staining blood smears as a thin biconcave disc, thicker around its perimeter where the elongated nucleus is present and thinner in the middle, giving the appearance of a ring. The ring eventually grows into the more rounded trophozoite stage.

(ii) Later trophozoites (by this stage sequestered on tissue endothelium in clinical infections) which initiate preparations for chromosome replication ($G_1$) and the start of DNA synthesis (S). Here, the parasite becomes a more rounded shape; it feeds more

actively, and forms a large pigment vacuole in which the degradation products resulting from haemoglobin digestion (hemozoin crystals-dark pigment) accumulate.

(iii) The schizonts stage starts when the single trophozoite nucleus begins to divide into two daughter nuclear bodies (M phase) (Arnot & Gull, 1998, Arnot et al., 2011). Approximately 16 nuclei are generated and appear in the merozoite located in the periphery. They blossom from the central mass containing the pigment vacuole, now full of compacted hemozoin crystals, and eventually egress from the RBC and parasite membrane surrounding the merozoites to carry on in invading new RBCs.

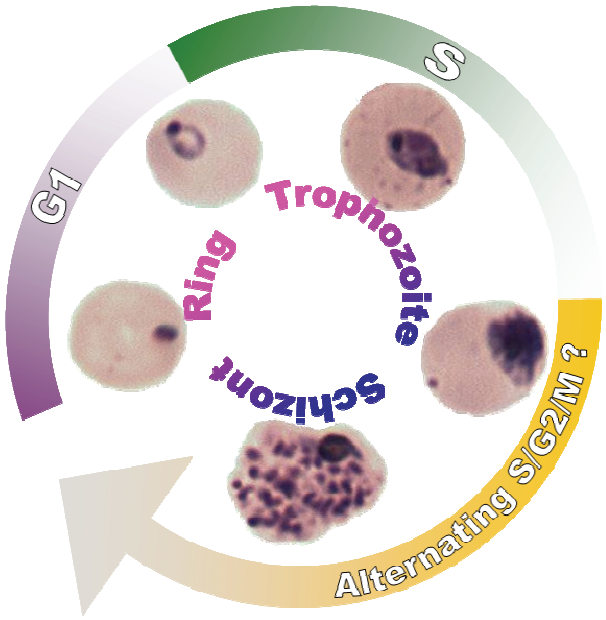



Figure 2 – Description of morphological stages as well as cell cycle replication in *P. falciparum* IDC. Note that the depiction of the cell cycle phases (G1, S, G2 and M) is an estimate.

### 1.4.2 Genome

The first attempt to sequence the genome of an eukaryotic pathogen was initiated in 1996 with the *P. falciparum* genome project (Waters & Janse, 2004). The determination of the genome sequence provided not only important information about the biology of the parasite, but also changed the way that many *Plasmodium* focused laboratories approach their research. The sequence of the genome facilitated research efforts that could potentially lead to the development of novel drugs. The haploid genome of *P. falciparum* clone 3D7 was found to have a size of ≈22.8 megabases with approximately 80% A and T nucleotide content, an obstacle in the study of this bug.

The nuclear genome consists of 14 chromosomes which encode at least 5400 genes. 60% of the encoded proteins are of unknown function, and furthermore, have presently little or no recognizable homologues in other organisms (Gardner *et al.*, 2002).

### *1.4.2.1 Genetic variation (SNPs and CNV)*

Genetic variation, variation in alleles of genes, occurs both within and among populations. Its magnitude is vital for providing the "raw material" for natural selection. It can appear in several forms, including single nucleotide polymorphisms (SNPs), variable numbers of tandem repeats, as well as larger structural rearrangements such as gene copy number variation (CNV), deletions, insertions, inversions and translocations.

SNPs are a type of polymorphism involving variation of a single base pair, and studying them can reveal their involvement with disease, drug response, or other phenotypes. The genome of *P. falciparum* clones and field isolates have shown a SNP density of approximately one every 500-1000bp (Mu *et al.*, 2002, Volkman *et al.*, 2007). Importantly for the theme of this thesis, variation in genes associated with phenotypes of decreased therapeutic drug susceptibility is expected to be subject to strong selective pressures. This is reflected as a reduction in SNP diversity found in the genomic regions surrounding known resistance loci (e.g. *pfcrt* and *pfdhfr*) as a result of recent antimalarial drug driven selective sweeps (Nair *et al.*, 2003, Wootton *et al.*, 2002).

Current studies have revealed gene CNV as a major source of genomic variability in *P. falciparum*, encompassing at least three times the total nucleotide content of SNPs. CNVs can range in size from thousands to millions of DNA base pairs. Since CNVs often encompass entire genes, they can influence gene expression levels as well as phenotypic variation, thereby revealing important functions both in disease and drug response and may also help to better understand genome evolution. In *P. falciparum,* a number of studies have shown examples of gene amplifications and deletions including genes associated with knob formation, cytoadherence and multi-drug resistance (e.g. *pfmdr1*) (Carret *et al.*, 2005, Ribacke *et al.*, 2007).

### 1.4.3 Transcriptome

In malaria parasites, the study of gene expression has increased in recent years with the release of the annotated sequence of the *P .falciparum* genome and the development and use of reporter gene constructs in transfection experiments. This has led to many advances in the field, as well as the ability to genetically modify the parasite. Gene transcripts can provide insight into the basic mechanisms of gene regulation and function, and may also lend knowledge to the mode of action of antimalarial drugs.

Comparisons of the gene expression profiles of genetically modified parasites such as drug-selected or gene knockout parasites with their parental wild-type parasites will allow the identification of other genes that interact with those functionally modified genes (Jiang *et al.*, 2008).

High-throughput transcriptome analysis using microarrays was available soon after the completion of the parasite genome (Bozdech *et al.*, 2003, Le Roch *et al.*, 2003), and has become a useful tool for investigating the biology of the malaria parasite. The complete asexual *P. falciparum* IDC transcriptome analysis demonstrated that at least 60% of its genome is transcriptionaly active in this stage, with the timing of mRNA expression for a given gene during the IDC correlating well with the expected function of the resultant protein in each specific time point of the cycle. For example, replication of the genome occurs in the early-schizont stage and correlates with the peak of expression factors involved in DNA replication and DNA synthesis. During the IDC the parasites undergo extensive morphological alterations, and these differences are tightly correlated with genetic expression clusters (Bozdech et al., 2003).

Studies analyzing the transcriptional responses to antimalarial drug challenges in *P. falciparum* have examined the drug's effect in multiple cell stages to identify changes in modulation of transporters, inhibition of protein synthesis, and perhaps activation of drug responsive protein networks (Ganesan *et al.*, 2008, Gunasekera *et al.*, 2007).

## 1.5 ANTIMALARIAL DRUG RESISTANCE

Chemotherapy, on top of vector control measures, plays a major role in combating malaria infection. However, this role is diminished by the paucity of currently available antimalarial drugs and by the apparent unstoppable emergence and spread of parasite drug resistance.

Malaria drug resistance is clinically defined as "the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject." Furthermore, "the form of the drug active against the parasite must gain access to the parasite or the infected RBC for the duration of time necessary for its normal action" (WHO, 1986).

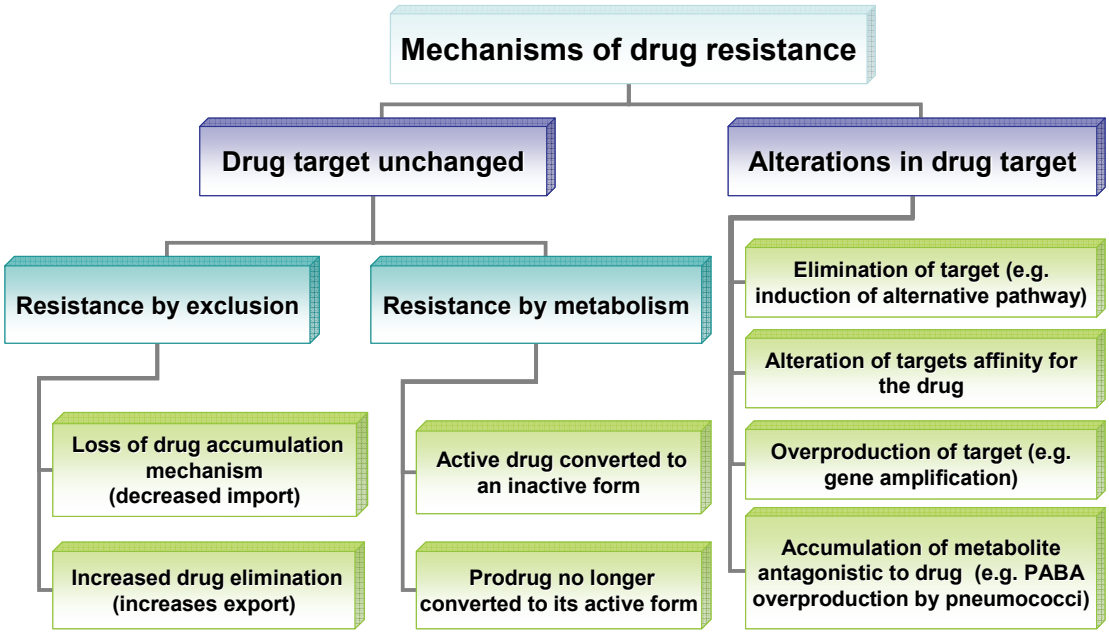
The emergence of resistance is likely the result of a complex intra- and interaction of factors between human, parasite, mosquito and the characteristics of the drug used. In the host population, it can include incomplete therapeutic treatment of infected patients. The lack of compliance, the involuntary use of counterfeit drugs and an immunity response can also affect the efficacy of chemotherapy. In the parasite population, it can include genetic and metabolic flexibility together with high rates of multiplication and dispersal of resistance genes via sexual recombination in the mosquito.

There are several ways to assess the susceptibility of *P. falciparum* to antimalarial drugs, including both *in vivo* and *in vitro* methods. Clinical failure following appropriate administration of a drug is an important paradigm in identifying drug resistance, as it defines the "real world". Besides a clear defect in drug failure (i.e. the non-elimination of the pathogen upon appropriate parasite drug exposure), other phenotypes have to be taken in consideration to identify the emergence of drug resistance. These include significant increases in parasite clearance time from the patient body, the identification of recrudescences and the selection of specific subpopulations, particularly when associated with the selection of genetic variants known or suspected to be linked to decreased parasite drug susceptibility. The identification of the latter are primarily the result of *in vitro* studies.
Studies based on *in vitro* culture of the parasite provide a significant advantage in studying the intrinsic parasite susceptibility to a drug without patient related confounding effects like immunity or pharmacokinetic factors modulating the bioavailability of the drug. These approaches have their own limitations, particularly those allowing the full allelic exchange targeted for genes suspected to be related with resistance. Therefore, caution must be exercised when drawing conclusions associating *in vitro* results with *in vivo* phenotypes. As a consequence, inhibition concentration values as a threshold of resistance for most antimalarial drugs have yet to be established. Also, not all field isolates and not all parasite genotypes present in one

isolate will survive adaptation to *in vitro* culture or even the *ex vivo* microtest performed in the field. Hence, some sub-populations may have an important effect on treatment failure, but by not surviving during *in vitro* experiments they will not contribute to the final measured phenotype, biasing the association between *in vivo* and *in vitro*. Additionally, sequestered parasites, absent from the peripheral circulation may be missed when sampling *in vitro* assays. Overall, it is important to have access to both *in vivo* and *in vitro* assays for a better understanding of the biological basis of parasite drug susceptibility.

### 1.5.1 Mechanisms of drug resistance

In general, development of drug resistance occurs in several discrete steps. When organisms or cells are exposed to suboptimal, and thus sublethal, levels of a drug, they tend to respond to the stress situation by adaptation involving one or more mechanisms of drug resistance.




In addition to single drug resistance, there is recent evidence for the development of multiple drug resistance mechanisms that allow the organism to survive not only to the exposure to one drug, but also to others with non related structures or mechanisms of

action. The exposure to sub-optimal drug levels through self medication in the management of fever in developing countries is probably one of the most important reasons for the presence of (multiple) drug resistant malaria.

## 1.6 ANTIMALARIAL DRUGS: MODES OF ACTION AND RESISTANCE

The effect of the bark of the cinchona tree on malaria was first uncovered by the native Indians of South America, while in China sweet worm-wood has been used for the treatment of malaria for a very long time (Aydin-Schmidt *et al.*, 2010). Different types of antimalarial drugs have had different histories, impact and although some with some similarities, they also differ in the molecular mechanism underling parasite drug resistance. Table 1 gives a brief overview of *P. falciparum* proteins with a proven or likely role in resistance to clinical antimalarial drugs.

### 1.6.1 Quinolines and related compounds

Introduced during the 17th Century, the use of extracts from the bark of the cinchona tree was the first effective chemotherapy available in Europe. Its principle active compound, isolated in the 19th Century, was named quinine (QN). Its structure is built upon a quinoline ring system. From this basic structure, the group of synthetic antimalarials collectively named quinolines was created during the 20th Century, including chloroquine (CQ), amodiaquine (AQ), piperaquine (PQ) and mefloquine (MQ). Based on more loosely related ring systems are the antimalarials halofantrine (HF) and lumefantrine (LUM) (Figure 3). These compounds are all thought to share a common target, with the most widely accepted hypothesis, based on studies with CQ, proposing the target as being in the parasite heme detoxification systems (Fitch, 2004).

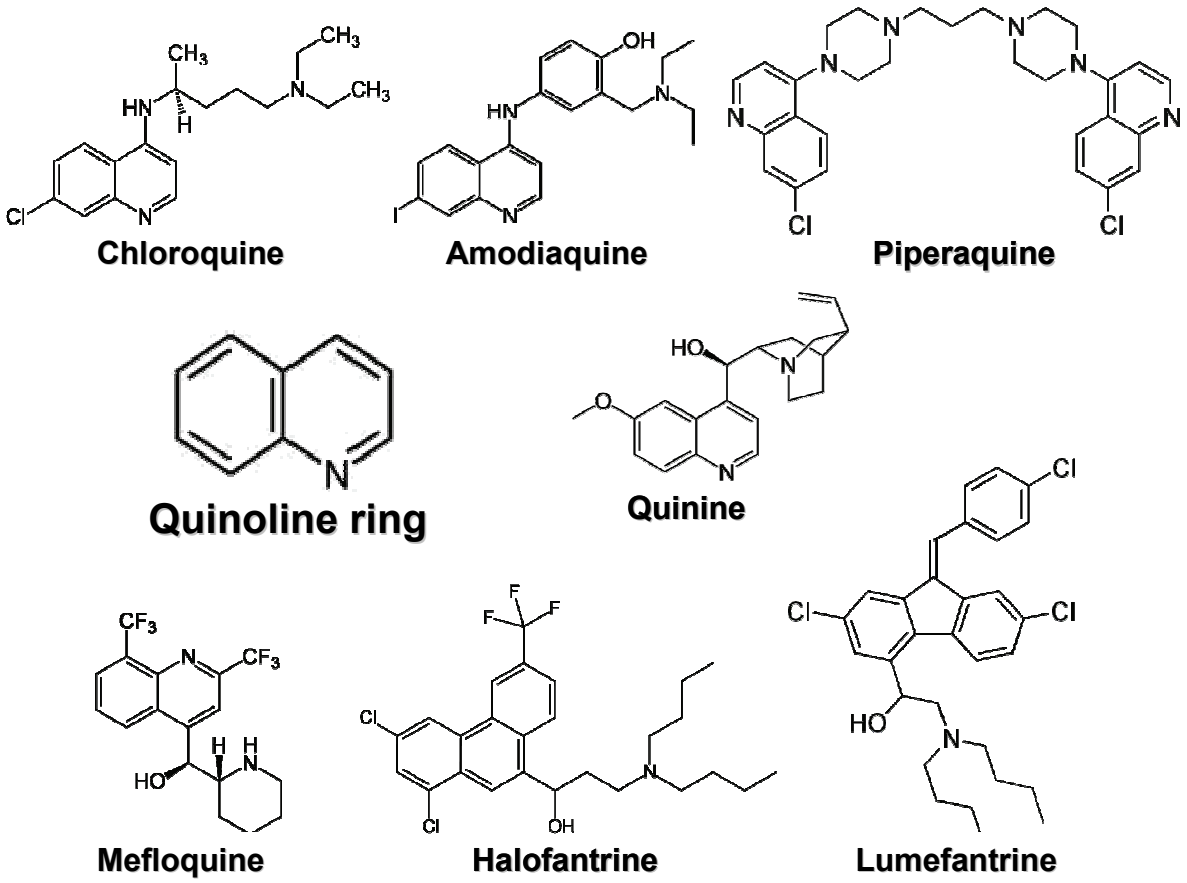



Figure 3 – Structure of the quinoline ring and antimalarial quinoline related compounds.

CQ, clinically available since 1947, is one of the most successful antimalarial drugs ever produced, being a safe and cheap compound that is estimated to have saved countless millions of lives (O'Meara *et al.*, 2010). *P. falciparum* resistance to this drug was identified approximately 10 years after its introduction, with the first pilot reports coming from the Thai-Cambodian borders in 1957, and the first formal complete records in Northeast South America. CQ resistance further expanded to Africa, where it first appeared in the late 1970s, further spreading to most sub-Saharan countries by the end of the 1980s.

CQ acts by interfering with the detoxification of the heme group (ferroprotoporphyrin IX) produced when haemoglobin is digested. This process occurs inside the *Plasmodium* parasite's food vacuole (FV), where CQ typically accumulates due to its di-protonation by the acidic environment of the FV lumen. There, it binds to the toxic heme, preventing the process of biomineralization towards hemozoin. The highly reactive free heme ultimately becomes lethal to the parasite.

Although the long-term accepted view for explaining CQ resistance resides in the accumulation of the drug inside the food vacuole, with higher accumulation of CQ in sensitive parasites compared with the resistant ones (Krogstad *et al.*, 1987), a direct

demonstration of the phenomenon at the molecular level occurred only recently (Martin *et al.*, 2009b). The discovery of the precise genetic basis of chloroquine resistance was also a long and difficult process, with more then 40 years between its initial clinical recognition and the identification of the chloroquine resistance transporter gene (*pfcrt*) (Fidock *et al.*, 2000). This gene, located on chromosome 7, is highly polymorphic with at least 20 variable codon positions reported to date (Cooper *et al.*, 2005). A specific polymorphism resulting in a lysine to threonine substitution at amino acid 76 (K76T) was shown to confer *in vitro* (Sidhu *et al.*, 2002, Fidock et al., 2000) and *in vivo* (Djimde *et al.*, 2001) CQ resistance to the extent that it became a molecular marker for predictive therapeutic efficacy (WHO, 2002).

Resistance to quinoline drugs, including the aforementioned CQ, has also been linked to other transporter proteins. This includes the long studied *P. falciparum* multidrug resistance 1 (PfMDR1), Na+/h+ exchanger (PfNHE) and multidrug resistance-associated protein 1 (PfMRP1). A more detailed description of the importance of *pfcrt* and the *pfmdr1* and *pfmrp1* ABC transporter genes in the development of drug resistance will be given in the next chapter.

### 1.6.2 Antifolates

The antifolate class of drugs consists of compounds that bind enzymes necessary for parasite folate biosynthesis. The main drugs used against malaria are the combinations sulphadoxine-pyrimethamine (SP) and chlorproguanil-dapsone (not as accepted due to the high risk of severe anaemia after treatment in patients with G6PD deficiency (Fanello *et al.*, 2008)). Pyrimethamine and chlorcycloguanil (the active metabolite of chlorproguanil), target the dihydrofolate reductase (DHFR) activity of the parasite's bifunctional DHFR-thymidylate synthase protein, whereas the sulfa drugs, sulphadoxine and dapsone, affect dihydropteroate synthase (DHPS). Their inhibition leads to decreased production of tetra-hydrofolate, a cofactor necessary for the production of a number of folate precursors which ultimately disturbs the biosynthesis of nucleotides and subsequent DNA synthesis (Ferone, 1977, Sridaran *et al.*, 2010). Resistance emerged rapidly when these drugs were introduced alone as a monotherapy, but synergistic combinations like SP (Fansidar™), first introduced in the late 1960s, have proved to be of long-term utility, especially as an inexpensive alternative to combat CQ-resistant parasites. Treatment with Fansidar™ is taken in a single dose

which results in high compliance. However, this has not stopped the spread of resistance, as recently witnessed in retrospective studies conducted in Mozambique (Raman *et al.*, 2010).

Nowadays, artemisinin-based combination therapy (ACT) has taken over the first-line regimen of choice in many areas of the world. Nevertheless, antifolates still play a key role in intermittent preventive treatment (IPT) in areas of high transmission of high risk groups (pregnant woman and infants) regardless of their infection status (Warsame *et al.*, 2010).

Mutations in the *dhfr* and the *dhps* genes of *P. falciparum* parasites have been associated with decreased parasite sensitivity to the antifolate drugs. A change from wild type Ser108 to Asn108 (S108N) in *pfdhfr* is sufficient to cause low level pyrimethamine resistance *in vivo* and by 100-fold relative to wild type *in vitro* (Cowman *et al.*, 1988, Peterson *et al.*, 1988). The triple *pfdhfr* mutant genotype consisting of N51I, C59R and S108N shows *in vitro* resistance to pyrimethamine with significantly higher inhibitory concentration values than with the single mutation at position 108 (Nzila-Mounda *et al.*, 1998) and have demonstrated strong association with *in vivo* SP treatment failure (Kublin *et al.*, 2002, Basco *et al.*, 1998, Happi *et al.*, 2005). Data from various malaria endemic areas suggest asymmetric selection of resistant genotypes starting with mutations in *pfdhfr* and followed by mutations in the *pfdhps* gene by which A437G and K540E SNPs have been associated with *in vivo* clinical failure (Happi et al., 2005). The quintuple mutant genotype consisting of the double *pfdhps* mutant mentioned above in combination with the *pfdhfr* triple mutant genotype (N51I, C59R, S108N) is a better predictor of clinical failure than either the multiple mutant genotype alone (Mugittu *et al.*, 2004, Happi et al., 2005, Kublin et al., 2002) as also demonstrated in paper I of this thesis.

Whereas the amino acid alterations mentioned above are to date the key factors associated with parasite antifolate resistance, other factors may play a role in the levels of clinical failure after SP treatment. Higher serum folate concentrations, as a result of a dietary folate supplementation in children and pregnant women have been reported to be associated with SP treatment failure (Dzinjalamala *et al.*, 2005, van Eijk *et al.*, 2008). The raised endogenous folate pools in parasites will compete with antifolate drugs at enzyme binding sites. As discussed further in this thesis, one endogenous substrate that is transported by MRPs (multidrug resistance-associated protein) are the folates (Deeley & Cole, 2006). In paper I we identified the first polymorphism selection (R1466K) in the *pfmrp1* transporter gene in recrudescent infections after SP treatment.

### 1.6.3 Artemisinins

For more than 2000 years, artemisinins have been used in traditional Chinese medicine for the treatment of febrile illness. But it was only in 1971 that Chinese scientists discovered its specific antimalarial properties extracted from the ubiquitous annual wormwood *Artemisia annua* (White, 2008).

Also known as qinghaosu, artemisinin (ART) and its derivatives (mainly utilized in ACT: artemether, artesunate and dihydroartemisinin (DHA)) are a group of sesquiterpene lactone endoperoxides that possess the most rapid action of all current drugs used against *P. falciparum,* being able to reduce the parasite biomass up to 10000 fold per asexual cycle. They also have a very short elimination half-life in the human body (around 1 hour), reducing the opportunity for the parasite to develop resistance.

Artemisinins are active in nearly all of the asexual stages of parasite development in the blood, and also affect the sexual stages of *P. falciparum* (gametocytes) which are essential for transmission. The specific mechanisms of action of the ART derivatives are still unsolved. Most studies agree that their activity involves the break of the intra-molecular endoperoxide bridge, and a recent study suggests that the digestive vacuole is an important initial site of endoperoxide antimalarial activity (del Pilar Crespo *et al.*, 2008). The active endoperoxide compound is assumed to interact with reduced heme (ferroprotoporphyrin IX) or iron to form free-radical by-products of both the drug and the heme (Jefford, 2001, Paitayatat *et al.*, 1997). The radicals are thought to react with susceptible groups within parasite enzymes and lipids; however, the exact sites of action are still unresolved.

The first protein to be suggested to be a target for ART was the *Plasmodium* translationally controlled tumor protein (TCTP) homologue (Bhisutthibhan *et al.*, 1998), a protein that binds heme. One report has shown that there is less incorporation of radiolabeled DHA in a resistant murine plasmodial strain *in vivo* and a 2.5 fold overexpression of TCTP in a rodent malaria model (Walker *et al.*, 2000), but no genetic alterations have been described regarding ART susceptibility.

Structural similarities of ART to thapsigargin, also a sesquiterpene lactone, led to the identification of another potential target related to the endoplasmic reticulum, PfATP6 (Eckstein-Ludwig *et al.*, 2003). This represents the only sarco/endoplasmatic reticulum calcium-dependent ATPase (SERCA) ortholog in *P. falciparum*, a central player in the crucial $Ca^{2+}$ homeostasis of the parasite. A mutation in the *pfatp6* gene, noted

exclusively in isolates from French Guiana, was reported to be associated with significantly increased $IC_{50}$ values for artemether in *ex vivo* tests (Jambou *et al.*, 2005).
More recently, *in vivo* artemisinin resistance has been proposed (Noedl, 2005) and identified by the presence of significantly decreased parasite reduction rates, manifested clinically by markedly longer parasite clearance times from the body (Dondorp *et al.*, 2009, Noedl, 2005, Noedl *et al.*, 2008, Noedl et al., 2010). With only a few individual cases matching as resistant: "adequate plasma drug absorption, prolonged parasite clearance times, increased ARTs $IC_{50}$s, and reemergence of parasites within 28 days" (Noedl et al., 2008, Noedl et al., 2010), the molecular basis for this phenomenon is uncertain, based on the fact that most of these observations are not clearly associated with altered artemisinin $IC_{50}$ *in vitro*. So far only minor determinants with likely roles in resistance, such as *pfmdr1* amplifications, correlate (Table 1) (Price et al., 2004, Imwong *et al.*, 2010).

#### *1.6.3.1 ACT- artemisinin based combination therapy*

In nearly all countries where malaria is endemic, ACTs are now the recommended first line therapy for uncomplicated *P. falciparum* malaria, a policy endorsed by the WHO in order to increase the efficacy of malaria chemotherapy while delaying the emergence of drug resistance.
Combination therapy is well established for the treatment of other diseases such as tuberculosis and infection by the human immunodeficiency virus (HIV). The rationale for combination therapy is based on the diminutive probability of resistance arising after simultaneous use of two or more antimalarials with different modes of action which do not share the same resistance mechanisms (White, 1999).
The commonly used ACTs are: artemether + lumefantrine (AL); artesunate + amodiaquine (AS-AQ); artesunate + mefloquine (AS-MQ); artesunate + sulphadoxine + pyrimethamine (AS-SP) and dihydroartemisinin + piperaquine (DHA-PQ).
These combination therapies work due to the ability of artemisinins to rapidly reduce the parasite biomass in the patient (producing a rapid clinical response), leaving the partner drug with relatively few parasites to eliminate. The partner drug, with its longer plasma half-life, is also responsible for preventing the recurrent parasitaemia associated with ART monotherapy. Nevertheless, for drugs with long half-life, blood levels can persist for days, a period during which a patient is essentially under antimalarial

monotherapy. The time interval when the drug concentrations are at sub-therapeutic levels but still significantly present has been referred as the "selective window". This is the period of time when the drug level is adequate to suppress the growth of susceptible parasites, but too low to prevent replication of less sensitive sub-populations, leading to their selection (Stepniewska & White, 2008, Hastings & Ward, 2005).

So far, the introduction of ACT as first line treatment has been very successful. Countries where ACT and other malaria controls activities have been introduced have witnessed significant reductions in endemicity (Dondorp *et al.*, 2010).

### 1.6.4 Atovaquone

While the scope of this thesis does not include these types of drugs, they are used as antimalarials, and are therefore mentioned briefly.

Atovaquone, the first antimalarial derived from rational drug design, acts by collapsing the mitochondrial membrane potential. Specifically, it inhibits the movement of the iron-sulfur protein sub-unit of cytochrome b (CYT b) which is necessary for the transfer of electrons in the respiratory chain (Mather *et al.*, 2005).

Atovaquone is administered as an antimalarial drug in combination with the antifolate proguanil (Malarone™) and is one of the few remaining effective drugs without an artemisinin derivative component. Its use has been limited to prevention and treatment in travellers, not because of a lack of efficacy or safety concerns, but because of its prohibitively high price (Dondorp et al., 2010). In addition, resistance to atovaquone seems to rise relatively easily through the accumulation of mutations in the *cytochrome b* gene, which appears not to be able to be completely prevented *in vivo* by the combination (Farnert *et al.*, 2003, Fivelman *et al.*, 2002, Korsinczky *et al.*, 2000). The limited use has so far minimized the drug selection pressure and prevented the appearance of resistance, but a SNP in codon 268 in *P. falciparum cytochrome b* gene has been shown to confer a high level of atovaquone resistance (Gebru *et al.*, 2006).

**Table 1: Protein and polymorphisms associated or likely to be associated with tolerance/resistance to antimalarial drugs**

| Protein | Function | Location | Principal drugs affected - Comments | | Polymorphisms | References |
|---|---|---|---|---|---|---|
| ATP6 (SERCA) | Membrane Ca2+-transporting ATPase | endoplasmatic reticulum | ART | putative determinant | S769N (French Guiana) | (Jambou et al., 2005) |
| CRT | Transporter | food vacuole membrane | CQ, AQ | major determinant | C72S, M74I, N75E, K76T, S163R, N326S/D, I356T/L, | (Djimde et al., 2001, Fidock et al., 2000, Foote *et al.*, 1990, Johnson *et al.*, 2004, Mu *et al.*, 2003, Reed *et al.*, 2000, Sidhu et al., 2002) |
| | | | MQ, HF, LUM, ART, QN, PQ | minor determinant | | |
| CYT b | electrons transfer - respiratory chain | mitochondrion | Atovaquone | | Y268S/N/C | (Gebru et al., 2006) |
| DHFR | Folate pathway enzyme | cytoplasm | PYR, PG, chlorproguanil | DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates | A16V, C50R, N51I, C59R, S108N/T, I164L | (Cowman et al., 1988, Peterson et al., 1988) |
| DHPS | Folate pathway enzyme | cytoplasm | SDX, dapsone | | S436A/F,A437GK 540E, A581G, A613S/T | (Happi et al., 2005) |
| MDR1 | Transporter | food vacuole membrane | MQ, HF, LUM, QN | major determinant | N86Y, Y184F, S1034C, N1042D, D1246Y, CNV | (Patel *et al.*, 2010, Sa *et al.*, 2009) |
| | | | CQ, AQ, ART | minor determinant | | |
| MRP1 | Transporter | cytoplasm membrane | CQ, QN, LUM | possibly also antifolates | H191Y,S436A,I876V,R1466K | (Dahlstrom *et al.*, 2009, Mu et al., 2003) |
| NHE1 | Transporter | cytoplasm and food vacuole | QN | limited in field studies to date | copy number of repeat motifs | (Ferdig *et al.*, 2004) |
| TCTP | Ortholog of human histamine-releasing factor | not yet established | ART | putative determinant | not yet known | (Bhisutthibhan et al., 1998) |

## 1.7 MEMBRANE TRANSPORTER PROTEINS AS MAIN MECHANISMS OF RESISTANCE

The malaria parasite infected RBC is a multi-compartment structure with numerous discrete membrane systems. The trafficking of solutes into and between these different compartments across the delineating membranes is mediated by membrane transport proteins: channels, transporters and pumps. Some of these proteins have been implicated in the phenomenon of antimalarial drug resistance, as well as being potential drug targets in their own right (Staines *et al.*, 2010). To date, well over 100 known and putative transporters sequences have been identified in the *P. falciparum* genome (termed the *Plasmodium* "permeome" (Martin *et al.*, 2005, Martin *et al.*, 2009a). However, linking the physiological functions of these proteins to their roles in drug resistance is not obvious. A full understanding of the roles of these proteins in infected RBCs requires knowledge of their subcellular localization and substrate specificity, as well as some knowledge of the effects on the parasite of modifying the sequence and/or

expression level of the gene involved. In *P. falciparum,* this type of information is scarce, with studies conducted on only a small number of proteins.

Among the potential candidate transporters that show altered expression in drug resistant and/or drug treated parasites are two key intracellular transporter players – the "*Plasmodium falciparum* chloroquine resistance transporter" (PfCRT) and the "P-glicoprotein homologue 1" (PfMDR1). The putative glutathione and glutathione conjugate transporter PfMRP1 (*P. falciparum* multidrug resistance-associated protein 1) was also recently shown to be involved in the modulation of parasite drug sensitivity. In the next sections, a more comprehensive overview of the specific transporters mentioned above will be presented, including their genetic and physiological characteristics.

### 1.7.1 *P. falciparum* chloroquine resistance transporter – PfCRT

The molecular mechanism of resistance to CQ was resolved through the analysis of a genetic cross between a chloroquine sensitive (CQS, HB3) and a chloroquine resistant (CQR, Dd2) clone. A locus mapped to chromosome 7 was shown to harbour a pivotal gene, *pfcrt* (Fidock et al., 2000), coding for a 45-kDa protein containing ten predicted transmembrane domains. This putative transporter was further localised to the membrane of the food vacuole of the parasite (Cooper *et al.*, 2002, Fidock et al., 2000).

The comparison between the Dd2 and HB3 *pfcrt* open reading frames revealed eight codon differences (Fidock et al., 2000). Among the various mutations, a specific codon at position 76 changing lysine to threonine was revealed to be the most reliable molecular marker of resistance through the analysis of a number of geographically distinct CQR and CQS clones (Bray *et al.*, 2005, Valderramos & Fidock, 2006). CQ efficacy trials in the field (Mali) clearly showed a total association between clinical failure and the presence of the 76T variant in the context of a strong selection of this allele among the recurrent infections compared to the baseline values (Djimde et al., 2001) (Figure 4).

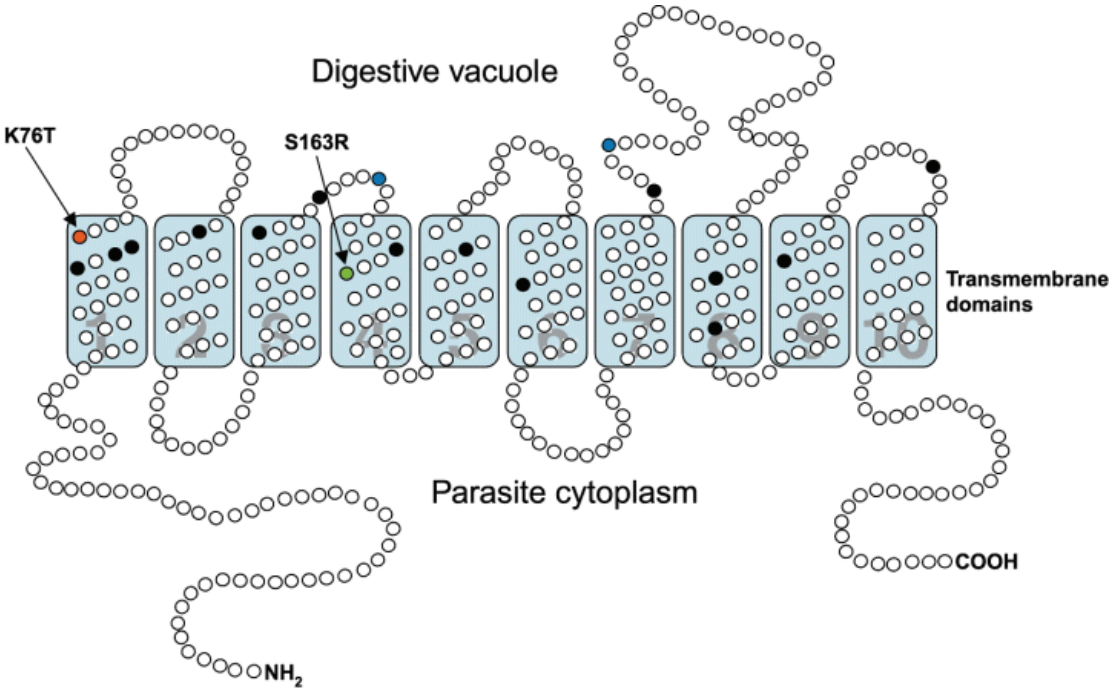



Figure 4 – Predicted protein structure of PfCRT. PfCRT is postulated to possess 10 transmembrane helices, with the N- and C-termini extending into the parasite cytoplasm. Black and red filled circles indicate the positions of mutations published from full-length pfcrt cDNA sequences identified in CQR parasites from field samples. The critical K76T mutation is indicated in red. The green filled circle indicates the position of the S163R mutation in amantadine- and halofantrine-resistant parasites, while blue circles represent additional mutations in these drug-pressured parasite lines. Reprinted from Molecular Microbiology (Bray et al., 2005) with permission from John Willey and Sons.

Lastly, from previous preliminarily data using episomal expression systems (Fidock et al., 2000), allelic exchange approaches demonstrated the central influence of this SNP in the *in vitro P. falciparum* CQ response (Lakshmanan *et al.*, 2005, Sidhu et al., 2002). Nevertheless, it has not been possible to introduce this single mutation into a CQS parasite, suggesting this mutation might have a detrimental functional effect that is compensated for by other *pfcrt* mutations.

Beside CQ, the PfCRT transporter can also influence the parasite's *in vitro* susceptibility to other antimalarial drugs, mainly quinoline-based but also artemisinin (Cooper et al., 2002, Lakshmanan et al., 2005, Sidhu et al., 2002, Sisowath *et al.*, 2009).

PfCRT is believed to be an essential protein for cell function, since PfCRT knock-out parasites do not survive. This lethality constitutes an obstacle for the precise understanding of its natural function and endogenous substrates.

In trying to clarify the mechanism of drug resistance in the malaria parasite, significant debate has been ongoing regarding PfCRT function (Summers & Martin, 2010). Recent

studies have revealed PfCRT homologues in plants which are most likely involved in glutathione homeostasis and associated with stress responses (Maughan *et al.*, 2010).

### 1.7.2 ABC transporters genes

ATP-binding cassette (ABC) transporters are best known for their role in multi-drug resistance. They can actively pump out a wide range of structurally and functionally diverse drugs, thereby decreasing intracellular drug accumulation and ultimately resulting in drug resistance (Borges-Walmsley *et al.*, 2003). The structure of a typical complete ABC transporter consists of two transmembrane domains (TMDs) each containing of six transmembrane helices (TM) that form the pathway for the transport of substrates, and two cytosolic nucleotide binding domains (NBDs) able to hydrolyse ATP to provide energy for this process. Sixteen ABC family members have been identified in the *P. falciparum* genome (Gardner et al., 2002, Kavishe *et al.*, 2009, Koenderink *et al.*, 2010, Martin et al., 2005), three of which, PfMDR1, PfMRP1 and PfMRP2, will be more extensively described in the next section concerning evidence of their role in *P. falciparum* drug resistance.

#### *1.7.2.1 P. falciparum multidrug resistance 1 - PfMDR1*

Long before the discovery of PfCRT as the main cause of CQ resistance, attention was given to *P. falciparum* multidrug resistance 1 (PfMDR1; also known as Pgh-1), the ortholog of mammalian P-glycoproteins that mediate resistance to multiple drugs in mammalian cancer cells. The gene *pfmdr1*, located on chromosome 5, encodes for a 162-kDa protein localized predominantly in the parasite's digestive food vacuole membrane (Cowman *et al.*, 1991).

Due to the similarity of this protein with the human MDR, it was originally hypothesized that PfMDR1 could be a major candidate for conferring resistance to CQR (Foote *et al.*, 1989, Wilson *et al.*, 1989). Support for this hypothesis came from the observation that the calcium channel blocker verapamil was able to reverse chloroquine resistance, in the same fashion as observed in MDR cancer cells (Watt *et al.*, 1990). Although this hypothesis was not completely accurate, it is now accepted that *pfmdr1* mutations differentially affect the CQ responses in CQR parasites and their

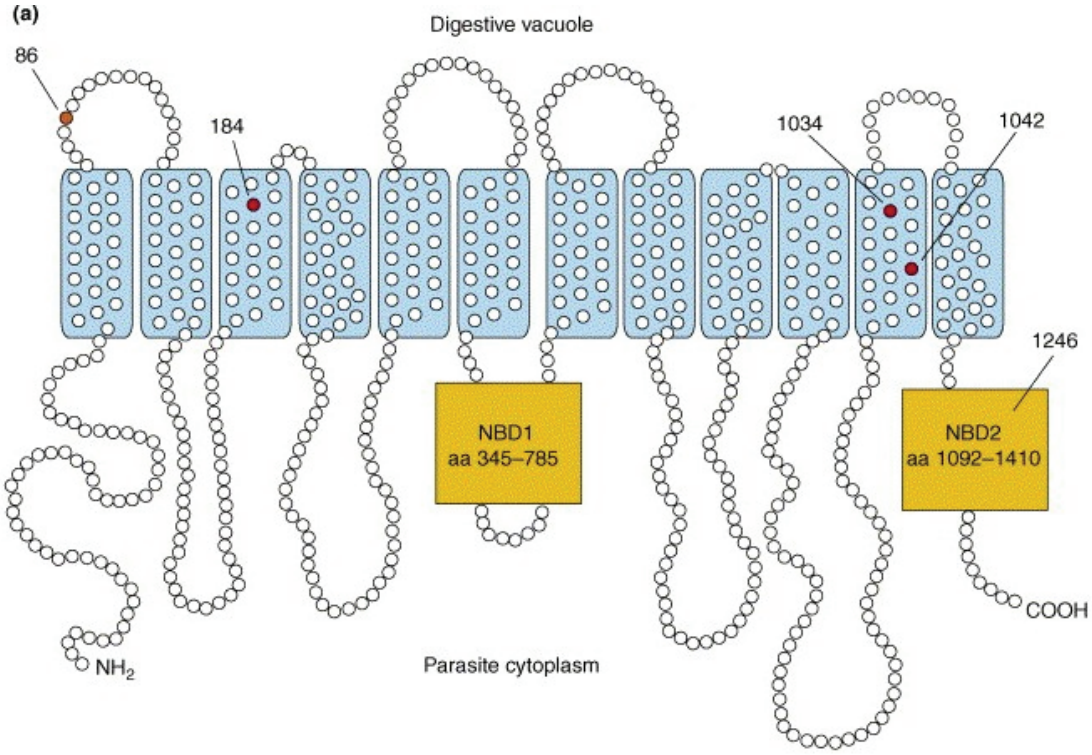
activities depending on the *pfcrt* haplotype to which they are associated (Patel et al., 2010, Sa et al., 2009).

The first polymorphism in *pfmdr1* that was correlated with drug resistance were gene copy number amplifications (Foote et al., 1989, Triglia *et al.*, 1991, Wilson et al., 1989). In the last few years, *pfmdr1* copy number has proven to be a pivotal molecular marker of resistance against MQ in the field, both *ex vivo* (Price *et al.*, 1999) and *in vivo*, where a comprehensive study performed in Western Thailand revealed a high association with treatment failure with MQ and AS-MQ (Price et al., 2004). Additionally, an increased risk of failure of short-term AL treatment (4 doses) has been documented to be significantly associated with increased *pfmdr1* copy number (Price et al., 2006). These results confirmed earlier laboratory data showing that *in vitro* development of MQ resistance was strongly associated with increases in copy number (Cowman *et al.*, 1994). Consistent with most previous data, knock-down experiments involving the genetic disruption of one of the two native *pfmdr1* copies present in the FCB drug resistance strain also showed increased *in vitro* susceptibility to MQ, LUM, halofantrine, QN and ART (Sidhu *et al.*, 2006), albeit no change in CQ sensitivity.

A set of five *pfmdr1* canonical point mutations were identified soon after the cloning of the gene: N86Y, Y184F, S1034C, N1042D and D1246Y (Figure 5). These have been linked in variable degrees to altered drug susceptibility both *in vitro* and *in vivo* to a broad range of drugs. Ongoing data support 5' located N86Y as being involved in the parasite's *in vitro* response to the quinolines (Lopes *et al.*, 2002). The *in vivo* importance of this SNP is demonstrated by the clear selection of distinctive alleles upon drug administration. Specifically, the 86N allele is selected by AL (Sisowath *et al.*, 2007, Sisowath et al., 2009, Sisowath *et al.*, 2005), an action believed to be mainly driven by the long half life component. Conversely, the 86Y allele has been observed to be selected by both CQ (Djimde et al., 2001) and AQ treatments (Holmgren *et al.*, 2006, Holmgren *et al.*, 2007).

Allele exchange studies have pointed toward the importance of the 3' distal S1034C, N1042D and D1246Y alleles as particularly important in the response to MQ and QN, and contributing for higher levels of CQ resistance in certain genomic environments (Reed et al., 2000). A second allelic modification study (Sidhu *et al.*, 2005), found little shift of CQ susceptibility with changes in *pfmdr1* encoded amino acids (1042D to N or introduction of the three residues 1034C, 1042D and 1246Y) in a progeny clone from the HB3xDd2 cross consistent with recent observations (Sa et al., 2009). This study supports the hypothesis that *pfmdr1* 3'point mutations can significantly affect parasite

susceptibility to a wide range of antimalarials in a strain-specific manner that depends on the parasite's genetic background.




(b)

*pfmdr1* variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

| | | PfMDR1 position and amino acid | | | | | Copy number |
|---|---|---|---|---|---|---|---|
| Region | Reference line (origin) | 86 | 184 | 1034 | 1042 | 1246 | |
| All | Wild type (3D7, Netherlands) | N | Y | S | N | D | 1 |
| Asia and Africa | FCB (Southeast Asia) | N | Y | S | N | D | ≥2 |
| | K1 (Thailand) | Y | Y | S | N | D | 1 |
| South America | 7G8 (Brazil) | N | F | C | D | Y | 1 |

Gray shading indicates residues that differ from the wild-type allele.

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Figure 5 – Predicted structure and genetic polymorphisms in PfMDR1. (a) PfMDR1 has two homologous halves, each with six predicted transmembrane domains and a nucleotide-binding pocket. The nucleotide-binding domains (NBD1 and NBD2; orange boxes) are each formed by large cytoplasmic domains. Polymorphic amino acids found in the K1 allele (N86Y) and the 7G8 allele (Y184F, S1034C, N1042D and D1246Y) are indicated. The D1246Y mutation is located in the predicted NBD2. (b) Representative haplotypes, including the one most commonly associated with amplification of *pfmdr1* copy number. Reprinted from Trends in Pharmacological Sciences (Valderramos & Fidock, 2006) with permission from Elsevier.

Expression of the *pfmdr1* gene has been shown to be induced after treatment with CQ, MQ and QN, but not after treatment with pyrimethamine, suggesting that induction of *pfmdr1* might be a drug specific mechanism of resistance (Myrick *et al.*, 2003).

Like PfCRT, the physiological function of PfMDR1 is unknown and we can only carefully extrapolate findings from mammalian and plant MDR1 homologues. Whatever the function of PfMDR1 may be, it seems to be essential, based on the findings that, like *pfcrt*, this gene is necessary for the survival of the organism.

#### *1.7.2.2 P. falciparum multidrug resistance associated proteins – PfMRPs*

In pioneering work conducted in 1969 (Srivastava & Beutler, 1969), it was reported that elimination of GSSG (oxidised glutathione) from human erythrocytes is a unidirectional and energy-dependent process. The discovery of a GS-X pump that requires GSH (reduced glutathione) as a cofactor for transporting a group of endogenous substrates and exogenous xenobiotics resulted in the cloning of a cDNA encoding a protein of 190 kDa, which they named MRP (Cole *et al.*, 1992). The class of transporters defined as the multidrug-associated proteins (MRP) have long been implicated in multidrug resistance in mammalian cells.

The MRP proteins define a sub-class of the ABC protein super-family. The canonical structure of a complete ABC transporter contains two ATP-binding cassettes in the intracellular part and two core membrane-spanning domains, MSD1 and MSD2. MRP transporters contain consensus regions named as Walker A, Walker B, and Signature C motifs that are required for ATP binding (Toyoda *et al.*, 2008).

The typical substrates of MRPs are amphiphilic organic anions. These can be transported unconjugated or conjugated with glutathione, glucuronate or sulphate groups. Some examples of the variety of molecules that MRPs can transport include endogenous substrates such as folates, anticancer drugs such as methotrexate, heavy metals such as arsenite and antibiotics such as difloxacin (Deeley & Cole, 2006).

Since CQ and potentially MQ were also reported to be transported by human MRP1 and 4, it has been speculated that *P. falciparum* MRP could do the same and thus contribute to drug resistance (Vezmar & Georges, 1998, Wu *et al.*, 2005).

The isolation of MRP coding genes in *P. falciparum* was reported 11 years ago, using the ABC signature targeting degenerate primer approach (Gil *et al.*, 2000). This approach revealed two genes, *pfmrp1* and *pfmrp2*, later identified by the *P. falciparum* genome sequencing project as (PFA0590w) and (PFL1410c), respectively. *Pfmrp1* is localized to chromosome 1 and *pfmrp2* to chromosome 12, and both encode for proteins localized to the cytoplasmic membrane of the parasite's asexual stages, while

exhibiting differential expression profiles (Kavishe et al., 2009, Bozdech & Ginsburg, 2004). *Pfmrp1* is transcribed mainly during the trophozoite stage and is suggested to encode a *P. falciparum* GSH/GSSG pump. This is supported by the coincident transcription of glutathione synthetase and the de novo synthesis of glutathione (Bozdech & Ginsburg, 2004, Klokouzas *et al.*, 2004, Bozdech et al., 2003). On the other hand, *pfmrp2* transcript levels peaks at a very early stage (early rings) and also in mature schizonts (Bozdech et al., 2003, Nogueira *et al.*, 2008).

Single nucleotide polymorphisms present in *pfmrp1* (also named G2) and *pfmrp2* (also named G56) were screened in parasite lab strains (Mu et al., 2003) in search of antimalarial susceptibility association. No association was found for any *pfmrp2* SNPs, but the SNPs H191Y and S437A in *pfmrp1* were found to be associated with decreased *in vitro* susceptibility to CQ and QN. A comprehensive biodiversity study performed in our lab revealed *pfmrp1* to be significantly polymorphic with distinct geographic patterns (Dahlstrom et al., 2009) (Figure 6).

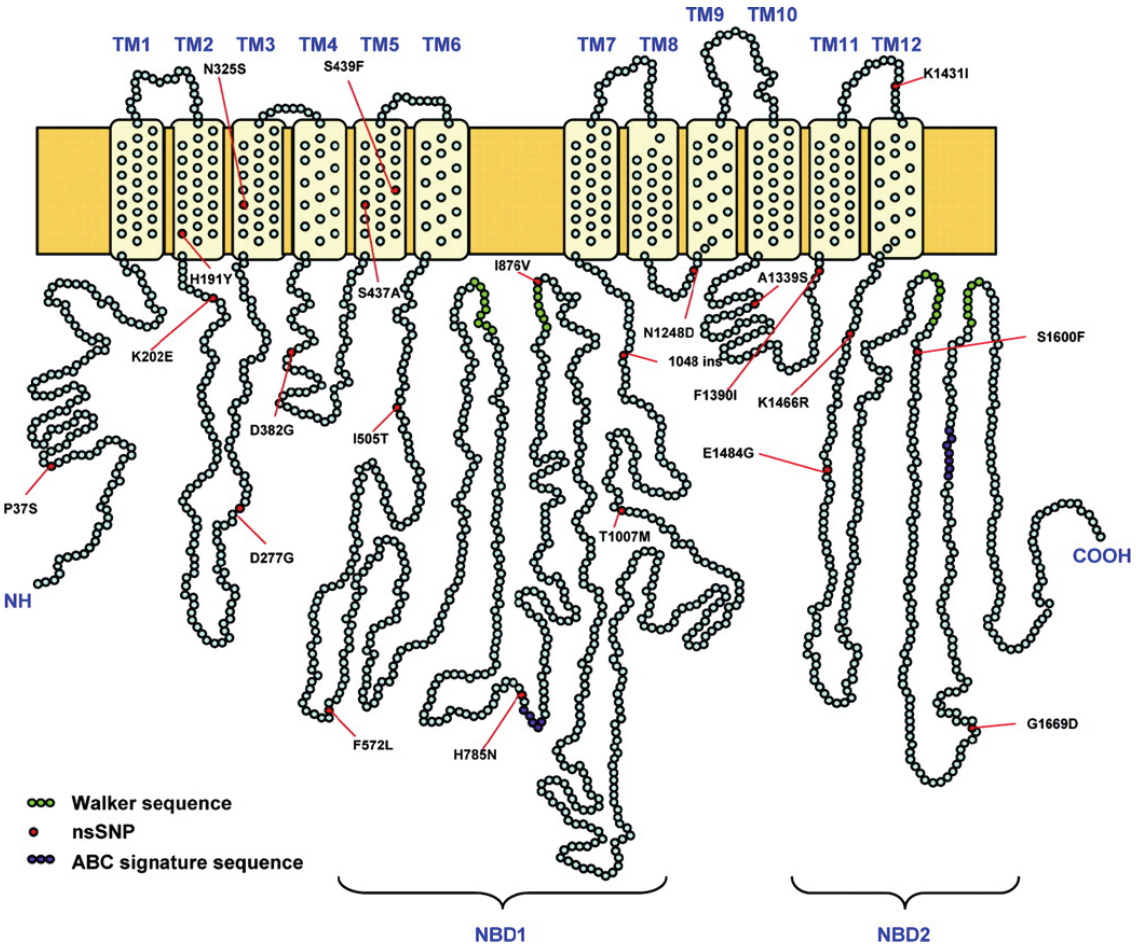


Figure 6 – Predicted 2-dimensional transmembrane domain organization by the HMMTOP algorithm (version 2) and single-nucleotide polymorphism (SNP) distribution in pfMRP1. Reprinted from The Journal of Infectious Diseases (Dahlstrom et al., 2009) with permission from Oxford University Press.

In Africa, the most prevalent SNPs are I876V and K1466R and from a clinical efficacy trial using AL, significant positive selection of 876I was detected in recurrent infections (Dahlstrom et al., 2009) (Figure 6). The SNP F1390I was found, in the above mentioned biodiversity study, to be of high prevalence in Southeast Asia and with only one case (Gambia) in Africa.

Recently, a genetic disruption study demonstrated that the gene encoding PfMRP1 was not essential for asexual stage development, although its deficiency did incur a significant fitness penalty, as knock-out parasites could not grow to a parasitaemia higher than 5%. Furthermore and likely related to this decrease in multiplication capacity, this study revealed increased intracellular glutathione accumulation in knock-out parasites, paralleled by an enhanced susceptibility to several antimalarial drugs, including CQ, QN and ART (Raj *et al.*, 2009).

# 2 AIM OF THE THESIS

## 2.1 OVERALL OBJECTIVE

To better understand the involvement of drug transporter genes in the molecular mechanisms underlying drug resistance in *Plasmodium falciparum* malaria.

## 2.2 SPECIFIC AIMS

**Paper I:** Contribution of identified polymorphisms in *pfmrp1* for the parasite response against antifolate antimalarials.

**Paper II:** The determination of the *in vitro* simultaneous transporter gene expression responses after drug challenges of *P. falciparum* parasites in the intra-erythrocytic cell cycle.

**Paper III:** *In vitro* antimalarial susceptibility response analysis of *P. falciparum* isolates– correlations with natural SNP diversity of drug transporter genes.

**Paper IV:** A first comprehensive account of the *pfmrp2* genetic variability and its association with *in vitro P. falciparum* antimalarial responses.

# 3 MATERIAL AND METHODS

## 3.1 STUDY SITES

The clinical studies analysed in this thesis have been performed in East Africa and Southeast Asia. The SP and AL efficacy trial was performed in Fukayosi, Tanzania (study I). Concerning studies III and IV, a set of parasites originated from the Mae Sot region, Western Thailand were culture adapted and studied for *in vitro* drug susceptibility. The original Thai infections corresponded to samples collected from patients before routine chemotherapy (AS-MQ).

### 3.1.1 Tanzania – Fukayosi village

Malaria is transmitted in Tanzania throughout the year with seasonal peaks during rainfalls in March-May and October-December. The main malaria species is *P. falciparum*. The clinical trial, analysed in paper I, was conducted in Fukayosi primary health care centre in April to July 2004, located in Bagamoyo district, on mainland Tanzania. At the time of the study, SP and AQ were used as first line and second line treatment respectively. In 2006 Tanzania adopted AL as first line treatment.

### 3.1.2 Thailand

Malaria is endemic in some regions of Thailand, especially in the forest regions as well as its border areas. *P. falciparum* and *P. vivax* are the main malaria species. For paper III and IV *P. falciparum* fresh isolates were collected between the year 2002 to 2008 from patients in Mae Sot, in the Tak Province. The isolates were provided from the Shoklo Malaria Research Unit. Thailand Western boarder with Burma/Myanmar, is an epicenter of emerging antimalarial drug resistance. To counteract the steep rise in MQ resistance, AS-MQ was introduced in Mae Sot in 1995 in areas with high MQ resistance (Na-Bangchang & Congpuong, 2007, Wongsrichanalai & Meshnick, 2008).

At present in Thailand, AS-MQ is first line treatment for all uncomplicated confirmed *P. falciparum* malaria (Carrara *et al.*, 2009).

## 3.2 ETHICAL CONSIDERATIONS

For paper I, ethical approval was obtained from Muhimbili University College of Health Sciences, Tanzania and Karolinska Institutet, Sweden (KID nr 03-684). For paper III and IV, the collection protocols were approved by the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok and Oxford Tropical Research Ethics Committee (OXTREC 027-04) at University of Oxford, UK. The attending physician assured provision of written informed consent in the local language in both studies.

## 3.3 CLINICAL DRUG EFFICACY TRIALS

In paper I, the clinical trial was included in the assessment of *P. falciparum* polymorphisms and treatment outcome. It was conducted in April-July 2004 in Fukayosi and included two arms comparing AL (n=50) and SP (n=56) (Martensson *et al.*, 2007). The trial involved children with uncomplicated *P. falciparum* malaria. The inclusion criteria for this study were: age of 6-59 months and bodyweight of ≥6 kg, parasitaemia levels of 2000-200 000 asexual parasites/μL of blood and axillary temperature of ≥37.5ºC or a history of fever the last 24 hours. Children with severe malaria, serious underling disease or known allergy to the drugs used were excluded from the study. Enrolled children were assigned to receive a fixed combination of 20mg/120mg AL (Coartem$^{TM}$; Novartis) twice daily for three days or 500mg/25mg SP (Fansidar$^{TM}$; Roche) as a single dose, according with their body weight. The children were followed up and routinely checked for parasitaemia on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 of the study, or at any day of recurrent illness. Parasitaemia was calculated by quantifying the number of parasites per 200 leucocytes by microscopy of Giemsa-stain thick blood films and multiplied by 40 to obtain the number of parasites/μL. Blood samples were collected on filter paper (3MM; Whatman) for molecular analysis.

## 3.4 PARASITE *IN VITRO* CULTURE STUDIES

*P. falciparum in vitro* culturing was performed for paper II and III. The laboratory clones were acquired from the Malaria Research and Reference Reagent Resource Center (MR4, ATCC Massanas Virginia), as well as generously provided by the late Prof. D. Walliker (Department of Animal and population genetics, University of Edinburgh, UK).

All parasites studied were cultured in human $O^+$ RBCs and Malaria Culture Medium, containing RPMI 1640 culture medium supplemented with 10% L-glutamine, 0.05% gentamicine (Gibco® / Invitrogen™) and 10% human $AB^+$ serum. Parasites were incubated at 37ºC in air-tight environment achieved either by conventional candle-jar technique (Trager & Jensen, 1976) or involving the use of gas (5% O2 and 5% CO2 in N2). Parasites were grown in static manner or in suspension on an orbital shaker (50 revolutions per minute), according to the circumstances.

Parasite intra-erythrocytic cell cycle synchronization, when applied, was performed by the incorporation in the parasite culture of 5% sorbitol for 10 min (Lambros & Vanderberg, 1979) or using magnetic columns (MACS®, Miltenyi Biotec). The latest consist in passing the parasites in a magnetic column which will split different parasite stages, based in the principle that the hemozoin iron atoms present in the parasites will be trapped in the column, while the parasite with no hemozoin (ring stage) will pass through the column.

### 3.4.1 Drug susceptibility assays

Different antimalarial drugs inhibitory concentrations were determined for the laboratory strains, as well as for the fresh isolates, by relative quantification of *P. falciparum* Histidine-Rich Protein 2 (PfHRP2) based on a Double-Site Sandwich Enzyme-Linked Immunosorbent Assay (Noedl *et al.*, 2005), followed by nonlinear regression analysis (http://malaria.farch.net).

## 3.5 MOLECULAR ANALYSIS

In paper I, molecular analysis was performed from blood samples collected on filter paper while in paper II to IV the source for nucleic acids was fresh parasite *in vitro* culture

### 3.5.1 Nucleic acids extraction

#### *3.5.1.1 DNA*

The samples were extracted using the ABIPRISM®6100 Nucleic Acid PrepStation (Applied Biosystems™, Fresno, CA, USA). When few samples were to be extracted QIAamp® DNA Mini Kits (Qiagen, Hilden, DE) was preferred. DNA extraction in paper I was performed according to manufacturer's recommendations with some adjustments for blood spotted in filter paper. In these circumstances the filter paper was cut in small pieces and soaked in distilled water together with proteinase K and respective buffer. This mixture was incubated for 1 hour at 58ºC and laid overnight at 4ºC before performing the extraction.

#### *3.5.1.2 RNA*

Extraction of RNA was carried out for gene expression analysis as well as for sequencing from cDNA. ABIPRISM®6100 Nucleic Acid PrepStation® (Applied Biosystems™, Fresno, CA, USA) together with manufacturer's recommendation was the chosen method.

### 3.5.2 Genetic fingerprinting

- *pfmsp1* and *pfmsp2* genes for PCR adjusted drug efficacy clinical trial analysis

Numerous genes in *P. falciparum* have been shown to comprise extensive genetic polymorphism, which can be used for genetic finger printing. Their polymorphisms are found dissimilar in different geographical locations in malaria endemic areas. An

example of it is the merozoite surface protein 1(*pfmsp1*) and merozoite surface protein 2 (*pfmsp2*) (Mugittu *et al.*, 2006, Snounou *et al.*, 1999).

Several studies have reported that *pfmsp1* allelic variants fall under three major types—MAD20, K1 and RO333 and their frequency varies in different geographical areas, even in neighbouring villages. *pfmsp2* alleles, which differ in number and sequence of intragenic repeats, can be grouped into two allelic families, FC27 and 3D7/IC, according to the central dimorphic domain as first observed over a decade ago.

Therefore, these *loci* have been widely used to characterize *P. falciparum* field isolates, evidencing the multiplicity of infection (number of parasite clones per sample) as well as in many trials to distinguish recrudescence from new infections (Kiwanuka, 2009).

Accordingly, in paper I recrudescences and reinfections were defined based on the step-wise genotype of *pfmsp1* and *pfmsp2*. Only samples classified as recrudescences according to *pfmsp2* genotyping were analysed for *pfmsp1*. A recurrent infection was classified as recrudescence if there was at least one allelic band matching with the corresponding baseline sample in both genetic markers, or as a reinfection if there were no matching allelic band in at least one genetic marker (Martensson et al., 2007).

### 3.5.3 Restriction Fragment Length Polymorphism (RFLP)

Although now less used, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. In this thesis this method was used for allelic discrimination, more specifically to analyse SNPs. This technique, equipment wise, is characterized to be a simple method with no demands of particularly costly equipment. Hence being generally favoured for field use. RFLP analysis involves cutting a particular region of DNA with known variability with restriction enzymes, then separating the DNA fragments. The methodology herein used to separate the DNA fragments was by agarose gel electrophoresis with ethidium bromide incorporated and visualized by UV transiluminator in a BioRad GelDoc 2000.

In paper I this technique was used to analyse the quintuple mutant haplotype associated with SP resistance namely the *pfdhfr* N51I, C59R, N108S/T and *pfdhps* A437G, K540E SNPs. The genotype of *pfdhfr* SNPs was made in a multiplex PCR as described previously (Veiga *et al.*, 2006). For the *pfdhps* A437G and K540E SNPs a previously published PCR-RLFP method was used (Duraisingh *et al.*, 1998).

### 3.5.4 Pyrosequencing

Pyrosequencing is a method of DNA sequencing based on a "sequencing by synthesis" principle. It relies on the detection of pyrophosphate release upon nucleotide incorporation, rather than chain termination with dideoxynucleotides used in the conventional sequencing (www.pyrosequencing.com). The technique was developed at the Royal Institute of Technology in Stockholm in 1996 (Ronaghi *et al.*, 1996).

This technique is ideal for SNP analysis where short fragments of DNA are sequenced directly from a PCR amplified product. Also, due to the multiplicity of infection found in performed clinical trials and taken in consideration that the malaria parasite is a haploid organism when lodged in the host, different allele proportions can be found in the same analyzed sample, favouring this technique to be used in molecular malaria research (Zhou *et al.*, 2006). In paper I, the analysis of *pfmrp1* I876V and K1466R SNPs was performed with pyrosequencing. Results for I876V were adjusted against a standard curve derived from different proportions of mixed reference laboratory strains, 3D7 and Dd2. When this adjustment is made, pyrosequencing allele quantification can successfully be performed. The results from the SNP analysis of K1466R were not adjusted against a standard curve because of the lack of allele variance among the reference laboratory strains. The threshold for the identification of a mixed infection was set as a pyrosequencing result between 10% and 90% for each allele.

In paper III, this technique was also the preferred choice for the determination of the Thai isolates *pfmdr1* F1226Y and *pfmrp1* F1390I polymorphisms.

### 3.5.5 DNA Sequencing

- Sanger method – outsourced

DNA sequencing is the standard technique for unveil new mutations, having been herein used with that main objective. It can cover large DNA fragments and is also convenient to use for analysis of adjacent positioned SNPs (ex. *pfcrt* amino acid positions 72 to 76). Due to these characteristics, sequencing technique was the preferred method and extensively used in paper II-IV. The Open Reading Frames of the *pfmdr1, pfcrt, pfmrp1* and *pfmrp2* genes were fully sequenced to determine their genetic variability in laboratory strains in paper II and for addressing the studied sample of Thai field isolates referred in paper III and IV. Amplicon fragments were sequenced

by Macrogen Inc. (Seoul, Korea). Analyses of the chromatogram sequences are described in this section under bioinformatics chapter.
It is to note that the sensitivity of sequencing is limited in detecting allele carried by minority parasite populations in mixed infections (Zhou et al., 2006). It is therefore possible that mixed genotypes might be misjudged as a single genotype.

### 3.5.6 Real-time PCR

In molecular biology, real-time PCR, also called quantitative real time PCR (qPCR) is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It has been proven to be a reference method for accurate quantification of nucleic acids since its introduction.
This technique was used in the studies of this thesis to determine gene copy number variations in the parasite genome, as well as for analysis of transcript levels. All the analyses were carried out with TaqMan® probe based real-time PCR, using ABIPRISM® 7700 or 7000 Sequence Detection Systems (Applied Biosystems™, Fresno, CA, USA)).
Newly designed gene specific primers and probes, were created using the Primer Express® 3.0 software. The homology specificity of the designed primers was confirmed *in silico* through BLAST (Basic Local Alignment Search Tool), and experimentally through the performance of dissociation curves of the amplified products, using a serially diluted DNA as reference template. Amplification efficiencies were evaluated using the same dilution series. Reaction specific troubleshoot optimization steps were sporadically performed.

#### *3.5.6.1 Relative gene copy number variation*

Determination of gene copy number variation was performed using specific primers and probes for the gene of interest and an endogenous control gene *tubulin beta chain* (PF10_0084). Analysis of *pfmdr1* gene copy number was analysed as previously described (Price et al., 2004). For *pfmrp1*, new primers and probe (labelled with 6-FAM as reporter dye at the 5'-end, MGBNFQ as the quencher at the 3'-end) were designed (Applied Biosystems™, Fresno, CA, USA) to be performed as a multiplex

using the same endogenous control gene primers and probe employed in *pfmdr1* analysis. *P. falciparum* DNA from 3D7, K1 and FCB laboratory clones were used as calibrators and positive controls (known copy number variation for *pfmdr1* gene) throughout the works of this thesis. Multiplex amplification reactions were done in triplicate in 96 well plates with TaqMan® Gene Expression Mastermix (Applied Biosystems™, Fresno, CA, USA), 300nM of each forward and reverse primer, 100nM of TaqMan® probe from both target and housekeeping gene and approximately 2ng of template. The thermal cycle profile was 50˚C 2min, 95˚C 10min and forty-five cycles of 95˚C 15 s and 60˚C 1 min. The detection threshold was set above the mean baseline value for the first 6–15 cycles. Relative gene copy number were computed according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). This technique was used in the works described in paper II and III.

#### *3.5.6.2 Relative gene transcription level*

The determination of relative gene transcription levels was in-depth performed in paper II. The design was similar as for the analysis of gene copy number, now with the use of reverse transcribed RNA as template and an alternative endogenous control gene, *seryl-tRNA synthetase* gene (PF07_0073). This gene was considered a better choice as compared with the previously mentioned *tubulin beta chain* (PF10_0084), as it is known to display stable (unaltered) levels of transcription throughout the entire intra-erythrocytic cycle. The levels of *pfmdr1*, *pfcrt*, *pfmrp1* and *pfmrp2* transcripts were compared to this endogenous control.

The amplification efficiency estimated for each gene was used as a correction factor. All experimental threshold cycle values ($C_t$) were first transformed to adjust the RNA concentration adding to the $C_t$ value to the $\log_2$ RNA concentration of each sample. Relative gene expression was calculated as the ratio between the transformed $C_t$ values of the target gene and the endogenous control (PF07_0073), taking in account the amplification efficiency for each gene (Stahlberg *et al.*, 2003).

## 3.6 BIOINFORMATICS

Amino acid sequence retrievals, BLAST searches and transcription data were accessed via: PlasmoDB (www.plasmoDB.org); *P. falciparum* Database, the Broad Institute of MIT and Harvard (http://www.broad.mit.edu); *P. falciparum* Blast Server, the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk); NCBI Entrez database (http://www.ncbi.nlm.nih.gov).

In paper II and III, the Sequencher™ software versions 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA) was used to analyze the sequence output with the 3D7 gDNA as reference.

Chromatogram files from the sequenced sequences from the *pfmrp2* gene (paper IV) were analyzed with a different program due to the presence of micro-indels throughout the gene. Analysis were performed by base-call using phred version 0.020425.c (http://phrap.org) (Ewing *et al.*, 1998) and aligned to the *P. falciparum* 3D7 genome sequence (PFL1410c) using the alignment program ssaha2 version 2.5.1 (Ning *et al.*, 2001). A position was called polymorphic if either the position had more than one read to support the nucleotide difference, or if the quality score of this position was 20 or higher. The Tablet program was used to visualize alignments (Milne *et al.*, 2010).

The PfMRP2 structure described in paper IV, was derived from hydropathy plots generated with the HMMTOP algorithm (version 2).

Crystallography data enables structural studies *in silico* or by computational homology. In *P. falciparum* many genes are specific of the parasite, but a fraction of the ORFs is homologous to well studied proteins in prokaryotes, as well in other eukaryotes organisms. In paper III these strategy was used to unveil the importance of PfMRP1 F1390I based in the bacterial homologous Msba ABC transporter crystal 3B60 using HHpred (Homology detection & structure prediction by HMM-HMM comparison server), Modeller software to generate a model and Yasara software for 3D visualization (Krieger *et al.*, 2002).

## 3.7 DATA DEPOSITION

Nucleotide sequence of the ORF of the *pfcrt, pfmdr1, pfmrp1* and *pfmrp2* genes from the laboratory strains W2 and FCB are available in the National Center for

Biotechnology Information (http://www.ncbi.nlm.nih.gov) database with GenBank accession numbers: GU797309, GU797310, GU797311, GU797312, GU797313, GU797314, GU797315, GU797316.

## 3.8 STATISTICS

To statistically evaluate the difference in SNPs prevalence between baseline and recurrent infections in paper I, Fisher's exact two-tailed test was used with statistical significance threshold defined as $P < 0.05$.

The statistical analysis of association between the Thai isolates genotype and their correspondent *in vitro* drug susceptibility in paper III and IV were performed with t-test or Mann-Whitney Rank Sum Test when normality of the data failed, and adjusted for Bonferroni significance. Pearson correlation was used to check significant positive or negative correlation of the *in vitro* antimalarial drug susceptibility using rational numbers.

In paper II, statistical significance of specific disturbance in the intra-erythrocytic cell cycle progression time due to drug exposure was performed with Fisher's exact test. Only time-points between the drug exposed and non-exposed parasites with the same stage proportion (above the described Fisher's test with $P > 0.05$) were considered to the next analysis of determining the gene expression induction effect due to the drug exposure. This time-point gene expression fold differences significance was determined using the t-test. A wave function applied to the number of total parasites ring stage count as well as to the gene expression data throughout all time-points for each clone was formulated to perform a non-linear regression analysis. The calculated fold difference significance was achieved by the use of t-test.

# 4 RESULTS AND SPECIFIC DISCUSSION

## 4.1 PAPER I

**"Clinical evidence for a possible role of PfMRP1 contribution to antifolate resistance"**

The antifolate drug combination SP targets the DHFR and DHPS enzymes, acting as competitive inhibitors of their natural substrates, thereby disturbing endogenous parasite folate biosynthesis. Point mutations in the genes coding for these enzymes have long been known to be the main mechanism of *P. falciparum* resistance against SP, as discussed in the introduction of this thesis. Nevertheless, it has recently become more apparent that other factors may play a role in the levels of clinical failure after SP treatment. Common to all antifolate drugs are folate or folate-component analogues and therefore it is not startling to find that higher serum folate concentrations (for example, as a result of dietary folate supplementation) are significantly associated with SP failure, as has been reported in children and pregnant women (Dzinjalamala et al., 2005, van Eijk et al., 2008). Knowing that the MRPs transport folate, we hypothesized that the PfMRP1 may contribute to *P. falciparum* resistance to SP *in vivo*.

To test this hypothesis *in vivo*, a SP *vs.* artemether-lumefantrine drug efficacy clinical trial conducted in Fukayosi, Tanzania was analysed. The prevalence of *pfmrp1* I876V and K1466R SNPs (previously identified as the most frequent *pfmrp1* polymorphisms in Africa (Dahlstrom et al., 2009)), between baseline infections and recurrent infections was determined, searching for SP-driven SNP selection. The well known quintuple mutation, associated with SP treatment failure (N51I, C59R and S108N from *pfdhfr* and the A437G and K540E from *pfdhps*), was also analysed.

There was a statistically significant selection (P=0.02) of the pure *pfmrp1* K1466 allele among the recrudescences (12/14, 85.7%) compared to baseline (52/101, 51.1%), whereas no significant changes in the frequency of *pfmrp1* I876V SNP were noted. As expected, we detected significant selection of the established molecular marker for SP resistance, the *pfdhfr*/*pfdhps* quintuple haplotype, in recrudescences (P = 0.001) and in

reinfections (P = 0.002). Interestingly, no linkage between *pfmrp1* K1466 and the quintuple mutation was found, suggesting independent selections.

It is unclear why *pfmrp1* K1466R is selected under SP treatment. One potential explanation is that SP drugs are transported out of the cell by PfMRP1 once SP acts in the cytoplasm, and that PfMRP1 is an efflux pump located in the cytoplasm membrane of the parasite. However this is unlikely since these drugs are not organic anions, the typical MRP substrates. Alternatively, as MRPs have been shown to transport folates (Stark *et al.*, 2003), selection of *pfmrp1* K1466R may occur if it affects the MRP mediated efflux of folates out of the cell. A reduction in the MRP mediated transport of folates results in an increased endogenous folate pool, which can compete with antifolate drugs at the enzyme binding sites. This PfMRP1 haplotype probably is less active in acting as a folate efflux mediator, thereby decreasing SP susceptibility in the parasite, and contributing to the treatment failure.
Complementary to these results, PfMRP1 represents the first known protein that can putatively influence levels of resistance to both quinolines and the antifolates, as well as to ACTs (Dahlstrom et al., 2009, Mu et al., 2003, Raj et al., 2009).

## 4.2 PAPER II

**"Parasites exposed to mefloquine have their cell cycle progression delayed and drug transporters gene expression affected"**

Prior to this work, only a few studies had demonstrated that antimalarials could interfere with the cell cycle progression of the parasite (Nakazawa *et al.*, 1995, Nakazawa *et al.*, 2002, Thapar *et al.*, 2005) including a very recent study with CQ (Valderramos *et al.*, 2010). Using MQ as a relevant and convenient ACT antimalarial reference, we focused on monitoring changes in the cell cycle progression rate of *P. falciparum* lab strains with different MQ susceptibilities. In parallel, we analyzed the variation in the abundance of the transcripts encoding the transporter proteins PfCRT, PfMDR1, PfMRP1 and PfMRP2 throughout the cycle upon MQ exposure. It is expected that enhanced transcriptional activity for these transporter genes could influence the drug susceptibility of the parasite.

For this study we chose 3 *P. falciparum* lab strains: one sensitive (W2) and two with decreased sensitivity (3D7 and FCB) to mefloquine. Re-sequencing the aforementioned transporter genes showed two main *pfmdr1* polymorphisms (N86Y and gene CNV), and numerous SNPs differences in the three remaining genes (Table 2 - data deposit in the GenBank).

**Table 2: Haplotype of *pfcrt, pfmdr1, pfmrp1* and *pfmrp2* of 3D7, W2 and FCB strains.**

| Gene | Strain | Amino acid position and single-letter code | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MAL7P1.27 (*pfcrt*)** | | | | | | | | | | | | | | | |
| | | **74** | **75** | **76** | **220** | **271** | **326** | **356** | **371** | | | | | | |
| | **3D7** | M | N | K | A | Q | N | I | R | | | | | | |
| | **W2** | I | E | T | S | E | S | T | I | | | | | | |
| | **FCB** | I | E | T | S | E | S | I | I | | | | | | |
| **PFE1150w (*pfmdr1*)** | | | | | | | | | | | | | | | |
| | | **86** | **659** | | | | | | | | | | | | |
| | **3D7** | N | N | | | | | | | | | | | | |
| | **W2** | Y | ins N | | | | | | | | | | | | |
| | **FCB** | Y | del 3N | | | | | | | | | | | | |
| **PFA0590w (*pfmrp1*)** | | | | | | | | | | | | | | | |
| | | **37** | **191** | **202** | **437** | **876** | **1390** | | | | | | | | |
| | **3D7** | P | H | K | S | I | F | | | | | | | | |
| | **W2** | P | Y | K | A | V | I | | | | | | | | |
| | **FCB** | S | Y | E | A | V | I | | | | | | | | |
| **PFL1410c (*pfmrp2*)** | | | | | | | | | | | | | | | |
| | | **199** | **262** | **593** | **630** | **634** | **646** | **714** | **963** | **964** | **970** | **1184** | **1527** | **1531** | **1745** |
| | **3D7** | L | D | N | D | D | N | K | S | N | D | D | S | L | N |
| | **W2** | V | ins[a] | N | ins[b] | N | D | I | del | D | N | N | T | I | N |
| | **FCB** | V | D | D | ins[b] | D | D | I | S | N | D | N | T | I | - |

[a]: insertion of 7a.a. (DENDQND); [b]: insertion of 25 a.a. (DGYVDDYVDDYVNDYVDDYVNDYVD)

*In vitro* MQ $IC_{50}$ and $IC_{99}$ concentrations were chosen to study the parasites behaviour regarding cell cycle development and gene transcripts. The assays were performed for the three laboratory strains for 48 hours with sample collection at 6 hour intervals from both MQ exposure ($IC_{50}$ and $IC_{99}$) and non exposed parasites (control). Morphological analysis was performed by Giemsa-stained smears for examination of parasite stages. RNA was collected at the same time points.

The examination of the parasite stages by light microscopy revealed a MQ-induced delay in the cell cycle progression of the three tested parasites. The degree of delay was

drug dose and strain dependent. The more arrested parasites corresponded to the least susceptible to MQ.

Plotting the number of parasites that were in early ring stage *vs.* time we fitted a curve with an equation of a wave. This nonlinear regression approach allowed us to measure significant differences in the degree of cell cycle delay induced by MQ.

Considering that each gene has its own expression profile strictly correlated with stage morphology (Bozdech et al., 2003), the MQ induced cell morphology delay was also detectable through the analysis of the transcript accumulation patterns.

Copy number change is commonly reported in response to *in vitro* drug exposure (Chavchich *et al.*, 2010, Wilson et al., 1989). Comparing relative transcript abundance between the strains at the initial experimental time point (0h) we showed that the expression of *pfmdr1* gene in the FCB strain, which harbours 2 copies of *pfmdr1* in the genome, was the highest compared with the other analysed strains.

The delay in parasite stage development by MQ exposure made it difficult to discriminate changes to gene transcription levels due to cell morphology differences from those due to the direct action of MQ on the transcript accumulation.

MQ gene induction could be directly determined by comparing the time-points with the same proportion of the different stages. In this way, we removed the stage-confounding factor with gene expression modulation ranging from 0.6-5.8 fold. However, this type of analysis gave rise to very few analysable points. To complement these results, another approach based on non-linear regression analysis of gene expression over time was conducted for the MQ $IC_{50}$ assays. Curve fitting the gene expression data (in the same manner we used for the morphology curves) we were able to calculate the general drug transporter gene induction by MQ at $IC_{50}$ concentration exposure. The calculated fold difference confirmed that the drug pressure was generally associated with mild but significant changes in the expression of the genes. Similar low induction levels have been observed in the transcriptome after exposure to chloroquine (Gunasekera et al., 2007) as well as to an experimental antifolate compound (Ganesan et al., 2008).

Taken as a whole, this work shows that the standard antimalarial MQ induces a delay in parasitic cell cycle development, while simultaneously influencing the transcription of four transporter genes coding for drug efflux pumps. These observations prompt the discussion that the overall basis of *P. falciparum* drug resistance is a complex multi-factorial phenomenon that extends beyond the usually proposed subjacent mechanisms

of target modification or drug transport. The cell cycle delay upon MQ exposure, in addition to expanding our view of the parasite's capability, raises caution regarding the interpretation of previous *in vitro* findings concerning parasite gene expression under drug pressure which have not taken into account the possible parasitic un-phasing stage at a certain time points.
The work expands our knowledge of the range of *P. falciparum* strategies used to evade antimalarial drugs, with clinical implications.

## 4.3 PAPER III

**"Novel potential molecular markers of *in vitro* drug resistance located in *pfmdr1* and *pfmrp1* genes"**

Southeast Asia is a historical epicenter of emerging *P. falciparum* antimalarial drug resistance. This status has been recently reinforced by local evidence of decreased parasite drug susceptibility to the artemisinins, the core component of presently recommended antimalarial combination therapies (ACTs). These recent events underlie the urgency for understand the molecular basis of drug resistance, particularly those leading to broad range multidrug resistance phenotypes. Alterations in the parasitic membrane proteins PfCRT, PfMDR1, and PfMRP1 are believed to be major contributors to resistance through drug efflux mechanisms and have been previously associated with decreased parasite drug responses *in vivo* and *in vitro*.
In this context, our study aimed to investigate the association of *in vitro* phenotypes of drug susceptibility (ICs) with the complete genotype of the aforementioned transporter genes in a set of Southeast Asian adapted parasites from the Thai-Burma border. For that purpose, we sequenced the full ORF of *pfcrt*, *pfmdr1* and *pfmrp1* from 46 parasite field isolates, while testing for *in vitro* drug susceptibility for the central ACT antimalarials ART, DHA, as well as the partner drugs MQ and LUM.

A relatively large range of *in vitro* $IC_{50}$ and $IC_{90}$ values was observed for the antimalarial drugs tested. Interestingly, Pearson correlation applied between the ICs values of the drugs revealed ($P<0.05$) a cross response within all drugs tested even though some of these drugs are not structurally similar.

ORF sequencing of *pfcrt*, *pfmdr1* and *pfmrp1* revealed previously published, as well as newly described SNPs (*pfmdr1* A750T). From the 7 SNPs found in *pfmdr1*, F1226Y showed to be associated with higher ICs for ART, MQ and LUM. Among the 11 SNPs found in *pfmrp1*, F1390I showed also significant associations with ART, MQ and LUM. This SNP in *pfmrp1* is expected to be localized in transmembrane domain 11, which has been proposed to be part of a substrate pocket in several ABC transporters including PfMDR1. Even though *pfmdr1* F1226Y and *pfmrp1* F1390I alleles haves similar association profiles, they are not linked (P>0.05), suggesting that the actions of the respective proteins are not coordinated at the sub-cellular level. Copy number variation of *pfmdr1* and *pfmrp1* was also analyzed. No *pfmrp1* CNV was detected, while *pfmdr1* CNV was shown to be present in more that 50% of the isolates. This polymorphism was associated with a significant decreased *in vitro* susceptibility to ART, MQ and LUM.

All 46 field isolates carried the same *pfcrt* haplotype (Dd2-like), thereby preventing any associative analyses with the *in vitro* phenotype, and leading to the preliminary conclusion that this gene is not involved in modulating the parasite responses to drugs in this study.

By grouping the different *pfmrp1* F1390I, *pfmdr1* F1226Y and *pfmdr1* CNV haplotypes present in the Thai field isolates, we observe a progressive increase in the $IC_{50}$ of the tested antimalarial drugs. In particular, we observed that the *pfmdr1* CNV is associated with an abrupt leap in MQ $IC_{50}$ values, and showed a less dramatic but still significant effect with ART and LUM, confirming previous findings (Cowman, 1995, Price et al., 1999, Price et al., 2004, Price et al., 2006, Sidhu et al., 2005).

Though Thailand is characterized for low multiplicity of infections, recent studies have shown that the standard detection methods often underestimate the phenomenon, leading to misclassification (Juliano *et al.*, 2010). After the *in vitro* analysis in the laboratory for drug susceptibility, DNA was extracted from all isolates to check for variance and/or selection in the culture adapting procedures. The re-check was performed solely for polymorphisms with significant association with the *in vitro* phenotype (*pfmrp1* F1390I, *pfmdr1* F1226Y and *pfmdr1* CNV). Genotype variance was detected in the three examined polymorphisms. Two isolates lost copies in *pfmdr1* gene during the procedure, changing from 2 copies to 1 copy; a mixed infection in position 1226 and 2 mixed infections in position 1390 were also found. Although this highlights the importance of caution in interpreting *in vitro* data (especially when relating the results to the subjacent field reality of the original infections), it is of note that the

observed changes did not change any of the previously determined statistically significant genotype-phenotype association and associated conclusions.

In conclusion, this work unveiled new candidate molecular markers for drug resistance, particularly important for two of the most central ACTs used worldwide: AL and AS-MQ. The precise mechanistic contribution for decreased drug susceptibility of these mutations remains an open question. Structural analysis performed for polymorphism F1390I in PfMRP1 show it is localized within transmembrane 11, which corresponds to TM16 in human MRP1 that has been proposed to be part of a substrate pocket (Deeley & Cole, 2006). In this context, it is conceivable that the F1390I SNP located in TM11 is changing the specificity of interactions between the PfMRP1 and the antimalarials MQ, LUM and ART. Although we could not perform structural analysis for amino acid 1226 in PfMDR1, it is found at the 3' end of the protein in the same region as three of the mutations previously found to contribute to enhanced MQ and ART sensitivity (S1034C, D1042N and D1246Y) (Sidhu et al., 2005, Reed et al., 2000), emphasizing its involvement in antimalarial susceptibility.
The exploration of clinical drug efficacy trials is being planned to search for confirmatory selection events upon drug exposure, particularly in the context of AL and AS-MQ combination therapies.

## 4.4 PAPER IV

**"Full sequence of the *pfmrp2* ORF unveiled a relatively complex gene, with multiple SNPs and micro-indel regions"**

Transporter proteins belonging to the ATP-binding cassette (ABC) super-family are well known to be involved in drug extrusion and associated with resistance in a large variety of phylogenetically different biological systems. *P. falciparum* PfMRP2 is an ABC transporter protein located in the cytoplasm membrane that has not been well studied and deserves to be explored in relation to drug resistance.

This study is the first comprehensive analysis of *pfmrp2* gene diversity. The *pfmrp2* ORF was sequenced in the aforementioned (paper III) 46 *P. falciparum* field isolates originating from Thailand. This isolates were previously phenotyped (paper III) which

allowed us to directly test potential associations of the polymorphisms found with altered *in vitro* drug susceptibility.

*Pfmrp2* was observed to harbour significant biodiversity with the identification of 21 non-synonymous SNPs, 7 synonymous and 5 micro-indel regions. The frequency of SNPs varied from the presence of a single event (1/46) to all mutated (46/46), compared to the 3D7 reference sequence (www.plasmodb.org).

Secondary structure analysis by HMMTOPv2 software predicted that PfMRP2 consists of 12 transmembrane helices distributed in two transmembrane domains (TMDs), each followed by a nucleotide binding domain (NBD) typically found in short MRPs, confirming previous secondary structure predictions (Kavishe et al., 2009). Several of the identified SNPs are positioned near the regions encoding the transmembrane domains, NBDs and ABC signatures, which are functionally important for control of substrate specificity, ATP binding and hydrolysis (Deeley & Cole, 2006) (Figure 7).

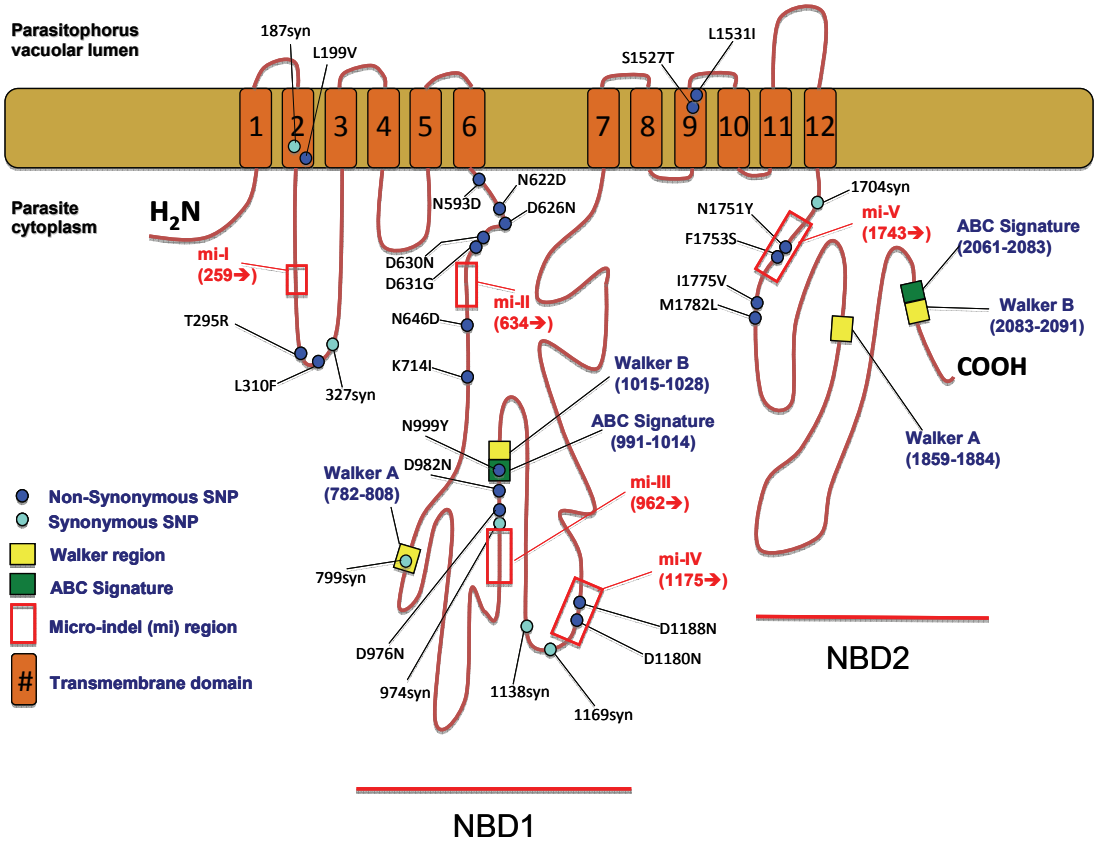


Figure 7 – Predicted structure of PfMRP2 with the approximate localization of the several types of polymorphisms. The numbering of a.a. follows the 3D7 reference genomic data, independently of the observed variable number of micro-indels. The dimensions of the protein are not exact, the representation solely serving the function of giving an overall view of the distribution of variable positions and regions in the primary structure.

Although unusual among *P. falciparum* ABC transporters, a significant number of micro-indels were identified in 5 distinct regions of the gene compared with the 3D7 reference. Two micro-indels were driven by insertions, two by deletions, while one micro-indel region included both insertions and deletions. The size polymorphisms lead to a notable length variation of the total protein in the analyzed field isolates (2088-2125 a.a.). The micro-indel regions were all localized outside of the TMDs. Their exclusion from such protein regions is probably related to the inability of the TMDs to accommodate this type of polymorphism, as they most likely alter the intra-membrane alpha-helix structure leading to functionally unacceptable distortions in the tertiary structure of the protein.

Since the isolates were previously characterized for *in vitro* susceptibility to ART, DHA, MQ and LUM (paper III) we also aimed to performed associative analyses with the *pfmrp2* genotype. Unfortunately, further association analysis was not possible due to the reduced sample size compared with the available degree of sequence diversity. The excluded polymorphisms were: polymorphisms with low frequency (<5 isolates), non-synonymous SNPs and SNPs found in the micro-indel regions (due to their variable presence depending on the indel size). In total only 3 SNPs (N622D, D631G and N646D) could be analyzed plus the 5 micro-indels sites, where no significant phenotype/polymorphism associations were detected. However, this lack of association might reflect solely the limitations on the power of sample study.

Conceptually, the localization of PfMRP2 in the plasma membrane suggests this ABC transporter is of potential relevance in the efflux of xenobiotics from the parasite cytoplasm, as previously reported for the structurally related PfMRP1 (Raj et al., 2009). The fact that *pfmrp1* and *pfmrp2* have essentially opposite transcriptional patterns throughout the *P. falciparum* IDC (as described in the study of paper II), so that each protein is expressed during different stages, suggests a complementary action that might also be reflected in the complex phenotypes of drug response. The large diversity found raises the hypothesis of the existence of PfMRP2 natural variants with variable transporting capacities, warranting further investigation in the context of drug resistance.

# 5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Drug resistance mechanisms in *P. falciparum* are turning out to be more complex than perhaps originally foreseen. The field has been dominated by the concept of a direct casual effect between the presence of specific mutations and the development of resistance phenotypes. This view had been supported by the particular case of resistance against chloroquine, since the CQR phenotype has been explained by the presence of one SNP. However, this is likely the result of decades of heavy selection through the long use of a declining drug.

Identification of relevant genes and mutations has been a key aspect of molecular analysis of drug resistance. Their usefulness in predicting the efficacy of different drugs in different regions has been evaluated and validated by comprehensive meta-analysis of the numerous studies into associations between clinical outcome and molecular markers. In accordance, in Paper I, we have observed a significant selection of the *pfdhfr/pfdhps* quintuple mutant haplotype after SP treatment in recurrent infections, and an association at baseline of this *in vivo* marker with subsequent treatment failure. Additionally, in this study we describe a mutation in a cytoplasm membrane efflux transporter gene (*pfmrp1* K1466R) that was selected for after SP treatment, demonstrating the likelihood of SP resistance involving other factors beyond the well known target genes. These results highlight the complexity of drug resistance mechanisms and the adaptability of efflux transporter genes.

Efflux transporters are long recognized players in the emergence and dissemination of resistant pathogens, and in the acquisition of additional mechanisms of drug resistance, mostly through decreased intracellular drug concentrations. *In vitro* expression of *P. falciparum* transporter genes may be a powerful approach for molecular characterization of substrate specificity, transport mechanism, influence of polymorphisms and drug cell-cycle stage specificity. Paper II shed some light on the complexity of this approach, demonstrating that confounding factors like the delay in parasite cell cycle development after drug exposure makes the comparison of transporter transcripts even more difficult to evaluate. We have documented a dose dependent delay of the parasite cell cycle after MQ exposure, particularly among the less sensitive *P. falciparum* strains. A delay in cell cycle progression could potentially

lead to a dormant stage with decreased parasite metabolic activity, thereby reducing the availability of the drug target, a mechanism known to exist in cancer cells (Roninson, 2003, Varna *et al.*, 2009). The existence of this phenomenon once more emphasizes the difficulty in understanding the molecular mechanisms that lead to drug resistance.

Through the search for drug resistance molecular markers in paper III and IV, we identified two SNPs associated with decreased drug susceptibility to ART, MQ and LUM, and reinforced the importance of *pfmdr1* copy number as a central factor in the response of *P. falciparum* to drugs of different structures. Considering the large diversity of polymorphisms detected in *pfmrp2*, the lack of association with the tested *in vitro* drugs might solely reflect the limitations of the power of the study. In Paper II, we found that *pfmrp2* had higher gene expression differences between sensitive and less susceptible strains when the parasites were exposed to MQ. The fact that *pfmrp1* and *pfmrp2* have essentially opposite transcriptional patterns throughout the IDC, so that each protein covers a different period, suggests complementary action between the two and warrants further investigation of the role of PfMRP2 in parasite drug resistance.

A thorough understanding of the complex interactions among antimalarial transport proteins, how these interactions influence parasite response to antimalarial drugs and the dynamics of antimalarial influx/ efflux is of extreme importance for improved and rationalized drug policies.

The fact that *P. falciparum* is an intracellular parasite makes its survival to antimalarial drugs a result of concerted complex events. PfMRPs, efflux transporters located in the cytoplasm of the parasite, expel substrates into the cytoplasm of the hosting RBC, which may perturb host cell integrity. Therefore, a balance between vacuolar accumulation and export of substances to the RBC cytoplasm may be vital for the parasite's survival. Since the parasite and its host exist essentially as one entity, the function of PfMRPs are far more complicated compared to MRPs of other organisms, and could be a reason why PfMRPs have not found to be major determinants of antimalarial resistance.

PfMRP1 and PfMRP2 potentially contribute to drug resistance in concert with the extensively studied PfMDR1 and PfCRT transporters located in the food vacuole membrane. The four transporters studied here encompass a broad spectrum of substrates enabling us to draw general mechanisms for antimalarial flux in *P. falciparum*, partly extending from previous findings (Figure 8).

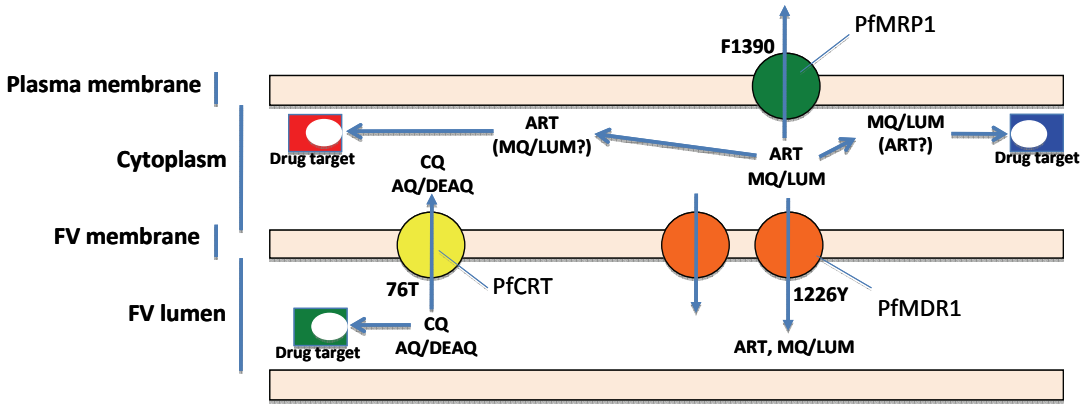


Figure 8 – Proposed physiological model for antimalarial transport in *P. falciparum*. The central assumption is that ART, LUM and MQ drugs have their main pharmacological targets located in the cytoplasm compartment. While PfMRPs located in the plasma membrane, pumps them out of the cell, PfMDR1 an importer inserted in the FV membrane, would contribute to further drug expulsion from the cytoplasm by transporting these drugs towards the lumen of the FV. Wildtype SNPs and copy number amplification of *pfmdr1* are associated with decreased susceptibility to these drugs conversely to aminoquinolines (CQ and AQ/DEAQ) which is believed to have their target inside the FV. CQ resistance is primarily determined by PfCRT, an exporter in the FV membrane. A threonine at position 76 in PfCRT is thought to have better transport capacities than the wildtype. This will decrease the susceptibility to CQ and AQ while increasing the susceptibility to MQ and LUM.

The studies described in this thesis were focused on the parasitic response to different antimalarials with the intention of contributing to our basic knowledge of antimalarial selection and resistance.

Unfortunately, the emergence of parasites that are clinically resistant to therapeutic drugs, specifically with respect to the currently used artemisinin and ACT, is inevitable. Combining different drugs, old and new, will help conserve the efficacy of these valuable antimalarials. One approach will be to combine drugs that target the same molecule, but which would independently select for mutually incompatible combinations of mutations. The ability to choose the right drug combinations will ultimately require the study of presently known and continually emerging resistance mechanisms.

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