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Role of PfATP6 and *pf*MRP1 in *Plasmodium falciparum* resistance to antimalarial drugs

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COVER ILLUSTRATION

Depicts the Wheel of Fortune and the *P. falciparum* life cycle representing the role of malaria in the world.

The Wheel of Fortune (Rota Fortunae) is a concept in medieval and ancient philosophy referring to the capricious nature of Fate. The wheel belongs to the goddess Fortuna, who spins it at random, resulting in that some suffer great misfortune, while others gain windfall.

There are other factors than Fate causing malaria, as will be described in this thesis. However, as Fortuna, malaria is unrelentlessly ruling the lives of millions of people. We need to increase our efforts to fight malaria, hopefully resulting in that we gain control over this devastating disease.

ILLUSTRATIONS
Cover: Illustration by Sabina Dahlström and Leopold Roos
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Till Mamma och till minne av Pappa

ABSTRACT

Half of the world's population live at risk for malaria and nearly one million people die from the disease every year. The malaria burden is greatest in children and pregnant women in sub-Saharan Africa. As effective treatment is crucial for malaria control, the spread of antimalarial drug resistance has contributed significantly to malaria attributed morbidity and mortality. The current cornerstones in malaria treatment are artemisinin-based combination therapy (ACT) for treatment of uncomplicated *Plasmodium falciparum* malaria and sulfadoxine-pyrimethamine (SP) for intermittent preventive treatment of pregnant women. While resistance to SP is already established, recent advances have provided the first evidence of decreased susceptibility or resistance to the ACT components; artemisinins (ART) and the key ACT partner drugs lumefantrine, amodiaquine and mefloquine. Development of resistance to ACTs and aggravation of SP resistance could be devastating, as there are presently no further well established treatment options. This stresses the importance to understand the molecular mechanism of resistance in order to potentially prevent its emergence and spread.

The sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *P. falciparum* (PfATP6) has been suggested to be a target for ART. Multidrug resistance proteins (MRPs) are known to be related with multidrug resistance in many organisms. The *P. falciparum* Multidrug Resistance Protein 1 (*pf*MRP1) has been suggested to have a role in the parasite response to several antimalarial drugs through drug efflux

The aim of this thesis is to understand the role of PfATP6 and *pf*MRP1 in decreased susceptibility/resistance to ACTs and SP *in vivo* and *in vitro*. In two comprehensive studies the global biodiversity of *PfATP6* and *pfmrp1* was determined, resulting in the identification of a large number of SNPs suggesting that both genes harbor significant diversity. The contribution of the identified *pfmrp1* polymorphisms in the parasite drug response *in vivo* was studied in *P. falciparum* infected patients from clinical drug trials. We observed a selection of parasites harboring *pfmrp1* I876 in patients with recurrent infections after treatment with artemether-lumefantrine and of *pfmrp1* K1466 in recrudescent infections after SP treatment, providing the first indications ever that *pf*MRP1 may have a role in *P. falciparum* drug response *in vivo*. *In vitro* associations between *pfmrp1* SNPs and decreased susceptibility to a large number of structurally unrelated antimalarial drugs, including artemisinin, lumefantrine, amodiaquine and mefloquine, were observed in *P. falciparum* fresh isolates. However, we could not detect any association with *PfATP6* SNPs.

In conclusion, in this work no further evidence was found supporting the hypothesis of PfATP6 as a target of ART. Nevertheless it was demonstrated that *PfATP6* harbors considerable sequence biodiversity which can be the basis for following studies investigating the association of *PfATP6* polymorphisms with artemisinin resistant or tolerant phenotypes, as they emerge. The association of *pfmrp1* polymorphism with *P. falciparum* response to a number of structurally unrelated drugs suggests that *pf*MRP1 may be a true multidrug resistance factor. Potential *pf*MRP1-based cross-resistance between ART and the partner drugs may have implications for development of resistance to ACTs.

LIST OF PUBLICATIONS

I. **Sabina Dahlström**, Maria I. Veiga, Pedro Ferreira, Andreas Mårtensson, Akira Kaneko, Björn Andersson, Anders Björkman, J. Pedro Gil. Diversity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *Plasmodium falciparum* (PfATP6).
Infection, Genetics and Evolution, 2008, 8, 340-5.

II. **Sabina Dahlström***, Pedro E. Ferreira*, M. Isabel Veiga, Nazli Sedighi, Lisa Wiklund, Andreas Mårtensson, Anna Färnert, Christin Sisowath, Lyda Osório, Hamid Darban, Björn Andersson, Akira Kaneko, Gwenaëlle Conseil, Anders Björkman, J. Pedro Gil. *Plasmodium falciparum* Multidrug Resistance Protein 1 (*pf*MRP1) and artemisinin-based combination therapy in Africa.
Accepted in the Journal of Infectious Diseases.

III. **Sabina Dahlström***, M. Isabel Veiga*, Andreas Mårtensson, Anders Björkman, J. Pedro Gil. Polymorphism in *pf*MRP1 (*Plasmodium falciparum* multidrug resistance protein 1) amino acid 1466 associated with resistance to sulfadoxine-pyrimethamine treatment.
Antimicrobial Agents and Chemotherapy, published ahead of print on 13 April 2009, doi:10.1128/AAC.00091-09

IV. Christin Sisowath*, **Sabina Dahlström***, Walther Wernsdorfer, J. Pedro Gil. Association between *pfmdr1*, *pfcrt*, *pfmrp1* and *PfATP6* mutations and in vitro susceptibility of fresh *Plasmodium falciparum* isolates to antimalarial drugs.
Manuscript.

* Shared first authorship.

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACT	artemisinin-based combination therapy
ART	artemisinins
ATP	adenosine triphosphate
CFTR	cystic fibrosis transmembrane conductance regulator
DBB	desbutyl-lumefantrine
DEAQ	desethyl-amodiaquine
DHA	dihydroartemisinin
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
EC	effective concentration
ER	endoplasmic reticulum
gDNA	genomic DNA
GSH	reduced glutathione
GSSG	oxidized glutathione
HBMF	home based management of fever
IC	inhibitory concentration
IDP	internally displaced person
IPTi	intermittent preventive treatment in infacyfor infants
IPTp	intermittent preventive treatment in pregnancy
IRS	indoor residual spraying
ITN	insecticide-treated net
LLIN	long lasting insecticide-treated net
MDA	multiple displacement amplification
MRP	multidrug resistance protein
MSD	membrane spanning domain
NBD	nucleotide binding domain
ORF	open reading frame
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PfATP6	the sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *Plasmodium P. falciparum*
PfATP6	*the sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of Plasmodium P. falciparum coding gene*
PfCRT	*P. falciparum* chloroquine resistance transporter
pfcrt	*P. falciparum chloroquine resistance transporter coding gene*
pfdhfr	*P. falciparum dihydrofolate reductase coding gene*
pfdhps	*P. falciparum dihydropteroate synthase coding gene*
pfmdr1	*P. falciparum multidrug resistance protein 1 coding gene*
PfMDR1	*P. falciparum* multidrug resistance protein 1

pfmrp1	*Plasmodium falciparum Multidrug Resistance Protein 1 coding gene*
*pf*MRP1	*Plasmodium falciparum* Multidrug Resistance Protein 1
pfmsp1	*Plasmodium falciparum* merozoite surface protein 1 coding gene
pfmsp2	*Plasmodium falciparum* merozoite surface protein 2 coding gene
PNG	Papua New Guinea
PPi	pyrophosphate
RBC	red blood cell
RT-PCR	real time-polymerase chain reaction
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SNP	single nucleotide polymorphism
SP	sulfadoxine-pyrimethamine
SUR	sulfonylurea receptor
TM	transmembrane domain
TSH	trypanothione
WGA	whole genome amplification
π	nucleotide diversity

1 INTRODUCTION

1.1 THE MALARIA PARASITE AND VECTOR

Malaria is a deadly disease threatening half of the global population. It is caused by protozoan parasites of the phylum Apicomplexa and the genus *Plasmodium*. There are more than 100 *Plasmodium* species that can infect mammals, birds and reptiles. Five *Plasmodium* species can infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. *P. knowlesi*, whose natural host is macaque monkeys, was recently suggested to be the fifth species that can infect humans (White, 2008). It has been shown to be a major cause of malaria in Malaysia (Cox-Singh *et al*., 2008). *P. falciparum* and *P. vivax* are the most common human species, while *P. falciparum* causes most severe disease and death. This thesis will focus on *P. falciparum*.

Human malaria is transmitted by the female mosquitoes of the *Anopheles* genus. Mosquitoes of the *Anopheles gambiae* complex are the main vector in sub-Saharan Africa, due to its adaptation to human settings. Other important vectors include the *A. funestus* complex in Africa and its close relative, the *A. minimus* complex, in South East Asia.

1.2 GLOBAL BURDEN OF MALARIA

It has been estimated that malaria transmission occurs in 109 countries putting 3.3 billion people at risk (figure 1). In 2006 there was an estimated 247 million malaria cases, resulting in nearly 900 000 deaths. This makes *P. falciparum* one of the leading causes of death worldwide, from a single infectious agent. The malaria burden is greatest in African children as 90% of the deaths occur in sub-Saharan Africa and 85% of the mortality affects children under five years. Although the number of deaths is similar to that of 2004, amelioration of the malaria situation has been achieved in seven African regions, where malaria control measures have resulted in 50% or more reduction in malaria incidence and mortality since 2000, and further 22 countries in the world have reached similar effects (WHO, 2008c). Morbidity and mortality are not the only consequences of malaria infection. The disease is estimated to be responsible for an average annual reduction of 1.3% in economic growth for countries with the heaviest malaria burden (Sachs & Malaney, 2002).

The great variation in malaria burden between different geographical regions can be driven by several factors. Infection with malaria is the result of a complex interplay

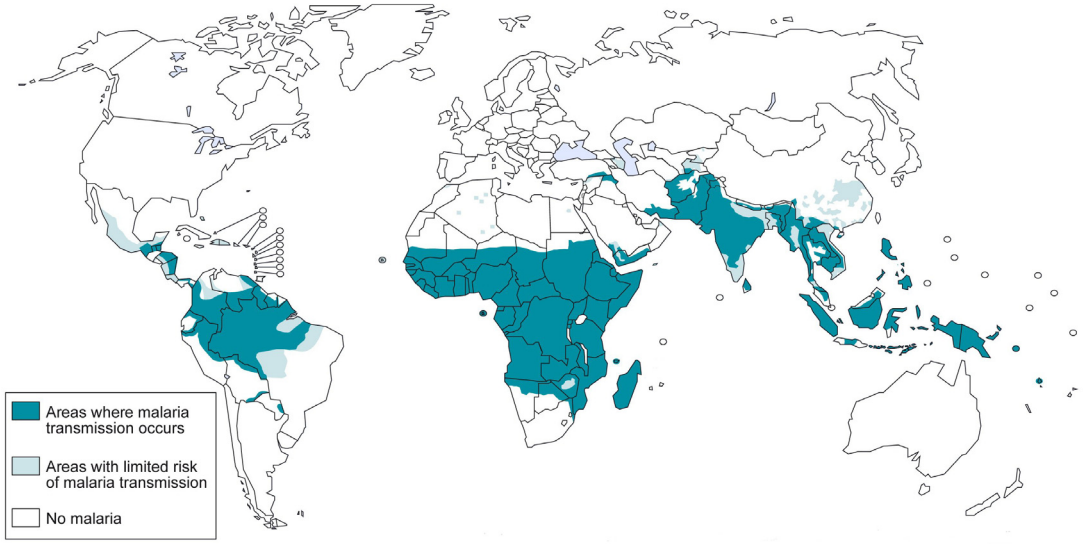

Figure 1 World wide malaria transmission 2008. Printed with permission from WHO. (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_ITHRiskMap.JPG)

between Man, parasite and the mosquito vector. The dynamics between these three elements is an important factor that affects malaria transmission (WHO, 2005b). Both the parasite and the main vector *A. gambiae* are sensitive to low temperature which can provide an explanation for the concentration of malaria in tropical and subtropical regions. Another key factor driving malaria transmission is the differences in levels of social and economical development. Poverty is concentrated in the tropical and subtropical zones where malaria transmission occurs (Sachs & Malaney, 2002). Socioeconomic factors include general poverty, quality of housing and access to health care and health education, as well as the existence of active malaria control programmes (WHO, 2005b). Furthermore malaria causes poverty by reducing the economical growth for countries with high malaria burden, resulting in a vicious circle. On the other hand, the trend could be reversed into a virtuous cycle, following the suggestion that reducing malaria transmission may be one of the most effective means to stimulate economic development in malaria endemic countries (Sachs & Malaney, 2002).

Malaria transmission can either be endemic or epidemic. An epidemic can be defined as an outbreak in a region where transmission normally does not occur or a sharp increase in malaria cases in a region with otherwise lower transmission. Endemic transmission can be either stable, continuous from year to year, or unstable, varying between years.

The intensity of malaria transmission affects the magnitude and nature of the malaria burden. Areas of stable transmission have many malaria cases . The two groups at

highest risk are children that have not yet acquired natural immunity and pregnant women whose immunity is compromised. In areas of unstable malaria there is less clinical immunity and malaria cases occur in all age groups (WHO, 2005b).

1.3 THE LIFE CYCLE OF *P. FALCIPARUM*

The life cycle of *P. falciparum* is extremely complex and requires that the parasite to go through numerous, highly specialized stages in both the human host and in the *Anopheles* vector, both intra- and extracellularly (figure 2). When a *P. falciparum* infected female *Anopheles* mosquito takes a blood meal, sporozoites are injected into the human host along with the anticoagulative saliva of the mosquito.

Sporozoites are entering the bloodstream from the site of injection and move to the liver where they invade hepatocytes. The sporozoites remain in the liver for 1-2 weeks and undergo asexual replication (tissue schizogony) in which each sporozoite can give rise to tens of thousands of merozoites.

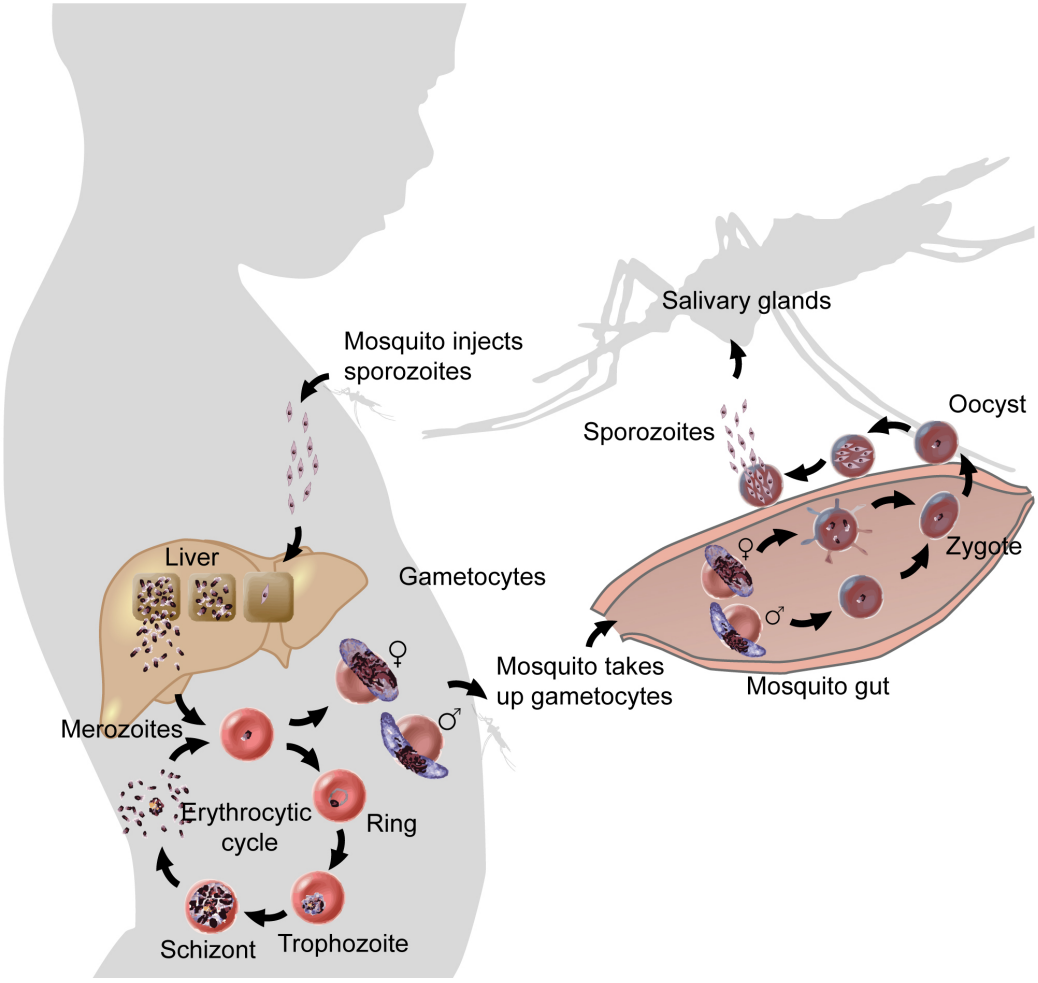

Figure 2 Life cycle of *P. falciparum*. Printed with kind permission from Dr. Christin Sisowath.

When the hepatocytes rupture, merozoites are released into the blood stream and can readily invade erythrocytes. Once inside the erythrocyte, asexual replication begins and the parasite develops through a series of specific stages inside the erythrocyte (erythrocytic schizogony). The parasite matures from merozoite to early trophozoite (ring stage), to the enlarged late trophozoite containing hemozoin pigmentation, to schizont containing around 20 merozoites. The infected erythrocyte is lysed and merozoites are released into the bloodstream, where they can infect further erythrocytes to continue the asexual replication. The intra-erythrocytic cycle of *P. falciparum* takes approximately 48 hours. Erythrocytes infected by mature stages can undergo sequestration, i.e. adhering to endothelial cells in deep blood vessels, to avoid clearance by the spleen. Therefore, only the earlier parasite stages can be seen in peripheral blood and not schizonts. A small proportion of the merozoites in the erythrocytes eventually differentiate to produce micro- (male) and macrogametocytes (female). These are the forms that enable the transmission of parasites to other hosts via the vector. Gametocytes can be ingested by *Anopheles* mosquitoes biting an infected individual, and form micro- and macrogametes in the midgut of the mosquito. The gametes undergo fertilization and form a zygote that is transformed into an ookinete that penetrates the wall of the midgut and develops into an oocyst. Sexual replication (sporogony) occurs within the oocyst and the produced sporozoites migrate to the salivary glands. This process takes 10-18 days and the mosquito is infective for 1-2 months afterwards. The cycle continues as the mosquito takes another blood meal.

1.4 THE CLINICAL DISEASE

Uncomplicated malaria can be defined as the absence of symptoms of severe malaria. Symptoms of uncomplicated malaria are non-specific (box 1) and difficult to distinguish from other febrile illnesses. If uncomplicated *P. falciparum* malaria is treated promptly with effective drugs, no more than 0.1% of the infections result in fatalities. However if uncomplicated infections are not treated they may result in severe malaria (box 2).

Box 1. Symptoms of uncomplicated malaria

Fever
Headache
Fatigue
Abdominal discomfort
Muscle and joint aches
Chills
Perspiration
Anorexia
Vomiting

Box 2. Symptoms of severe malaria

Cerebral malaria
Generalized convulsions
Severe anaemia
Hypoglycaemia
Metabolic acidosis with respiratory distress
Fluid and electrolyte disturbances
Acute renal failure
Acute pulmonary oedema
Circulatory collapse, shock
Abnormal bleeding
Jaundice
Haemoglobinuria
High fever
Hyperparasitaemia

Even if treated, mortality in patients with severe malaria is 15-20%. If untreated, severe malaria is almost always fatal (WHO, 2000, WHO, 2006).

There is a wide range of clinical manifestations of malaria mainly due to the level of immunity in the infected individual which is strongly correlated with the transmission pattern in the area. Naïve individuals that are infected with malaria will become ill. In areas of stable malaria transmission, such as many regions of sub-Saharan Africa, clinical immunity to malaria can be acquired. Infants in these areas seem to be protected from the disease, probably by antibodies from their mother. Severe malaria seems to affect mainly the very young children and it has been suggested that protection is acquired after only 1-2 severe infections (Gupta *et al.*, 1999). Clinical immunity to symptomatic infection with maintained high parasitaemias is built up during childhood. Hence mostly children are suffering from uncomplicated malaria infection. During adolescence antiparasite immunity is finally acquired, reducing the parasite burdens within individuals and on a population level (Schofield & Mueller, 2006). Women become susceptible to malaria infection upon pregnancy due to compromised acquired immunity (Doolan *et al.*, 2009). Immunity to malaria is referred to as premunition, which means that exposure to the parasite is necessary to maintain immunity and that immunity can be lost if exposure is interrupted (Schofield & Mueller, 2006). Therefore immunity in areas of low transmission is difficult to build up and both children and adults may suffer from clinical malaria disease.

1.5 MALARIA CONTROL

After the Second World War, following the discoveries of DDT and chloroquine, WHO launched a global programme for malaria eradication. Although elimination of malaria was achieved in some areas, the programme generally did not succeed as intended, partly due to development of resistance against DDT by the vector and chloroquine by the parasite. Subsequently, efforts were reoriented from malaria eradication and elimination to control. Malaria control can be defined as reducing the disease burden to a level at which it is no longer a public health problem (WHO, 2008a). The main current tools in malaria control are antimalarial drugs, including artemisinin-based combination therapy (ACT) and intermittent preventive treatment (IPT), and vector control measures, including insecticide-treated nets (ITN) and indoor residual spraying with insecticides (IRS).

1.5.1 ACT

Malaria is a preventable and curable disease. The requirement for effective malaria control therefore includes prompt access to affordable and efficacious antimalarial drugs. ACT is used for treatment of uncomplicated *P. falciparum* malaria with the

objective to cure the infection and prevent progression to severe disease. (For definition and rationale for ACT, see section 1.6). Deployment of ACT on Zanzibar resulted in dramatically reduced malaria attributed morbidity and mortality within two years (Bhattarai *et al.*, 2007). In 2008, all countries worldwide, except four, had adopted ACT as the first-line treatment for uncomplicated *P. falciparum* malaria However, access to treatment is inadequate in most countries (WHO, 2008c).

1.5.2 IPT

In Africa intermittent preventive treatment in pregnancy (IPTp) is recommended since pregnant women are especially vulnerable to malaria due to infection of the placenta. In IPTp full treatment doses are administered at least twice during the pregnancy as prophylaxis. Drugs suitable for preventive use must have a long half-life and a very good safety profile, as they are given to healthy subjects, and the only current option fulfilling these criteria is sulfadoxine-pyrimethamine (SP) (WHO, 2008a). It has been demonstrated that the use of IPTp reduces the incidence of maternal anaemia and improve infant birth weight (ter Kuile *et al.*, 2007), which possibly leads to improved child survival (Menendez *et al.*, 2007). At the end of 2006 a majority of the countries in Africa had adopted IPTp as national policy (WHO, 2008c). The benefits with intermittent preventive treatment for infants (IPTi) are also being investigated.

1.5.3 ITNs

Untreated bed nets form a protective barrier around persons using them. However, application of an insecticide greatly enhances the protective efficacy of bed nets. If there is high ITN coverage in a community, the numbers and longevity of mosquitoes will be reduced, resulting in protection also of individuals that do not sleep under ITNs. Studies have demonstrated that the use of ITNs can reduce child mortality by one fifth and malaria incidence by half (Lengeler, 2004). Nearly all African countries have adopted the policy of providing ITNs free of charge to children and pregnant women, but the coverage is still not adequate in most countries (WHO, 2008c). Insecticide resistance is a threat to the sustainability of ITNs, since most nets are impregnated with pyrethroids, to which vectors are already resistant in some areas of the world (WHO, 2008a). More recently, long-lasting insecticide-treated nets (LLINs) that retain effective concentrations of insecticide for at least three years have been developed.

1.5.4 IRS

IRS is the application of long-acting insecticides on the walls and roofs inside houses to kill the mosquito that land on these surfaces. A number of insecticides are used for IRS, including DDT, and some can also repel mosquitoes and prevent that they enter in a sprayed room. IRS has been shown to reduce malaria incidence and malaria

transmission (Mabaso *et al.*, 2004). The use of IRS has declined and is now generally used in *foci* of high malaria transmission (WHO, 2008c). Both ITN and IRS are most effective against mosquitoes that bite and rest indoors, such as *A. gambiae* and *A. funestus*.

1.5.5 Impact and consequences for malaria control

The impact of the described malaria control tools can be illustrated by the success in specific regions were the application of interventions has been strengthened. In Eritrea, Rwanda, Sao Tomé and Principe, and Zanzibar the malaria burden has been reduced by 50% or more between 2000 and 2007 (WHO, 2008c). The recent advancements in malaria control and availability of new tools, combined with the improvement of social and economic standards of people living in endemic areas have inspired the governments of malaria-endemic countries and major international donors to aim for a new goal: elimination of malaria. Malaria elimination is defined as interruption of malaria transmission in a defined geographical area. This goal may be feasible for an increasing number of countries where malaria is already under control (Greenwood, 2009).

1.6 ANTIMALARIAL DRUGS

Uncomplicated *P. falciparum* malaria is predominately treated with ACT that is now adopted as first-line treatment in almost all countries worldwide. Artemether-lumefantrine and artesunate-amodiaquine are the most used ACTs in Africa, while artemether-lumefantrine and artesunate-mefloquine are the leading ACTs in Asia (WHO, 2008b). Quinine is the most used drug for treatment of severe malaria. Recently, however, the artemisinin derivates artesunate and artemether represents valid alternatives (Pasvol, 2005). SP is the main present option for IPTp for pregnant women. Another preventive measure is chemoprophylaxis for travellers to malaria-endemic countries. The recommended choices for chemoprophylaxis include chloroquine, chloroquine-proguanil, atovaquone-proguanil, mefloquine and doxocyclin. The focus of this thesis lies on the key antimalarial drugs, ACTs and SP, which will therefore be presented in detail.

1.6.1 SP

SP started to be used due to the widespread resistance to chloroquine. The drug is easy to handle since only one single oral dose is needed for treatment and it is inexpensive. Resistance to SP developed rapidly in Southeast Asia, South America and several areas of Africa (Kublin *et al.*, 2002, Nzila *et al.*, 2000a, Omar *et al.*, 2001, Wongsrichanalai *et al.*, 2002). Due to the rapid development of resistance, SP is now recommended to be reserved for IPT in Africa.

SP acts by interfering with two enzymes in the biosynthesis of folate. Sulfadoxine is an analogue to p-aminobenzoic acid and competitively inhibits dihydropteroate synthase (DHPS). Pyrimethamine is a competitive inhibitor of dihydrofolate reductase (DHFR). The inhibition of these two key enzymes affects the synthesis of tetrahydrofolate, which is needed in the production of dTTPs and the amino acids methionine and glycine (Sibley *et al.*, 2001). Consequently the parasite is killed because of impaired synthesis of DNA and amino acids.

1.6.2 ACT

Development of resistance to the commonly used antimalarial drugs, chloroquine and SP, emphasized the need of new effective treatments. Combination therapy had been used successfully in the treatment of cancer, tuberculosis and HIV prior to being introduced in malaria. The beneficial effects of combination therapy include potential synergy between the drugs and delay in emergence of resistance. Resistance to two antimalarial drugs with different mechanisms of action rarely occurs since the risk of resistance to develop simultaneously would be the product of the mutation rates of the genes associated with resistance to each drug, multiplied by the number of parasites in the infection (White, 1999).

ACT is a combination of a rapidly eliminated artemisinin derivative and a slowly eliminated partner drug, typically an amino-quinoline. Artemisinin and its derivatives artesunate, artemether and dihydroartemisinin (DHA) will in this thesis be referred to as ART (ART). ART are very active drugs that can reduce the parasite biomass up to 10 000-folds per asexual cycle, which is much faster than any other antimalarial drug. Furthermore they are gametocytocidal, which can result in reduced malaria transmission (White, 1999). Because of their activity and their short elimination half-life, ART are less prone to development of resistance. In ACT, ART rapidly reduces the parasite biomass leaving relatively few parasites for the partner drug to eliminate. The residual parasites are meeting a higher concentration of the partner drug than if it would have been used in monotherapy. As a result parasites which are also partially resistant to the partner drug may be cleared. The partner drug, in turn, may protect ART against resistance by clearing the remaining parasites (figure 3) (White, 1999). Hence, ACT may sometimes improve treatment efficacy in an area where resistance to the partner drug has already developed. An example of the application of this concept is the introduction of artesunate-mefloquine to counteract the problem of mefloquine resistance in Thailand border. It resulted in reduced incidence of *P. falciparum* malaria and halted progression of mefloquine resistance (Nosten *et al.*, 2000). The delay in development of resistance to the partner drugs can be expected to be greater in low

transmission areas than in high transmission areas, where there are more reinfections that can be selected by the partner drug when ART is eliminated.

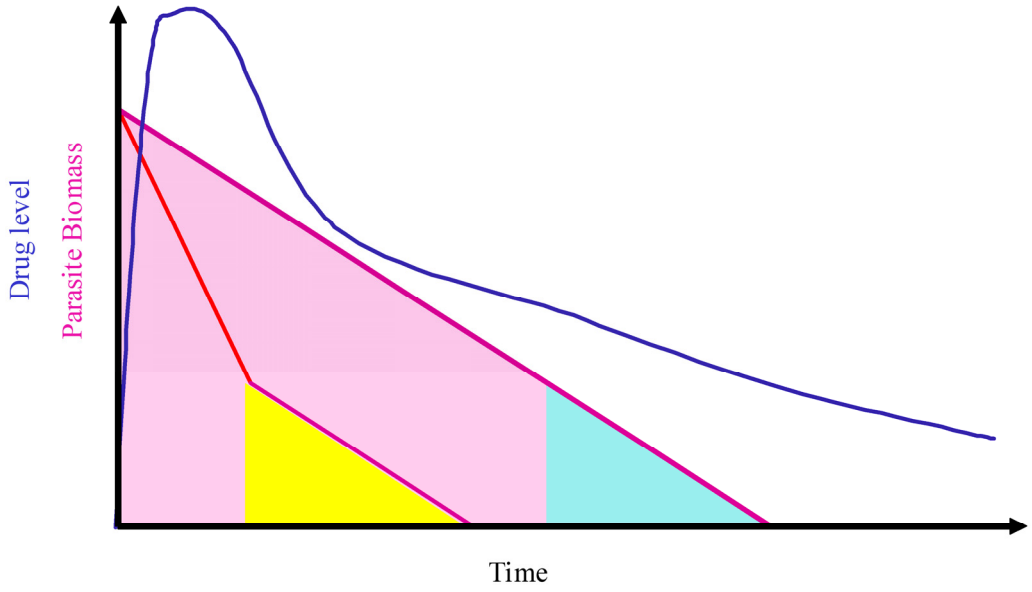

Figure 3. The principle of ACT. The large purple triangle represents the parasite biomass when exposed to a partner drug (blue line) in monotherapy. In ACT, ART rapidly reduces the parasite biomass (red line) and only a small number of residual parasites (yellow triangle) will be exposed to the partner drug. These parasites meet a much higher concentration of the partner drug than the same parasite biomass exposed to the partner drug in monotherapy (turquoise triangle). Adapted from (White, 1999).

1.7 RESISTANCE TO ANTIMALARIAL DRUGS

Resistance to antimalarial drugs can be 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 active form of the drug must be able to gain access to the parasite or the infected red blood cell (RBC) for the duration of time necessary for its normal action (WHO, 1986). Hence, antimalarial drug resistance is not strictly defined and many factors can change the perception of resistance.

Two separate events are required for drug resistance to develop; *de novo* mutations and drug selection pressure. The frequency of mutations in parasite populations that are not exposed to drugs are low, since mutations occur rarely and populations with mutations that affect parasite fitness may not survive or manage to replicate. One or several mutations may be required for reduced drug susceptibility. When the initial mutation has occurred, the parasite may be subject to drug-derived selection pressure. Parasite populations can be selected either when they cause a primary infection and survive the treatment (recrudescences) or as newly infecting parasites which are infecting shortly

after a treatment (reinfections). Selection of reinfecting parasites occur when a resistant/less susceptible parasite can infect a patient that has been undergoing treatment and survive. Hence, it has the opportunity to be transmitted, while a sensitive parasite would be cleared. The selective pressure mainly derives from sub-therapeutic levels of a slowly eliminated drug within this individual. Since reinfecting parasites often meet a lower drug level than recrudescences, also low grade resistant (tolerant) parasites may be selected. Resistance can spread as the selected recrudescences and reinfections are better transmitted to other hosts. The factors affecting the emergence and spread of resistance to antimalarial drugs can be numerous (figure 4) and the importance of each factor is not fully understood.

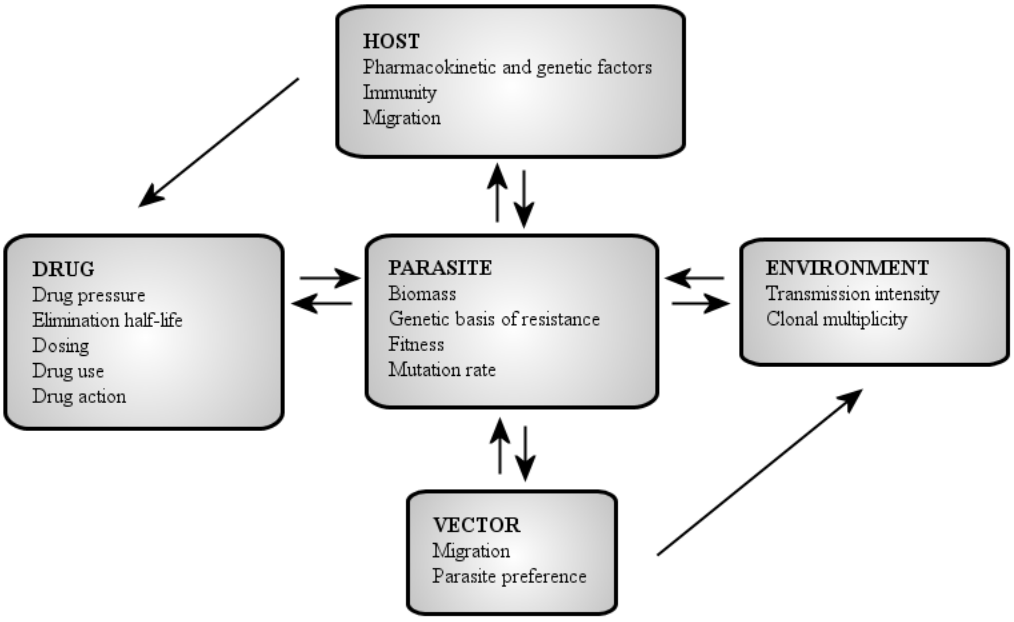

Figure 4. Factors affecting the emergence and spread of antimalarial drug resistance and their interactions.

1.7.1 Impact of antimalarial drug resistance

The importance of ACT as a cornerstone in malaria control is described in section 1.5. Consequently the impact of resistance to ACT, as well as to chloroquine and SP, the drugs that malaria treatment was previously relying on, may be considerable. In low transmission areas people have little or no immunity and consequently drug resistance will be visible by increasing rates of treatment failures (Bjorkman & Bhattarai, 2005). Drug resistance may be a contributing factor to malaria epidemics in these areas (Talisuna *et al.*, 2004). The impact of drug resistance in areas with high malaria transmission may be difficult to determine because most individuals have partial

immunity to malaria. The manifestations of drug resistance include persistent parasitaemia and increased risk of severe disease, initially in individuals with low immunity. Cerebral malaria may increase the incidence of neurological deficits and severe anaemia may increase the risk of HIV following blood transfusions. Thus drug resistance in high endemic areas may not be visible mainly in the malaria attributed mortality, but in the all-cause mortality (Bjorkman & Bhattarai, 2005, Talisuna *et al*., 2004). Therefore the impact of chloroquine resistance in Africa may have been underestimated, resulting in continued usage of chloroquine after wide-spread resistance in many countries. However it has been shown that chloroquine resistance had major public health impact in terms of increased malaria attributed mortality in Africa (Trape, 2001).

1.7.2 Resistance to SP

Resistance to SP spread rapidly in Southeast Asia and South America (Wongsrichanalai *et al.*, 2002) and in several areas in Africa (Kublin *et al.*, 2002, Nzila *et al.*, 2000a, Omar *et al.*, 2001, Wongsrichanalai *et al*., 2002). While chloroquine could be used for several decades, resistance to SP occurred even before the drug was widely used (Wongsrichanalai *et al.*, 2002). Several factors could contribute to the fast development of resistance to SP. Both sulfadoxine and pyrimethamine have quite long elimination half-lives, 10 and 4 days respectively, and are subsequently present in sub-therapeutic concentrations for a long time in the blood. In high transmission areas this could result in selection of reinfections with deceased SP susceptibility (Curtis *et al.*, 1998, Nzila *et al.*, 2000b). Furthermore, SP resistance could have been selected by other antifolates that could confer cross-resistance to SP. Sulfa drugs has been used as antibiotics since the 1930s (Triglia & Cowman, 1999) and cross-resistance between sulfadoxine and other sulfa drugs has been demonstrated (Triglia *et al.*, 1997). Another most relevant drug reducing the efficacy of SP treatment is considered to be trimethoprim-sulfamethoxazole, a drug used as prophylaxis against opportunistic infections in AIDS and HIV infections in Africa. SP is widely used in several of the countries where HIV infection rates are highest (Iyer *et al.*, 2001).

1.7.3 Resistance to ACT

1.7.3.1 Resistance to partner drugs

In Africa clinical efficacy to the main ACTs employed, artemether-lumefantrine and artesunate-amodiaquine was demonstrated to be high (Martensson *et al.*, 2007, Martensson *et al.*, 2005). However selection of reinfections after treatment with artemether-lumefantrine (Dokomajilar *et al.*, 2006b, Sisowath *et al.*, 2007, Sisowath *et al.*, 2009, Sisowath *et al.*, 2005, Happi *et al.*, 2009) and artesunate-amodiaquine

(Holmgren *et al.*, 2007) have been reported, indicating development of tolerance to the partner drugs.

In South East Asia both recrudescences and reinfections after artesunate-mefloquine (Price *et al.*, 2004) were observed, indicating resistance/tolerance to both drugs. However it was suggested that this observation was due to decreased efficacy of mefloquine since mefloquine resistance was established in the area of Thailand where the study was performed and the parasite clearance time was normal (Woodrow & Krishna, 2006). Several recent studies reported declining efficacy of artesunate-mefloquine on the Thailand-Cambodia and Thailand-Myanmar borders and in southern Cambodia (Carrara *et al.*, 2009, Wongsrichanalai & Meshnick, 2008, Rogers *et al.*, 2009). Although the treatment failures were initially attributed to mefloquine also in these studies, it has been suggested that ART may have a role in the declining ACT efficacy because of the increased parasite clearance times after ART monotherapy and ACT in the area (Wongsrichanalai & Meshnick, 2008, Maude *et al.*, 2009) (Rogers *et al.*, 2009)

1.7.3.2 Resistance to ART

When introduced artemisinin was regarded as a magic bullet in antimalarial treatment, to which resistance would essentially not develop, due to the short elimination half-life and the rapid action of the drug. A number of recent observations however leave this wishful thinking in the past. In fresh isolates from French Guiana highly elevated IC_{50} (the drug concentration where 50% of the parasite growth is inhibited) of artemether was observed (Jambou *et al.*, 2005). In a rodent model stable artemisinin resistant *P. chabaudi* could be acquired after selection under drug pressure (Afonso *et al.*, 2006). In a clinical efficacy study in Central African Republic with artesunate monotherapy it was demonstrated that recrudescent infections had increased IC_{90} to DHA (Menard *et al.*, 2005). Recently two patients were suggested to have resistant *P. falciparum* infections after artesunate monotherapy in Cambodia (Noedl *et al.*, 2008). These infections were classified as resistant to ART according parameters previously suggested by H. Noedl (Noedl, 2005) where treatment failures are defined as resistant in the presence of: adequate plasma drug concentrations, prolonged time to parasite clearance and reduced *in vitro* susceptibility to DHA (Noedl *et al.*, 2008). In addition treatment failures after artesunate-mefloquine with increased parasites clearance times was observed, as described in the previous section, suggesting that decreased susceptibility to ART has a role in the failing treatment. In summary all these studies suggest that artemisinin resistance can and will develop upon selection by ART monotherapy and ACT. This stresses the importance of understanding the associated molecular mechanism of resistance in order to potentially prevent its emergence and

spread and supply data for future evidence based development of new therapies, like drugs targeting ART resistant parasites. If not prevented, the spread of ART resistance will have dramatic public health consequences world wide, due to the essential contribution of ACT for malaria control (Bhattarai *et al.*, 2007).

1.7.4 Assessment of *P. falciparum* drug resistance

There are different ways to study the *P. falciparum* susceptibility to antimalarial drugs. Clinical efficacy trials can be seen as a reference method since they reflect the actual susceptibility pattern in the studied area, where treatment failures represent the *P. falciparum* resistant phenotypes *in vivo*. However there are several host factors, including immunity and pharmacokinetics that can confound the association of *P. falciparum* drug phenotype and clinical outcome. To exclude the effect of host factors, *P. falciparum* susceptibility to various antimalarial drugs can be studied *in vitro* that provides a quantitative assessment of drug resistance. Further benefits with *in vitro* studies include evaluation of single drugs within a combination, cross-resistance and synergy/antagonism between various drugs, as well as new or failing drugs, that cannot yet/still be studied *in vivo* (Laufer *et al*. 2007). However it is difficult to mimic the *in vivo* drug exposure *in vitro*. Fresh isolates may be more representative than *in vitro* adapted isolates, which may have changed genetically in the adaptation. On the other hand, field methods for susceptibility testing may be more insecure than the laboratory methods, especially since measurements cannot be repeated. In summary both *in vivo* and *in vitro* studies are important to obtain a full picture of the spread of *P. falciparum* drug resistance.

1.8 MECHANISMS OF DRUG RESISTANCE

General mechanism of drug resistance can be summarized as in box 3 (Scholar, 2000). In *P. falciparum* the most well studied mechanisms of resistance include decreased affinity for the drug of the target (e.g. SP resistance through mutations in DHFR and DHPS) and altered drug transport (e.g. chloroquine resistance through mutations in the *P. falciparum* chloroquine resistance transporter (PfCRT)). Genetic alterations include gene amplification, single nucleotide polymorphisms (SNPs) as well as insertions or deletions.

Box 3. General mechanism of resistance:

Altered transport of the drug
Enzymatic inactivation of the drug
Decreased conversion of the drug to the active compound
Increased amount of a metabolite antagonizing the drug action
Altered amount of the drug target
Decreased affinity of the drug target

In this section the genes presently documented to contribute for the mechanism of resistance to ACTs and SP will be described, initiating with the well studied *pfdhf*, *pfdhps*, *pfcrt* and *pfmdr1*, the genes encoding DHFR, DHPS, PfCRT and *P. falciparum* multidrug resistance protein 1 (PfMDR1), respectively. However, the focus of this thesis will be on *pfmrp1* and *PfATP6*, encoding the *P. falciparum* multidrug resistance protein 1 (*pf*MRP1) and the *P. falciparum* sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) orthologue (PfATP6), describing what can be learnt from their mammalian homologues and how they can be related to *P. falciparum* drug resistance. These will be described in section 1.9 and 1.10, respectively.

1.8.1 *Pfdhfr* and *pfdhps*

P. falciparum resistance to pyrimethamine and sulfadoxine has been well documented as associated with point mutations in their respective target genes, i.e. *pfdhfr* and *pfdhps*. However, these genes do not seem to explain the full resistance mechanism to SP (Wang *et al.*, 1997b, Wang *et al.*, 1999).

Early studies had demonstrated that pyrimethamine resistance in *P.falciparum* was caused by decreased affinity of the drug to a structurally modified DHFR (Chen *et al.*, 1987). Subsequent sequencing of *pfdhfr* in parasite isolates with different pyrimethamine susceptibilities identified the following alterations as associated with resistance: S108N, N51I, C59R, and I164L (Cowman *et al.*, 1988, Peterson *et al.*, 1990, Thaithong *et al.*, 1992). Further experiments confirmed that mutations in these positions modified the structure of DHFR so that pyrimethamine binding was impaired (Sirawaraporn *et al.*, 1997).

The basis of sulfadoxine resistance started to be investigated with the characterization of the *pfdhps* gene and the sequencing of *pfdhps* in parasite isolates with various levels of sulfadoxine susceptibility. The mutations found to be associated with resistance were

S436A/F, A437G, A581G and A613S (Brooks *et al.*, 1994, Triglia & Cowman, 1994). Later the K540E mutation was detected in field studies (Wang *et al.*, 1997a). In cultures with low concentration of folate the mutations were correlated to resistance to sulfadoxine *in vitro* (Wang *et al*., 1997b).

In vivo combinations of mutations in *pfdhfr* and *pfdhps* has been shown to be correlated with SP treatment failure (Wongsrichanalai *et al*., 2002). In particular a quintuple mutant (*pfdhfr*: S108N, N51I, C59R + *pfdhps*: A437G, K540E) has been strongly associated with SP treatment failure on the African continent (Nzila *et al*., 2000a, Happi *et al.*, 2005, Kublin *et al*., 2002).

1.8.2 *Pfmdr1*

PfMDR1 is an ATP-binding cassette (ABC) transporter that localizes to the membrane of the food vacuole. As its mammalian homologue, the P-glycoprotein, PfMDR1 can modulate the response to various structurally diverse drugs including ART and partner drugs. Recent observations suggest that PfMDR1 is an importer of antimalarial drugs and other substrates into the food vacuole (Rohrbach *et al.*, 2006) and that SNPs in *pfmdr1* can alter the transport by affecting substrate specificity (Sanchez *et al.*, 2008).

Gene amplification of *pfmdr1* has been seen to be associated with increased *in vitro* susceptibility to mefloquine and artesunate in field isolates (Wilson *et al.*, 1993, Price *et al.*, 1999). In accordance, parasites with decreased *pfmdr1* copy number have been shown to have decreased *in vitro* susceptibility to mefloquine, lumefantrine and artemisinin (Barnes *et al.*, 1992, Peel *et al.*, 1994, Rohrbach *et al*., 2006). *In vivo*, *pfmdr1* amplification has been associated with treatment failures after mefloquine and artesunate-mefloquine treatment in Asia (Price *et al*., 2004, Lim *et al.*, 2009, Rogers *et al*., 2009).

SNPs in *pfmdr1* may also alter drug response. In transfection based experiments, leading to specific allele changes, and by analyzing field isolates, *pfmdr1* SNPs N86Y, Y184F, S1034C, N1042D and D1246Y have been shown to be associated with alterations of *in vitro* susceptibility to mefloquine, lumefantrine, artemisinin, artesunate and dihydroartemisinin (Duraisingh *et al.*, 2000, Reed *et al.*, 2000, Sidhu *et al.*, 2005, Anderson *et al.*, 2005). *In vivo pfmdr1* N86, 184F and D1246 alleles have been selected in recurrent infections after treatment with artemether-lumefantrine (Sisowath *et al*., 2005, Dokomajilar *et al*., 2006b, Sisowath *et al*., 2007, Happi *et al*., 2009) while the opposite alleles *pfmdr1* 86Y, Y184 and 1246Y have been associated with recurrent infections after amodiaquine or artesunate-amodiaquine treatment (Holmgren *et al.*, 2006, Dokomajilar *et al.*, 2006a, Holmgren *et al*., 2007).

1.8.3 *Pfcrt*

PfCRT is located in the membrane of the food vacuole where chloroquine has been suggested to act by binding to hematin, a toxic byproduct from the digestion of hemoglobin, thereby preventing synthesis of non toxic hemozoin (Bray *et al.*, 1998). The K76T mutation in *pfcrt* has been demonstrated to be the main determinant for chloroquine resistance (Djimde *et al.*, 2001, Sidhu *et al.*, 2002, Lakshmanan *et al.*, 2005). It has been suggested that mutant *pfcrt* can transport chloroquine out of the food vacuole (Valderramos & Fidock, 2006, Sanchez *et al.*, 2007).

PfCRT has also been shown to influence the effect of both ART and the partner drugs. Transfection of mutant *pfcrt* resulted in increased susceptibility to artemisinin, DHA and mefloquine, as well as some decrease in amodiaquine susceptibility (Sidhu *et al*., 2002). *In vivo* studies have shown that artemether-lumefantrine treatment selects for K76 (Sisowath *et al*., 2009) whereas amodiaquine and artesunate-amodiaquine selects for 76T (Dokomajilar *et al*., 2006a, Happi *et al.*, 2006, Holmgren *et al*., 2006).The selection pressure in these studies are thought to be derived from the partner drug.

1.9 SARCO/ENDOPLASMIC RETICULUM CA^{2+}-ATPASE (SERCA)

1.9.1 Mammalian SERCA

In eukaryotes calcium (Ca^{2+}) is an important intracellular second messenger controlling a range of cell functions, including muscle contraction, exocytosis, cell division and cell differentiation. Intracellular calcium concentration is well regulated by influx and efflux of calcium through the plasma membrane and various organelles (Moreno & Docampo, 2003). There is a steep calcium concentration gradient between the high concentration in the extracellular space and intracellular stores and the low concentration in the cytoplasm of the cell. Consequently the rapid release of calcium from stores into the cytosol can result in in potent signaling. After the release calcium is rapidly cleared from the cytosol to stop the signal and avoid cell toxicity.

The endoplasmic reticulum (ER) contains a large store of calcium in the cell that is the primary source of calcium for signaling (Nagamune *et al.*, 2008). Influx of calcium into the ER is mediated by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In vertebrates there are three SERCA genes coding for large membrane proteins with 10 transmembrane (M) domains and three cytoplasmic domains: the actuator domain that transmits conformational changes to the M domains, the nucleotide binding domain (NBD) and the phosphorylation domain (Garcia *et al.*, 2008). The NBD domain binds ATP, while the domains M4, M5, M6 and M8 domains binds calcium (Kimura *et al.*, 1993). During the hydrolysis of one ATP molecule, two calcium molecules are

transported from the cytoplasm into the ER. A residue in the phosphorylation domain of the SERCA becomes phosphorylated after ATP hydrolysis (Garcia *et al*., 2008). The activity of mammalian SERCA can be abolished by the highly specific inhibitor thapsigargin (Lytton *et al.*, 1991) by locking the protein in a conformation that cannot bind calcium (Xu *et al.*, 2004).

1.9.2 *P. falciparum* SERCA

Calcium has been shown to regulate several processes in apicomplexan parasites including host cell invasion and motility (Nagamune *et al*., 2008). In *Plasmodium* it has been suggested that similar mechanisms may be involved in host cell invasion (Billker *et al.*, 2004, Green *et al.*, 2008). Calcium may also be important for *Plasmodium* gametocyte differentiation (Billker *et al*., 2004) and for synchronization of the parasite life cycle in response to the host melatonin production (Garcia *et al*., 2008).

The calcium concentration in the cytosol of *Plasmodium* parasites is likely to be low and the uninfected RBCs also have low calcium concentration (Nagamune *et al.*, 2008). To explain how the parasite can overcome the absence of the essential extracellular calcium it has been suggested that the intraerythrocytic calcium concentration increases during malaria infections since the RBCs become more permeable and reduce export (Garcia, 1999) and/or that there is a relatively high calcium concentration in the parasitophorous vacuole, in which *Plasmodium* reside (Gazarini *et al.*, 2003). Calcium storage in a compartment similar to the mammalian ER has been demonstrated in *Plasmodium* (Garcia *et al*., 2008).

Only one *P. falciparum* SERCA orthologue, PfATP6, has been identified (Kimura *et al*., 1993, Varotti *et al.*, 2003). The overall homology between mammalian SERCA and PfATP6 is relatively low. However, all key residues previously shown to be related with calcium transport, e.g. calcium binding and ATP binding, in rabbit SERCA, were conserved in PfATP6 (Kimura *et al*., 1993). Further, it was demonstrated that the SERCA inhibitor thapsigargin could induce calcium release into the cytosol from intracellular stores, probably ER, by inhibition of the PfATP6, suggesting that PfATP6 is essential for *P. falciparum* calcium homeostasis (Varotti *et al*., 2003) and that PfATP6 in functionally related with higher mammal homologues.

1.9.3 PfATP6, a target for ART?

The SERCA inhibitor thapsigargin is a sesquiterpene lactone, as are ART. From these structural similarities the hypothesis emerged that ART act by inhibiting PfATP6. This was supported by the demonstration that artemisinin specifically inhibited PfATP6 expressed in *Xenopus laevis*, as thapsigargin. The two drugs showed an antagonistic

interaction in *P. falciparum* cultures and similar localization in the parasite. Hence PfATP6 was suggested to be a target of ART (Eckstein-Ludwig *et al.*, 2003). In human SERCA thapsigargin binding is determined by residues in the transmembrane domains M3, M5 and M7. Mutations within these domains can reduce thapsigargin affinity and/or inhibitory effect (Xu *et al.*, 2004). Results from homology modeling of PfATP6 and docking simulation artemisinin to PfATP6 suggest that residues in M3, M5 and M7 are important also for artemisinin binding (Jung *et al.*, 2005). Investigations of the differences in the thapsigargin-binding cleft of mammalian and *Plasmodia* SERCA revealed that mutations introduced in residue 263 in PfATP6 reduced artemisinin inhibition, suggesting that this amino acid is involved in artemisinin binding to PfATP6 (Uhlemann *et al.*, 2005). However natural variation in this amino acid has not been found (Cojean *et al.*, 2006, Ferreira *et al.*, 2007, Price *et al.*, 2004), which might indicate a very conserved and hence functionally important region of the protein.

Natural variation in PfATP6 and association with artemisinin susceptibility has recently started to be investigated. In French Guiana an S769N SNP was associated with decreased *in vitro* susceptibility to artemether in fresh isolates (Jambou *et al.*, 2005). This challenging initial report could not be confirmed since the S769N SNP has not been found in subsequent studies (Mugittu *et al.*, 2006b, Price *et al*., 2004, Sisowath *et al*., 2007), with the exception of one sample that was fully sensitive to DHA *in vitro* (Cojean *et al*., 2006). The combination of two additional SNPs, E431K and A623E, was identified in a fresh isolate from Senegal with increased IC_{50} to artemether (Jambou *et al.*, 2005).Through partial or full sequencing of *PfATP6*, only three additional SNPs have been identified in field samples: I89T in Thailand (Price *et al*., 2004), H243Y in Africa (Cojean *et al*., 2006) and a synonymous SNP in nucleotide position T2694A in São Tomé and Príncipe (Ferreira *et al*., 2007). In only two studies *PfATP6* has been comprehensively sequenced in a significant number of clinical samples, i.e. by Jambou and colleagues (Jambou *et al*., 2005) that fully sequenced the gene in 60 samples, but did not report the location of further variations, and Cojean and colleagues (Cojean *et al*., 2006) that partially sequenced the gene in 154 samples. Further studies were needed to describe the biodiversity of *PfATP6*.

1.10 MULTIDRUG RESISTANCE PROTEINS

1.10.1 ABC transporters

Multidrug resistance proteins (MRPs) are members of the ABC transporter superfamily (Dassa, 2003), one of the largest protein families in many organisms (Higgins & Linton, 2003). ABC transporters are mainly transmembrane proteins which are able to transport substrates across various cell membranes against a concentration gradient in an energy-dependent reaction requiring ATP hydrolysis. They can transport a large

variety of substrates including endogenous substrates as well as drugs and exogenous chemicals. ABC transporters are involved in a range of different physiological functions, such as protection against exogenous toxins, uptake and distribution of therapeutic drugs, antigen processing and lipid homeostasis.

A typical ABC transporter consists of two membrane spanning domains (MSDs) each with six predicted transmembranes helices (TMs) that form the pathway for transport of substrates and two NBDs that hydrolyze ATP to provide energy for this process (Varadi *et al.*, 2003). Each NBD contain the highly conserved Walker A, Walker B and C signature primary sequence motifs that play a critical role in the mechanism of ATP binding and hydrolysis.

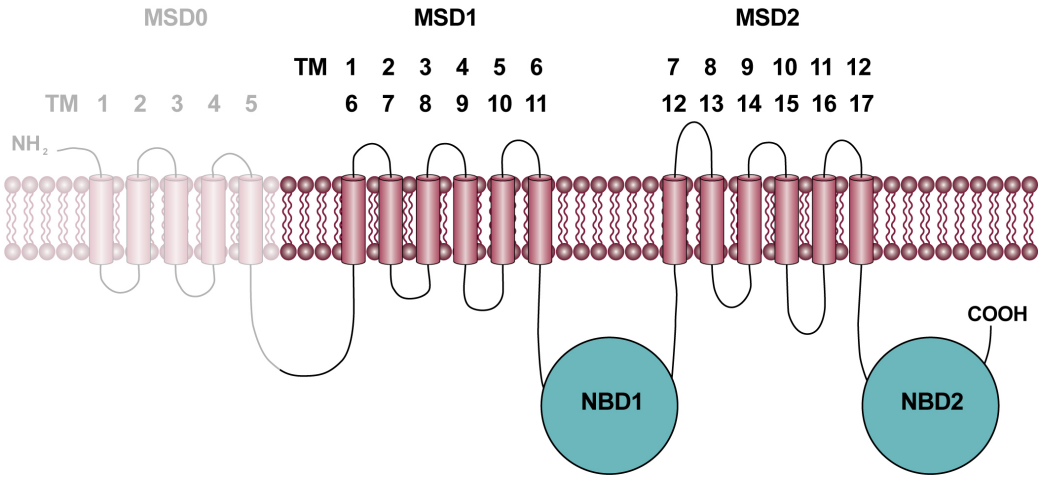

Figure 5. MRP topology of long MRPs (TM1-17), including human MRP1, -2, -3, -6 and -7, and short MRPs (TM1-12), including human MRP4, -5, -8 and -9.

1.10.2 Mammalian MRPs

The MRPs of higher mammals and particularly human MRPs are most well studied. Information gathered *in vitro* and *in vivo* concerning these transporters has been representing the basis of information and educated hypothesis concerning MRPs of other organisms. A brief summary of relevant data mostly based on studies of human MRPs is presented in this section.

MRPs belong to the ABCC branch of ABC transporters (according to the Human Gene Nomenclature Committee, described in (Dassa, 2003)). The human ABCC branch has 13 members and includes MRPs, as well as non drug transporters like the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptors (SURs) (Deeley *et al.*, 2006). MRPs can either have the typical ABC structure described above (two MSDs and two NBDs) or belong to the group of "long" MRPs that have an

additional MSD (MSD0) in the N-terminal region containing five predicted TMs (Figure 5) (Conseil *et al.*, 2005, Deeley *et al*., 2006). MSD0 seems to have a function in trafficking of the protein from the endoplasmic reticulum (ER) to the plasma membrane, but only if other trafficking signals are absent or mutated (Deeley *et al*., 2006).

An additional structural characteristic of MRPs, different from other ABC transporters, is the asymmetry between the NBDs, where the NBD1 of an MRP can be more structurally related to another MRP than to the NBD2 of that particular protein. In general NBD2 has a more typical ABC structure while structural particularities of NBD1 include a gap between the Walker A and the ABC signature motifs, which seems to be present in all identified MRPs (Deeley & Cole, 2003, Deeley *et al*., 2006). Supporting the observed structural asymmetry NBD1 has been shown to have higher ATP-binding affinity than NBD2 while NBD2 have higher ATPase activity than NBD1 (Conseil *et al*., 2005).

1.10.2.1 Substrate specificity and physiological role – what can be learnt from human MRPs

The typical substrates (or allocrites) of MRPs are amphiphilic organic anions. These can be transported unconjugated or conjugated with glutathione, glucuronate or sulfate groups. Substrates can be dependent on reduced glutathione (GSH) for their MRP-mediated transport (Deeley & Cole, 2003). Examples of the variety of molecules that MRPs can transport include endogenous substrates: leukotrienes, prostaglandins, bilirubin, folate, GSH and oxidized glutathione (GSSG); anticancer drugs: vinca alkaloids, doxorubicin and methotrexate; heavy metals: arsenite and antimonite; and antibiotics difloxacin and grepafloxacin (Sharom, 2008, Deeley & Cole, 2003). Interesting, it has also been demonstrated that human MRPs can transport antimalarial drugs. MRP1 was shown to transport chloroquine and overexpression of MRP1 resulted in cells that were more resistant to chloroquine than the parental cells (Vezmar & Georges, 1998). Futhermore chloroquine, quinine and primaquine were shown to reverse MRP-mediated resistance to doxorubicin by direct binding to MRP1, which may suggest that they could be transported by MRP (Vezmar & Georges, 2000). In erythrocytes it was shown that mefloquine could inhibit MRP1- and MRP4-mediated transport and leading to the suggestion that this drug could be an MRP substrate (Wu *et al.*, 2005).

As can be understood from the great diversity of substrates, MRPs are involved in numerous physiological processes of which a few are presented here. Human MRPs participate in the elimination of Phase II products through transport of conjugated

exogenous drugs/toxins. By this capacity MRPs have also been shown to have a role in protection of sensitive sites of the body as it is present in the blood-brain-barrier, the passage from blood to cerebrospinal fluid and the placenta. MRPs also have a role in the cellular protection against oxidative stress. GSH is a main antioxidant that can reduce reactive oxygen species. Increased cellular oxidative stress will result in accumulation of the oxidized form of glutathione, GSSG, which can be eliminated from the cell through MRP-mediated efflux. The highest affinity substrate of MRP1 is leukotriene C4, which is produce by eosinophils and mast cells in inflammation, indicating an endogenous role for MRP1 in immunity (Deeley *et al*., 2006).

1.10.2.2 Transport mechanism

Substrate transport of MRPs involves two connected cycles, the catalytic cycle of ATP hydrolysis that drives transport and the transport cycle in which the substrate is transferred across membranes. The transport mechanism has been difficult to study and details are still unclear. A hypothetical model of the transport cycle of MRP1 has been adopted from Deeley and colleagues (Deeley *et al*., 2006) (Figure 6). This transport cycle is based on the recent finding that it is the binding of ATP that induces the conformational changes in the MSDs that result in substrate release (Deeley *et al*., 2006).

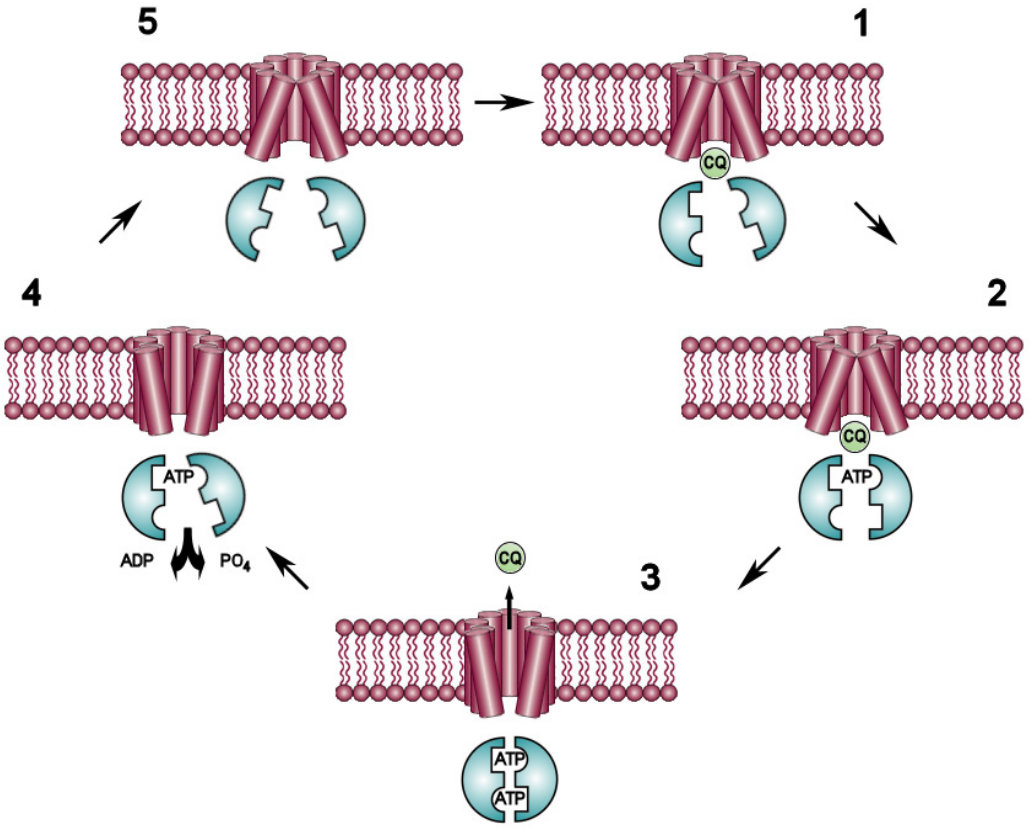

Figure 6 Model of the transport cycle of human MRP1.The MRP substrate (green) is represented by chloroquine (CQ). Substrate transport involves the following steps:

1) Substrate binding to high affinity sites in the protein induces conformational changes in NBD1 facilitating ATP binding.
2) ATP binds to NBD1 and the C signature in NBD2.
3) A second ATP molecule binds NBD2 and the C signature in NBD1. In result conformational changes in the MSDs are induced which leads to decreased affinity for the substrate, that is subsequently released.
4) The ATP in NBD2 is hydrolyzed and ADP is released.
5) The MRP resets to a high-affinity substrate binding state. It is not known whether this step requires ATP hydrolysis and/or ADP or ATP release from NBD1.

1.10.2.3 Functional important domains

1.10.2.3.1 Catalytic ATP cycle

The NBDs show a high degree of conservation within the ABC transporter superfamily, especially in the Walker A, B and C signature motifs, indicating the functional significance of these regions. Mutagenesis of residues in the NBDs show that the they are important for ATP-dependent substrate transport (Center *et al.*, 1998). The importance of residues in the NBDs is illustrated by the numerous naturally occurring mutations in these regions, which are leading to human diseases. Cystic fibrosis is caused mainly by mutations in the NBDs of the CFTR in the ABCC

subfamily, which are often related to severe forms of the disease. Likewise, the diseases Dubin-Johnhson syndrome, pseudoxanthoma elasticum and persistant hyperinsulinemic hypoglycemia of infancy are predominately caused by mutations in NBD2 of MRP2, ABCC6 and SUR1, respectively (Conseil *et al*., 2005).

1.10.2.3.2 Substrate recognition

Although MRPs transport structurally diverse substrates that can be unconjugated or conjugated, most substrates compete reciprocally with each other suggesting that they are interacting with the protein in a similar way and may share binding sites (Deeley & Cole, 2003). Indeed the importance of TMs 10, 11, 16 and 17 in human MRP1 in substrate specificity have been confirmed experimentally by several methods and with many substrates. In mutagenesis studies also other TMs (6, 7, 8, 9, 12, 14, and 15) and the cytoplasmic loop connecting TM 15 and 16 have been demonstrated to contribute to substrate specificity. Finally, MRP1 has been shown to retain substrate transport capability also in the absence of MSD0, indicating that it is not of major importance in substrate specificity nor transport (Deeley & Cole, 2006).

1.10.2.3.3 Interaction between MSDs and NBDs

Communication between MSDs and NBDs is important for the induction and transmission of conformational changes in the respective domains during the transportation cycle. However, how these changes are transduced is still poorly understood. It has been suggested that TM 10, 11, 16 and 17 in human MRP1 could form a putative basket/pore close to the cytosolic interface, which would have the role of a gateway to the translocation pathway. The basket could be important for both substrate specificity and overall activity. Especially aromatic residues may be crucial in the initial interaction with substrates in the basket (Deeley & Cole, 2006). Mutations in residues in the cytoplasmic loop connecting TM 15 and 16 have been shown to affect both substrate specificity and catalytic activity of MRP1. Also residues in TM 11 seem to be important for substrate binding as well as ATP binding by NBD1 (Deeley *et al*., 2006, Deeley & Cole, 2006).

1.10.3 MRPs in drug resistance

From the human point of view, the function of MRPs can be seen as a double-edged sword. On one hand they protect sensitive tissues from toxic substances, but on the other hand they can cause resistance to xenobiotics in human tumor cells (Haimeur *et al.*, 2004) and in pathogenic organisms such as bacteria (Poelarends *et al.*, 2003) and parasites (Ouellette & Légaré, 2003).

1.10.3.1 Drug resistance in cancer

The phenomenon of multidrug resistance was discovered more than 30 years ago. It was observed that human tumor lines selected for resistance to one drug also acquired resistance to a broad range of structurally unrelated drugs. An important underlying mechanism of multidrug resistance is drug efflux performed by ABC transporters, the main ones being several MRPs as well as the P-glycoprotein (P-gp) and the breast cancer resistance protein (BCRP) (Schinkel & Jonker, 2003).

Beside drug efflux, MRPs can affect drug resistance by alternative mechanisms. It has been observed that repression of MRP genes impairs folate export resulting in increased intracellular folate concentration and resistance to pyrimethamine (Stark *et al.*, 2003, Assaraf *et al.*, 2003). MRPs can also contribute to drug resistance through export of oxidized glutathione (GSSG), which would relieve the cell from oxidative stress generated by radicals, which is produced by some chemotherapies, such as anthracyclins (Leier *et al.*, 1996).

Among MRPs, the "long" MRPs, including MRP1, has been shown to confer resistance *in vitro* to natural product drugs such as vinca alkaloids, anthracyclins and epipodophyllotoxins. The "short" MRPs primarily confer resistance to nucleoside and nucleotide analogues. (Deeley *et al*., 2006) In general, the key MRP contribution to resistance in cancer is overexpression of the protein (Deeley *et al*., 2006, Bates, 2003), although SNPs in this gene have also been shown to alter drug resistance (Sharom, 2008, Conseil *et al*., 2005, Center *et al*., 1998). MRP overexpression has been demonstrated in a variety of solid tumors, sometimes as an indicator of poor prognosis and/or poor response to chemotherapy (Deeley *et al*., 2006, Bates, 2003). However, the contribution of MRPs in clinical drug resistance is not yet clearly defined.

1.10.3.2 Parasite drug resistance

The *Leishmania tarentolae* ABC transporter PGPA was one of the first MRPs to be discovered (Ouellette *et al.*, 1990). In concert with enzymes involved in the synthesis of trypanothione (TSH), PGPA can contribute to resistance to antimony, which is the active compound of *Leishmania* treatments. PGPA seems to confer resistance by sequestration of antimony-TSH conjugates in intracellular vesicles (Ouellette & Légaré, 2003). The arsenical drug melarsoprol is one of the only treatment options for late stage sleeping sickness caused by *Trypanosoma brucei*. Overexpression of the *T. brucei* PGPA homologue *TbMRPA* resulted in a 10-fold increase in melaroprol IC_{50} (Shahi *et al.*, 2002).

1.10.4 PfMRPs

Two MRP homologues have been identified in the *P. falciparum* genome, *pf*MRP1 (PFA0590w) and *pf*MRP2 (PFL1410c) (Bozdech & Ginsburg, 2004, Ouellette & Légaré, 2003, Gil *et al.*, 2000). *pf*MRP1 is an 1822 amino acid protein situated in the parasite plasma membrane (Klokouzas *et al.*, 2004, Raj *et al.*, 2009). The *pf*MRP1 coding gene, *pfmrp1*, is transcribed mainly during the late trophozoite stage and was suggested to encode a *P. falciparum* GSH/GSSG pump, supported by the observation that its transcription coincides with that of glutathione synthetase in the *de novo* synthesis of glutathione (Bozdech & Ginsburg, 2004). *pf*MRP2 is transcribed earlier, mainly in the ring stages and peaks again in the end of the cycle, and thereby does not seem to be intimately connected with GSH as *pf*MRP1 (Bozdech & Ginsburg, 2004).

Since chloroquine and potentially mefloquine were demonstrated to be transported by human MRP1 and 4, it has been speculated if a *P. falciparum* MRP could transport chloroquine and mefloquine and might contribute to resistance to these drugs (Vezmar & Georges, 1998, Wu *et al*., 2005). Mu and colleagues sequenced the parasite isolates Hb3, Dd2, D10 and 7G8 and identified SNPs in multiple transporters, including *pf*MRP1 (named G2) and *pf*MRP2 (named G56), in comparison with 3D7. Association of the identified SNPs with IC_{50} to chloroquine and quinine was analyzed in 97 cloned isolates of different origins. The *pf*MRP1 SNP H191Y was associated with decreased susceptibility to chloroquine in Asian isolates and quinine in South American isolates, while the SNP S437A was associated with decreased susceptibility to chloroquine and quinine in South American isolates. No associations between genotype and phenotype were seen with *pf*MRP2 (Mu *et al.*, 2003). The associated SNPs were further analyzed in a number of isolates with defined drug susceptibility from Thailand. H191Y could not be evaluated in the study since it did not show any variation, while S437A was not shown to be associated with the parasite response to any of the tested drugs (Anderson *et al.*, 2005). In a relatively small clinical efficacy trial from Kenya H191Y and S437A were not associated with recurrent infections after amodiaquine monotherapy. However H191Y and S437A SNPs were shown to be rare in these region, present in 6/77 (8%) and 4/72 (6%) of the baseline infections respectively, so it is possible that the study did not have enough power to demonstrate an association (Holmgren *et al.*, 2006). In Iran H191 and S437 were found to be absolutely linked at a frequency of 13.6% (Ursing *et al.*, 2006).

A recent comprehensive study provided evidence for a role of *pf*MRP1 in antimalarial drug resistance. *pf*MRP1 was disrupted in the chloroquine resistant parasite W2, resulting in significantly increased sensitivity to chloroquine, quinine, artemisinin, piperaquine and primaquine and accumulation of chloroquine and quinine, suggesting

that *pf*MRP1 contributes to *P. falciparum* response to these drugs by transporting them out of the cell. The transport of chloroquine and quinine was enhanced with GSH. Furthermore GSH, GSSG and GSH-conjugates was also shown to accumulate providing evidence that *pf*MRP1 is a *P. falciparum* GSH/GSSG exporter. Parasites with the disrupted *pf*MRP1 could not grow to a density higher than 5% parasitaemia, if medium was not changed twice a day. The impaired growth was suggested to be due to limited capacity of the knock out to remove toxic substances (Raj *et al*., 2009).

2 AIM

The aim of this thesis was to identify new mechanisms of resistance to the most commonly used antimalarial drugs. Specific aims were to study: (a) the global biodiversity of *PfATP6* and *pfmrp1* and (b) the contribution of identified polymorphisms in the parasite response to these drugs *in vivo*, in *P. falciparum* infected patients from clinical drug trials, and *in vitro*, in *P. falciparum* fresh isolates with characterized drug susceptibility.

3 MATERIALS AND METHODS

The intention of this section is not to repeat information that can be found in the papers, but to give background, rational or deeper knowledge of the materials and methods used in the papers of the thesis.

3.1 STUDY SITES

In this section the geographical areas are described where the clinical efficacy trials of paper II and III were conducted and from which the fresh *P. falciparum* isolates tested *in vitro* in paper IV origin.

3.1.1 Tanzania

Malaria is transmitted in Zanzibar and mainland Tanzania throughout the year, with seasonal peaks during the rainfalls in March-May and October-December. The main malaria species is *P. falciparum*.

3.1.1.1 Zanzibar

Zanzibar is located outside the coast of mainland Tanzania and consists of two large islands, Unguja and Pemba, and numerous small ones. Paper II includes a clinical drug efficacy trial conducted in two sites; Kivunge Hospital on Unguja and Micheweni Hospital on Pemba. Both hospitals are located in densely populated rural areas. The trial was performed in October 2002 to February 2003, when chloroquine and SP was still supplied to the study sites by the government. Antimalarial drugs, but not ACTs, were available in the private sector. Later in 2003 Zanzibar became one of the first regions in Africa to implement ACT, with artesunate-amodiaquine as first line treatment and artemether-lumefantrine as second line treatment for uncomplicated *P. falciparum* malaria.

3.1.1.2 Fukayosi

Fukayosi is a village located in a relatively scarcely populated rural area in Bagamoyo district, on mainland Tanzania. Paper II and III include a clinical trial conducted in Fuakyosi Primary Health Care Centre in April to July 2004. At the time of the study SP was the first line treatment for uncomplicated *P. falciparum* malaria and amodiaquine the second line treatment in Tanzania and ACTs were not available in the governmental health care. In 2006 Tanzania adopted artemether-lumefantrine as first line treatment.

3.1.2 Thailand

Malaria transmission in Thailand occurs especially in the forest and forest fringes and along borders. *P. falciparum* and *P. vivax* are the main malaria species. In 2006 the

highest incidence of malaria in Thailand was in the Tak Province (Na-Bangchang & Congpuong, 2007), located in North West of the country, bordering Myanmar. For paper IV *P. falciparum* fresh isolates were collected from patients attending the Malaria Clinic in Mae Sot, in the Tak Province in May to June 2007. Resistance to chloroquine, SP and mefloquine developed in Thailand, particularly on the border between Thailand and Cambodia that is known as the epicentre of drug resistance. Mefloquine resistance developed simultaneously also on the Thailand-Myanmar border. To counteract the problem of mefloquine resistance, artesunate-mefloquine was introduced 1995 in areas with high mefloquine resistance, such as Mae Sot (Na-Bangchang & Congpuong, 2007, Wongsrichanalai & Meshnick, 2008). Today artesunate-mefloquine is first line treatment for all uncomplicated lab-confirmed *P. falciparum* malaria in Thailand.

3.1.3 Uganda

Malaria transmission occurs all year around in most parts of Uganda while transmission intensity is highly variable with very high transmission in the northern and eastern parts of the country and low transmission in southwest. The main malaria species is *P. falciparum*. Because of the conflicts in northern Uganda most of the population has been relocated into camps for internally displaced persons (IDP). Camps are often overcrowded with poor access to medical care. The study, included in paper IV, was conducted in Amuru IDP camp situated in the Gulu district in September 2007. Amuru IDP camp has a population of around 24 000 people and malaria transmission in the camp is appreciated to be high (Prugger *et al.*, 2008). As resistance to the previous first line treatment chloroquine + SP was increasing, artemether-lumefantrine was implemented as first line therapy in Uganda in 2004. In 2006 artemether-lumefantrine was started to be used in the Home Based Management of Fever (HBMF) a program based on pre-packaged drugs with instructions distributed to parents/guardians of children in Gulu and other districts (Prugger *et al*., 2008). The majority of patients with fever are treated based solely on clinical diagnosis (without microcopy) with artemether-lumefantrine, ampicillin and trimethoprim. Artemether-lumefantrine can also be bought in the local pharmacies, without medical prescription.

3.2 CLINICAL DRUG EFFICACY TRIALS

In this thesis two comparative clinical efficacy trials were included in the assessment of *P. falciparum* polymorphisms and treatment outcome. The first trial was performed on the islands of Zanzibar in October 2002-February 2003 and included two treatment arms comparing artemether-lumefantrine (n=200) and artesunate-amodiaquine (n=208) (paper II) (Martensson *et al.*, 2005). The second trial was conducted in April-July 2004 in Fukayosi on Tanzania mainland and included two arms comparing artemether-lumefantrine (n=50) and SP (n=56) (paper II and III) (Martensson *et al.*, 2007). Both

trials enrolled children with uncomplicated *P. falciparum* malaria. Inclusion criteria in the studies were; age of 6-59 months and bodyweight of ≥6 kg (with the exception of the artemether-lumefantrine arm in the Zanzibar study where inclusion criteria was age of 9-59 months and bodyweight of ≥9kg, since artemether-lumefantrine was not registered for use in smaller children at the time of the study), parasitaemia level of 2000–200,000 asexual parasites/μL of blood; and axillary temperature of ≥37.5°C at the time of enrolment or history of fever during the preceding 24 h. Exclusion criteria were; symptoms and/or signs of severe malaria, hemoglobin concentration of <50 g/L, and serious underlying disease or known allergy to the study drugs. Enrolled patients were assigned to receive a loose combination of artesunate (Plasmotrim 100 mg; Mepha S.A.) and amodiaquine (Flavoquin 153 mg; Roussel) once daily for three days, or a fixed combination of 20 mg/120 mg artemether-lumefantrine (Coartem; Novartis) twice daily for three days, or 500mg/25mg SP (Fansidar; Roche) as a single dose. The drugs were administered according to body weight. Both studies had 42 days follow up after treatment. At days 0, 1, 2, 3, 7, 14, 21, 28, 35 and 42 or on any day of recurrent illness clinical assessment and laboratory tests, including thick blood smears for microscopy, were performed and blood samples were collected and spotted on filter paper for parasite genotyping. Parasitaemia was quantified by microscopy of Giemsa-stained thick blood films by counting the number of parasites per 200 leukocytes and multiplied by 40 to obtain the number of parasites/μL.

3.2.1 Distinction of recrudescences and reinfections

In clinical efficacy trials conducted in high transmission areas in Africa it is inevitable that some of the enrolled patients are infected by new *P. falciparum* parasites during the follow up period. These cannot be separated clinically or microscopically from the recrudescences that cause treatment failures. The mechanism of resisting a drug may also be different if the parasite has actually managed to survive the treatment (recrudescence) compared to infecting at sub-therapeutic drug levels (reinfection). Therefore it is important to be able to classify the recurrent infections correctly either as recrudescences or reinfections.

It has been suggested that two polymorphic genetic markers should be used to discriminate between recrudescences and reinfections (Mugittu *et al.*, 2006). Accordingly, in paper III recrudescences and reinfections were defined based on stepwise genotyping of the highly diverse surface antigens *P. falciparum* merozoite surface protein 2 (*pfmsp2*) and 1 (*pfmsp1*). Only samples classified as recrudescences according to *pfmsp2* genotyping were analysed for *pfmsp1*. A recurrent infection was classified as a 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). *pfmsp2* and *pfmsp1* was analysed as previously described (Snounou *et al.*, 1999). Size polymorphisms classified in reference allelic type/family of the genes were used to distinguish between different *P. falciparum* infections.

3.3 *IN VITRO* DRUG SUSCEPTIBILITY STUDIES

In paper IV the *in vitro* susceptibility of fresh *P. falciparum* isolates was tested in Mae Sot, Thailand and Gulu, Uganda. In Uganda, inclusion criteria were individuals with *P. falciparum* infection with a parasitaemia of 1000-100 000 asexual parasites/μL. Exclusion criterion was treatment with antimalarial drugs within the preceding 4 weeks. Blood was collected from patients for drug susceptibility testing and blood spots were put on filter paper for genetic analysis. The isolates were grown on plates with serial diluted drugs for 23.5 hours and the *in vitro* susceptibility was evaluated by measuring the inhibition of schizont maturation by microscopy as described previously (Knauer *et al.*, 2003, Prugger *et al*., 2008) with minor modifications. Calculations of parasite drug susceptibility were performed using the log-concentration-response-probit model (Wernsdorfer & Wernsdorfer, 1995). The percentage of schizont maturation at the tested drug concentrations was used to construct a linear regression with the WHO log dose response software. Probits, representing the effective concentrations (ECs), were plotted against the logarithmic concentration of the analysed drug. A range of EC values between EC_{10} and EC_{90} were calculated by using the probit representing the EC of interest together with the slope and intercept values of the linear regression of the respective isolate.

In *in vitro* studies EC_{50} and EC_{90} are generally analysed. We chose to evaluate a range of effective concentrations between EC_{10} and EC_{90}, to properly understand the role of a genotype in drug response. Analyses of solely a single EC value, i.e. EC_{50}, can result in that associations in the lower or higher EC ranges are missed or that the importance of an association only present in EC_{50} is overestimated. We interpreted an association throughout the whole range of EC values as a significant contribution of the genotype to the drug susceptibility. On the other hand if association was only seen in a part of the EC range, our interpretation was that the analysed genotype was not the only determinant for the drug response, suggesting a multifactorial or alternative mechanism of resistance. Mixed infections, e.g. isolates with mixed alleles in a genotype, were excluded from the analysis of that particular genotype.

3.4 ETHICAL CONSIDERATIONS

Ethical approval was obtained from ethical committees in the endemic countries and from Karolinska Institutet, Sweden (KI Dnr 94:230; 03-536; 03-684; 03-753;

2009/387-31/2). All samples were obtained upon informed consent of the patients or their guardians.

3.5 LONG PCR

A long PCR method for amplification of the complete *pfmrp1* gene was developed using Takara LA® Taq (Takara Shuzo, Japan) based on a previously described method (Sakihama *et al.*, 2001b). The benefits of this method was to limit the amount of DNA needed from the patients samples and to facilitate further PCR amplifications, especially in samples with low DNA concentration. Long PCR could also be useful in cloning and transfection experiments. This method was used in paper II, while in paper IV whole genome amplification (WGA) was employed for the same purpose.

3.6 WHOLE GENOME AMPLIFICATION

One of key limitations when working with clinical samples is that the samples are often available only in small amounts, limiting the possibility of extensive and repeatable analysis. In paper IV we addressed this issue by using WGA. This method allows abundant supplies of high-fidelity DNA to be produced from small amounts of genomic DNA (gDNA). There are several WGA techniques and multiple displacement amplification (MDA) is currently the most used method (Lovmar & Syvanen, 2006) because of its beneficial outcome. MDA is based on the DNA polymerase of the bacteriophage φ29, which amplifies DNA isothermally at 30°C, has remarkably high processivity generating extremely long DNA products and has a proof-reading activity resulting in low error rate (Lasken & Egholm, 2003). Genotyping results of human SNPs from MDA were shown to be concordant with those from gDNA (Lovmar & Syvanen, 2006, Silander & Saarela, 2008) and it has also been used in microorganisms (Lovmar & Syvanen, 2006). It has been shown that MDA exhibit little amplification bias when cultured *P. falciparum* parasites were analysed (Carret *et al.*, 2005). As clinical samples often contain both human and *P. falciparum* DNA, there have been worries that the amplification of *P. falciparum* DNA could be compromised due to the high A+T content of the *P. falciparum* genome compared to the human. However recent studies confirm that sequence representation is preserved also in samples containing both human and *falciparum* DNA and it can be used in several downstream applications (Volkman *et al.*, 2007, Wang *et al.*, 2009). Since the MDA reaction is performed isothermally, the preference for some genetic regions due to nucleotide content can be avoided (Lasken & Egholm, 2003). In paper IV we used the Illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, Little Chalfont, UK), a MDA kit adjusted for low amount of DNA (<10ng). When we compared gDNA and MDA DNA in SNP analysis with pyrosequencing in mixed infections, the two methods showed notable concordance. However SNPs in single samples were identified by

sequencing of samples after MDA that were not confirmed when repeated from gDNA. These SNPs could derive from MDA, but it is more possible that they originate from downstream applications such as PCR or sequencing. These SNPs were clearly not included in the SNP analysis. Further studies need to be done to confirm that MDA represents gDNA also when analysing field samples containing mixed infections from filterpaper. All results up to this point indicate that MDA can be used to extend the supplies of valuable DNA stocks also in *P. falciparum*.

3.7 PYROSEQUENCING

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

Advantages	Disadvantages
Sensitive	Not applicable for all SNPs
Specific	Sometimes skewed without standardization
Can be standardized	Standardization can be difficult without a mutant lab strain
Easy	
Inexpensive	
Quantitative	
Detects several alleles/SNPs in one run	
Repeatable results	

Table 1. Advantages and disadvantages with pyrosequencing in *P. falciparum*. Content based on experience from paper I-IV and (Zhou *et al.*, 2006)

Pyrosequencing was used in paper I-IV to genotype the SNPs E431K in *PfATP6* and I876V and K1466R in *pfmrp1*. For I876V the results were adjusted against a standard curve derived from different proportions of mixes of the reference laboratory strains 3d7 and Dd2. When this adjustment is made, pyrosequencing allele quantification can successfully be performed. The results of the SNPs analysis of *PfATP6* E431K and *pfmrp1* K1466R were not adjusted against a standard curve because of the lack of a mutant among the reference laboratory strains. The definition of a mixed infection was a pyrosequencing result between 10% and 90% for both alleles.

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3.8 SNP ANALYSIS OF MIXED INFECTIONS

In high transmission areas a large portion of the malaria patients carry multiple *P. falciparum* populations. Mixed infections may constitute an obstacle in the analysis of associations between polymorphisms and drug response *in vivo* and/or *in vitro*. Detection of minority populations within mixed infections may be difficult as well as how to handle the statistical analysis of mixed infections.

3.8.1 Detection of mixed infections

Detection of minority populations within mixed infections can be difficult, but is important especially *in vivo* where a minority population can be selected and expand under drug pressure. There are several methods used for *P. falciparum* SNP analysis with varying success to distinguish mixed infections.

Direct sequencing is not a very sensitive method to detect mixed infections (Zhou *et al*., 2006). Efforts have been made to try to ameliorate the analysis of mixed infections by normalizing against a pure clone or standard curve (Hunt *et al.*, 2005). However background noise from sequencing of PCR products from mixed population from the field can disturb these analyses. Sequencing can however be very useful to identify new polymorphisms and analyse DNA fragments containing many potential SNPs.

PCR-RFLP (restriction fragment length polymorphism) can be quite sensitive in the detection of mixed infections. However mixed infections and incomplete digestions can be difficult to separate, making the method less trustworthy and it often needs to be repeated (Zhou *et al.*, 2006). Furthermore the method requires visual interpretation of the results, which can lead to intra- and interindividual discrepancies. Real time PCR (RT-PCR) seems to be a quite sensitive method to detect mixed infections, but the parasite DNA concentration of each sample needs to be determined before analysis since the method can give different results depending on DNA concentration (Alker *et al.*, 2004, Ochong *et al.*, 2008). More studies are needed to determine the usefulness of RT-PCR in mixed infections.

Pyrosequencing, described in section 3.7, is as good as PCR-RFLP or better in distinguishing mixed infections even when the results are not adjusted against a standard curve (Zhou *et al*., 2006). From our analyses we have learnt that pyrosequencing can be more robust and even quantitative if adjusted against a standard curve. Thus pyrosequencing can be recommended as a sensitive method to detect mixed infections and have been the preferred option for the analysis of associations between *pfmrp1* I876V and K1466R and drug response *in vivo* in paper II and III. In paper I, II and IV sequencing was used since the diversity of the genes in the studied areas had not been extensively explored previously. Hence in paper IV some mixed genotypes may have been interpreted as pure, which could blur the association between genotype and phenotype. However, the majority of the isolates from Thailand are expected to be single infections due to the characteristics of the malaria setting.

3.8.2 Statistical analysis of mixed infections

Drug-derived selection is based on the assumption that a more resistant parasite population can grow at clinically relevant drug concentrations, while a more sensitive population cannot grow at the same drug levels, and will be eliminated.

In the example of *pfmrp1* I876V *in vivo*, selection of I876 is based on that parasite populations carrying the I876 allele survive, while all parasites carrying 876V tend to be eliminated. Elimination of parasites carrying 876V will occur independently of if only these parasites are infecting (pure) or if there are also parasites with I876 in the infection (mixed). Mixed infections with I876 and 876V should consequently become pure I876 after selection. Thus we suggest that *in vivo* the selected genotype should be analysed against the mixed infections together with the non-selected genotype. In paper II data was presented accordingly, while in paper III three options were presented; the mixed infections was counted as either the selected or the non-selected genotype or the mixed infections were completely removed from the calculations. In the frame of the thesis we have chosen to present what we think is the optimal way of counting, namely with mixed infections as the non-selected genotype. This method of analysing mixed infections has also been applied by others (Dokomajilar *et al.*, 2006b).

Statistical calculations of mixed infections in *in vitro* studies are more difficult since all populations in an isolate are likely to contribute to the phenotype. Therefore we choose to remove the mixed infections from the association analyses of the particular genotype that was mixed, not to blur the results.

3.9 STATISTICAL ANALYSES

3.9.1 Nucleotide diversity

To have a measure of DNA polymorphism in the three different analysed genetic regions of *PfATP6* in paper I we used the concept of nucleotide diversity (Nei, 1987) that was estimated with DnaSP software version 4.0 (Rozas & Rozas, 1999). Nucleotide diversity (π) can be described as the average proportion of nucleotide differences between all possible pairs of sequences in the sample and can be simplified accordingly:

π = [(total # differences) / (# pairwise comparisons)] / size of sequence

3.9.2 *In vivo* studies

In paper II and III Fisher's exact two-tailed test was used to evaluate the difference in SNP prevalence between the baseline and recurrent infections. Statistical significance was defined as $p < 0.05$. All enrolled patients were included in the baseline independent of which treatment arm they were allocated to. In paper II the mixed infections were grouped with the non-selected genotype and analysed against the selected genotype. Paper III presented three methods of analysing the mixed infections; the mixed infections were counted as either the selected or the non-selected genotype or the mixed infections were completely removed from the calculations. In the frame of the thesis the results were presented with the mixed infections analysed together with the non-selected genotype, since we consider this being the optimal way of analysing mixed infections (see section 3.8).

In paper II and III the χ^2 test was used to compare observed and expected frequencies to test for an association between genotypes. In paper II mixed infections were removed from this analysis not to blur the association. In paper III association between residue 1466 and the *pfdhfr/pfdhps* quintuple was evaluated and the mixed genotypes in position 1466 were accounted as K, the selected genotype, since this is according to the premises that the *pfdhfr/pfdhps* haplotype is defined after (Kublin *et al.*, 2002). All statistical analyses were performed with GraphPad QuickCalcs (GraphPad Software Inc. San Diego, CA).

3.9.3 *In vitro* studies

The statistical analyses of association between genotypes and *in vitro* drug susceptibility in paper IV were performed with the non-parametric test Mann-Whitney U. A non-parametric test should be performed when the data is not normally distributed. As the test is based on ranking, the absolute EC values are not of importance, which moderate the effect of outliers and provide robust results. In this study the test is ranking the drug susceptibilities and comparing the sum of ranks of the

wild type and the mutant group, and based on U statistics an exact *p*-value of the difference between the groups is provided. For larger samples the U statistics is approximating normal distribution and an asymtotic *p*-value can be used. Therefore the asymptotic *p*-value was used when the size of each genotype group was larger than 10 (Motulsky, 1995). Statistical significance was defined as $p < 0.050$. The statistical analyses were performed in SPSS.

4 RESULTS AND SPECIFIC DISCUSSION

4.1 *PFATP6* DIVERSITY (PAPER I AND IV)

Since little was known about the natural diversity of *PfATP6*, the proposed target of ART, we analysed the diversity of *PfATP6* in a large number of *P. falciparum* samples of different geographical origins including locations in Africa, Southeast Asia, Pacific and South America. We chose to study three genetic regions and one SNP in *PfATP6* based on earlier findings: (a) region 1, the homologue sequence of *PfATP6* of the thapsigargin-binding cleft in mammalian SERCA including the amino acid 263, (b) region 2, the interspecies variable region surrounding amino acid 623, (c) region 3, the region surrounding amino acid 769 and (d) the SNP E431K.

We have showed for the first time that *PfATP6* harbour considerable sequence biodiversity, probably due to the large sample size analyzed, in comparison with previous studies. Sequencing of 388 *P. falciparum* samples resulted in identification of 33 SNPs, 29 non-synonymous and 4 synonymous (paper I). One additional synonymous SNP in residue 490 was identified after the sequencing of fresh *P. falciparum* isolates from Uganda (n=30) and Thailand (n=48) (paper IV). Most SNPs were found in low frequency (≤2%) and originated from Zanzibar and Uganda. The SNPs E431K, N569K and A630S were present in comparably high frequencies in Zanzibar, Tanzania and Uganda and were also observed in samples from several other African countries. In South East Asia the only SNP found was N683K, present in samples from Thailand and Cambodia.

No variation was found in *PfATP6* amino acid 263, suggested to be involved in artemisinin binding (Uhlemann *et al.*, 2005), a result consistent with previous observations (Cojean *et al*., 2006, Ferreira *et al*., 2007, Ibrahim *et al.*, 2009, Menegon *et al.*, 2008, Price *et al*., 2004). Since natural variation in this amino acid has never been found it is currently impossible to evaluate its importance *in vivo*.

Variation was not found in residue 769, previously associated with decreased artemisinin susceptibility in fresh isolates from French Guiana (Jambou *et al.*, 2005). S769N has not identified elsewhere (Ferreira *et al*., 2007, Ferreira *et al.*, 2008, Ibrahim *et al*., 2009, Jambou *et al*., 2005, Menegon *et al*., 2008, Mugittu *et al*., 2006b, Price *et al*., 2004, Zhang *et al.*, 2008) except for in one isolate sensitive to DHA (Cojean *et al*., 2006). A fresh isolate from French Guiana with high IC_{50} for artemether and both S769 and 769N alleles was put in culture *in vitro*. After three weeks in culture the mutant was not detected and the IC_{50} had decreased markedly, suggesting that the mutant had poor fitness (Legrand *et al.*, 2008). If 769N confers a loss of fitness to the parasite, this

SNP may not be detected in a parasite population as long as the population is not under substantial artemisinin selective pressure which could explain why the SNP is not yet spreading. Alternatively S769N may not be important in other settings and in parasites with other genetic backgrounds than in French Guiana.

Most of the identified SNPs in Zanzibar where located in region 2 (see figure 1 in paper I) in which the nucleotide diversity was one order of magnitude greater ($\pi = 1.7 \times 10^{-3}$) than regions 1 ($\pi = 1.1 \times 10^{-4}$) and 3 ($\pi = 2.7 \times 10^{-4}$). In Uganda 9/10 identified SNPs were located in region 2 as well. Region 2 is unique to *P. falciparum* and is located in the interspecies variable region, which is conserved in several *Plasmodium* species. This protein region has been suggested to influence *Plasmodium* regulation of SERCA activity (Kimura *et al.*, 1993, Tanabe *et al.*, 2004). One way to interpret this observation is that SNPs can easily accumulate in this less conserved region, since they will not significantly affect the function of the protein, hence not influencing the fitness of the parasite. On the other hand, all the 20 SNPs identified in region 2 were non-synonymous, which may raise the question if they are under positive selection (Hughes & Verra, 2002).

Although the studies in Zanzibar and Tanzania were conducted in 2002-2004 before the implementation of ACT when artemisinin pressure was probably minimal in the area, the diversity of *PfATP6* was surprisingly similar to the study in Gulu, Uganda, which was performed in 2007, where artemisinin pressure was probably substantially higher because of uncontrolled usage of ACT. These results indicate that artemisinin may not yet have selected for any of the analysed SNPs in Uganda.

4.2 PFATP6 AND *IN VITRO* SUSCEPTIBILITY TO ARTEMISININ (PAPER IV)

In vitro susceptibility to artemisinin was tested in 48 fresh isolates from Mae Sot, Thailand and 30 fresh isolates from Gulu, Uganda. In both sites the susceptibility to artemisinin was highly variable, ranging from 1.5-40.5nM in Thailand and 5.0-175.9nM in Uganda. The observed variability in artemisinin susceptibility could not be explained by association with the analysed *PfATP6* SNPs. However only N569K and A630S in Uganda and N683K in Thailand showed enough variation to be evaluated. The most artemisinin resistant sample in Uganda was a 569K and 747Y double mutant, and further investigation is needed to determine its importance.

4.3 *PFMRP1* DIVERSITY (PAPER II AND IV)

Paper II is the first comprehensive study of *pfmrp1* diversity. The *pfmrp1* open reading frame (ORF) was sequenced in 103 *P. falciparum* infections originating from most malaria endemic regions (paper II) and in 47 fresh *P. falciparum* isolates from Mae Sot, Thailand and 30 from Gulu, Uganda (paper IV). *pfmrp1* was observed to harbor significant biodiversity with 23 non-synonymous SNPs, 8 synonymous SNPs and one insert were identified in the gene. The distribution of the SNPs showed distinct geographic patterns (table 2). In Africa the most common SNPs were I876V and K1466R, with no other SNP seen in more than two samples. K1466R was only found in Africa and Papua New Guinea (PNG).

The majority of SNPs were identified in samples with origin in Asia and Oceania. There the most common SNPs were H191Y and S437A, which were linked and mutated in almost all samples. Distinct haplotypes of *pfmrp1* SNPs in amino acid positions 785, 876, 1007 and 1390 were observed. Either they were all wildtype, mutated in positions 876 and 1390 or mutated in positions 785, 876 and 1007 with or without mutation in position 572. Interestingly, mutations in 1390 and 1007 were mutually exclusive, suggesting a potential functional implication. Several SNPs were specific for PNG and Vanuatu and diversity in the latter seem to be low, as previously described for other loci (Sakihama *et al.*, 2001a). I876V was the most spread SNP worldwide, present in all parasite populations except for in South America where no SNPs in *pfmrp1* were observed. Since the diversity is varying so much between different geographical regions it can be recommended to analyse phenotypic associations with *pfmrp1* SNPs within a region. Otherwise the region can be a confounder in the relation between *pfmrp1* SNPs and parasite drug susceptibility.

		*pf*MRP1 polymorphisms											
Origin	**N**	**37**	**191**	**202**	**325**	**437**	**572**	**785**	**876**	**1007**	**1390**	**1431**	**1466**
Africa													
Africa	19	P	H	K	N	S	F	H	I	T	F	K	K
Benin	1	P	H	K	N	S	F	H	I	T	F	K	**R**
Gambia	1	P	H	K	N	S	F	H	**V**	T	**I**	K	K
Ghana	1	P	H	K	N	S	F	H	I	T	F	K	**R**
Guinea Conakry	1	P	H	K	N	S	F	H	I	T	F	K	**R**
Kenya	1	P	H	K	N	S	F	H	**V**	T	F	K	**R**
Malawi	1	P	H	K	N	S	F	H	**V**	T	F	K	**R**
Uganda	9	P	H	K	N	S	F	H	I	T	F	K	K
	9	P	H	K	N	S	F	H	**V**	T	F	K	**R**
	9	P	H	K	N	S	F	H	I	T	F	K	**R**
	5	P	H	K	N	S	F	H	**V**	T	F	K	K
Middle East													
Iran	2	P	**Y**	K	N	**A**	F	H	I	T	F	K	K
	1	P	**Y**	K	N	**A**	F	H	**V**	T	F	K	K
	1	P	**Y**	K	N	**A**	F	H	**V**	**M**	F	K	K
Yemen	1	P	H	K	N	S	F	H	I	T	F	K	K
South East Asia													
Cambodia	3	P	**Y**	K	N	**A**	F	**N**	**V**	**M**	F	K	K
	2	P	**Y**	K	N	**A**	**L**	**N**	**V**	**M**	F	K	K
	2	P	H	K	N	S	F	H	I	T	F	K	K
	1	P	**Y**	K	N	**A**	F	H	**V**	T	**I**	K	K
	1	P	**Y**	K	**S**	**A**	F	H	I	T	F	K	K
Thailand	14	P	**Y**	K	N	**A**	**L**	**N**	**V**	**M**	F	K	K
	8	P	**Y**	K	N	**A**	F	H	**V**	T	**I**	K	K
	7	P	**Y**	K	**S**	**A**	F	H	I	T	F	K	K
	6	P	**Y**	K	N	**A**	F	**N**	**V**	**M**	F	K	K
	2	P	**Y**	K	N	**A**	F	H	I	T	F	K	K
	2	P	H	K	N	S	F	H	I	T	F	K	K
	1	P	**Y**	K	**S**	**A**	**L**	**N**	**V**	**M**	F	K	K
	1	P	**Y**	K	N	**A**	F	**N**	**V**	T	**I**	K	K
	1	P	**Y**	K	**S**	**A**	**L**	**N**	**V**	T	F	K	K
	1	P	**Y**	K	**S**	**A**	F	H	**V**	T	**I**	K	K
	1	P	**Y**	K	**S**	**A**	F	H	I	T	F	K	K
Oceania													
Papau New Guinea	3	**S**	**Y**	**E**	N	**A**	F	H	**V**	T	**I**	K	K
	1	P	**Y**	K	N	**A**	F	H	**V**	T	F	K	**R**
	1	P	H	K	N	S	F	H	I	T	F	K	**R**
Vanuatu	6	P	**Y**	K	N	**A**	F	H	**V**	T	**I**	**I**	K
	1	P	**Y**	K	N	**A**	F	H	**V**	T	**I**	K	K
South America													
Colombia	13	P	H	K	N	S	F	H	I	T	F	K	K
Surinam	1	P	H	K	N	S	F	H	I	T	F	K	K

Table 2. *pf*MRP1 haplotype diversity in different geographical regions. Mutations are marked in bold and highlighted in grey. This table is based on mutations that were present in more than two samples.

Secondary structure analysis by HMMTOPv2 software predicted that *pf*MRP1 is constituted by 12 transmembrane helices distributed in two MSDs, each followed by an NBD as a typical short MRP (paper II), which confirms previous secondary structure predictions (Klokouzas *et al.*, 2004). Several of the identified SNPs are positioned near predicted functionally important protein regions; transmembrane domains, expected to control substrate specificity (Deeley & Cole, 2006) and NBDs, important for ATP binding and hydrolysis of the protein (Center *et al.*, 1998) (figure 7). Natural variation in these regions suggests the existence of *pf*MRP1 proteins with variable transporting capacities.

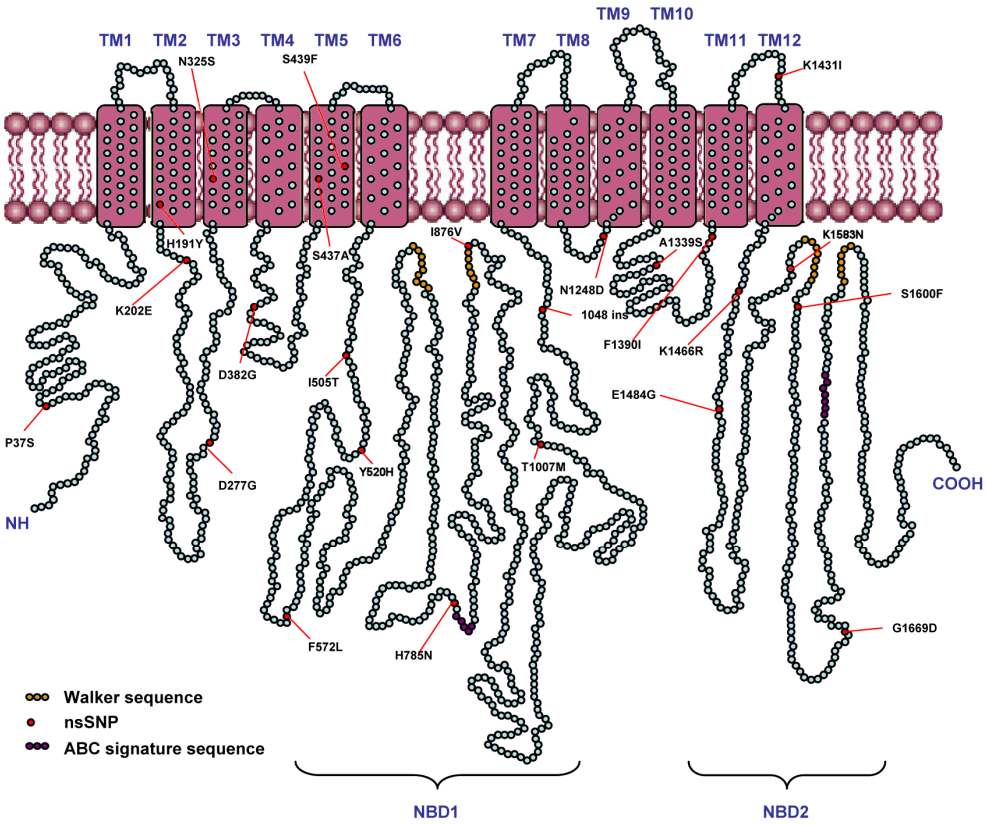

Figure 7. *pf*MRP1 predicted 2D transmembrane domain organization by the HMMTOPv2 software, and positions of non-synonymous SNPs identified in paper II and IV. TM = transmembrane domain, NBD = nucleotide binding domain.

4.4 *PF*MRP1 IN CLINICAL EFFICACY TRIALS (PAPER II AND III)

4.4.1 ACTs (paper II)

The most common *pfmrp1* polymorphisms identified in Africa, I876V and K1466R, were analyzed in *P. falciparum* samples from malaria-infected patients, in two clinical drug efficacy trials in Tanzania (n=106) and Zanzibar (n=408), treated with artemether-lumefantrine vs. SP or artemether-lumefantrine vs. artesunate-amodiaquine, respectively. There were no statistically significant changes in frequency of K1466R after artemether-lumefantrine or artesunate-amodiaquine treatment. We observed a

statistically significant positive selection of the pure I876 allele in the recurrent infections (92.1%) of the artemether-lumefantrine arm compared to the baseline (76.9%) (p= 0.038) in Zanzibar. In Tanzania the same tendency was detected after artemether-lumefantrine treatment, although it was not statistically significant. Pooling the two independent studies supplied robust evidence for a strong selection of the pure I876 in recurrent infections (89.5%) compared to the baseline (75.9%) (p= 0.007). As the majority of the artemether-lumefantrine recurrent infections in these studies were reinfections (Martensson *et al*., 2005, Martensson *et al*., 2007), we believe that the observed selection is mainly due to selection of reinfections by sub-therapeutic levels of lumefantrine. No evidence of selection was observed after artesunate-amodiaquine treatment.

The localization and the predicted function of residue 876 may provide an insight in the difference in selection observed between the tested drug combinations. Residue 876 is located in NBD1, immediately downstream of the Walker B motif (figure 7), an important region for ATP binding and hydrolysis. *In silico* analysis suggested that variation in residue 876 may influence ATP docking and consequently the catalytic cycle and potentially the overall protein function (paper II). It is unlikely, but not impossible, that 876 would also affect substrate specificity of *pf*MRP1. It is more probable that the observed difference in selection of 876 between the two treatments depends on that *pf*MRP1-mediated transport is more important for the parasite response to lumefantrine than to amodiaquine/DEAQ. This may be due to the location of the target of the respective drugs. The main target of amodiaquine/DEAQ is not known, but it is probable that it can be located in the food vacuole, as the target for chloroquine, another 4-aminoquinoline. The target for lumefantrine is not yet identified, however it has been suggested to be located outside the food vacuole (Sisowath *et al.*, 2009), similarly to the target of the related arylaminoalcohol, mefloquine(Famin & Ginsburg, 2002). Hence, *pf*MRP1, that is located in the plasma membrane, may be more important for drugs with their respective targets outside the food vacuole, like lumefantrine, while drugs with a target in the food vacuole, like amodiaquine/DEAQ, can be more dependent on transporters in the membrane of the food vacuole, e.g. PfCRT and PfMDR1. However also drugs acting in the food vacuole may be associated with plasma membrane transporters as *pf*MRP1 for the final extrusion from the parasite, after efflux from the food vacuole.

4.4.2 SP (paper III)

Since MRPs can contribute to antifolate resistance in mammalian cells (Stark *et al*., 2003, Hooijberg *et al.*, 1999, Zeng *et al.*, 2001) we wanted to determine the role of *pf*MRP1 in the *P. falciparum* response to antifolate antimalarials, by testing if the most

common SNPs in *pfmrp1*, I876V and K1466R, were under SP selection *in vivo*. In a clinical efficacy trial conducted in Tanzania (n=106) which included an arm with SP treatment, the frequencies of I876V and K1466R were compared between baseline infections and the recrudescences and reinfections after SP treatment. There was a statistically significant selection of the pure K1466 allele among the recrudescences (12/14, 85.7%) compared to the at baseline (52/101, 51.1%) (Fisher, two-tailed, p = 0.020). Furthermore 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 (Fisher, two-tailed, p = 0.002). Importantly, there was no association between K1466 and the *pfdhfr/pfdhps* quintuple, indicating that K1466 is selected independently of the quintuple haplotype.

An explanation for the observed selection could be that sulfadoxine and pyrimethamine are transported out of the cell by *pf*MRP1. However this is a less likely explanation since these drugs are not organic anions, the typical MRP substrates. Alternatively, the *pfmrp1* SNP can be selected if it affects MRP mediated efflux of folates out of the cell. In mammalian cells MRPs have been shown to transport folates (Assaraf *et al.*, 2003, Zeng *et al.*, 2001). When MRP mediated transport of folates is impaired there is an expansion of the intracellular folate pool, which can compete with pyrimethamine in the binding to its target DHFR (Assaraf, 2006). Hence impaired MRP activity can cause resistance to SP (Stark *et al.*, 2003). The *pf*MRP1 protein harbouring the selected K1466 allele probably transports folates less well, thereby contributing to SP resistance in the parasite.

4.5 *PF*MRP1 *IN VITRO* (PAPER IV)

The influence of the identified polymorphisms in *pfmrp1* on drug susceptibility *in vitro* was assessed in fresh *P. falciparum* isolates from Mae Sot, Thailand (n=48) and Gulu, Uganda (n=30). The tested antimalarial drugs included artemisinin, chloroquine, quinine, lumefantrine and its metabolite desbutyl-lumefantrine (DBB) in both study sites, mefloquine, atovaquone and pyronaridine only in Thailand and amodiaquine solely in Uganda. The complete range of effective concentrations from EC_{10} to EC_{90} was analysed to properly understand the association of the evaluated genotype in drug susceptibility. We demonstrated that polymorphisms in *pf*MRP1 were associated with *P. falciparum* responses to all the studied drugs, except for pyronaridine, in either Thailand or Uganda (table 3), suggesting that *pf*MRP1 may be a true multidrug resistance factor. Importantly, artemisinin, mefloquine, DBB and atovaquone susceptibilities were associated with some common *pfmrp1* SNPs, suggesting related mechanism of resistance and potential cross-resistance. In addition, susceptibility to

Study site	Less susceptible genotype	Drug	*p*-value	Association (*p*-values)								
				EC_{10}	EC_{20}	EC_{30}	EC_{40}	EC_{50}	EC_{60}	EC_{70}	EC_{80}	EC_{90}
Thailand	H191	Mefloquine	e			0.048	0.036	0.031	0.031	0.026	0.036	
	N325	Chloroquine	e	0.048	0.03	0.03	0.009	0.039				
	S437	Mefloquine	e			0.048	0.036	0.031	0.031	0.026	0.036	
	F572	Artemisinin	a				0.031	0.016	0.024	0.021	0.041	
		Atovaquone	e		0.038							
	H785	Artemisinin	a					0.033	0.042	0.03	0.03	
		DBB	a	0.008	0.008	0.013	0.013	0.013	0.013	0.014	0.014	
		Atovaquone	a	0.009	0.009	0.018						
		Mefloquine	a		0.032	0.035	0.032	0.032	0.049			
	I876	Artemisinin	e					0.027	0.033			
		Atovaquone	e	0.014	0.014	0.029						
	876V	Chloroquine	e		0.03	0.027	0.014	0.045				
	T1007	Artemisinin	a					0.024	0.031	0.02	0.02	0.04
		DBB	a	0.013	0.012	0.017	0.016	0.017	0.019	0.019	0.016	
		Atovaquone	a	0.008	0.007	0.014						
		Mefloquine	a		0.028	0.033	0.028	0.028	0.042			
	1390I	Lumefantrine	e								0.041	
	wt+double mutant	Artemisinin	a					0.029	0.045	0.035	0.032	
		DBB	a	0.004	0.004	0.007	0.007	0.007	0.009	0.009	0.012	
		Atovaquone	a	0.005	0.01	0.022						
		Mefloquine	a		0.033	0.037	0.027	0.025	0.033	0.04		
Uganda	876V	Quinine	e	0.029	0.029	0.039	0.029					
	K1466	Amodiaquine	e	0.022	0.019	0.016	0.034					

Table 1. *pf*MRP1 polymorphisms associated with decreased drug susceptibility *in vitro*

these drugs, with the exception of atovaquone, was associated with *pfmdr1* gene amplification.

Of the drug response-associated SNPs in *pf*MRP1, amino acids 191, 325 and 437 were located in the predicted transmembranes domains, 572, 785, 876, 1007 and 1466 in the NBDs and 1390 in a cytoplasmic loop (figure 7). Residues in transmembranes domains and cytoplasmic loops of human MRPs may affect the substrate specificity (Deeley & Cole, 2006) while residues in the NBDs can affect the catalytic cycle of the protein and thereby its overall function (Center *et al.*, 1998). In paper II the T1007M alteration was predicted to affect *pf*MRP1 function, which supports the associations between T1007M and drug response observed in this study.

The *in silico* results from paper II suggest that variation in residue 876, positioned immediately downstream of the Walker B motif in NBD1 of *pf*MRP1, can influence the ATP hydrolysis cycle. We observed an association between I876 and decreased susceptibility to artemisinin, but between 876V and chloroquine and quinine. These results are incoherent with previous data suggesting that chloroquine, quinine and artemisinin can all be transported by *pf*MRP1 (Raj *et al.*, 2009), and that residue 876 can participate in the catalytic cycle and thereby the overall function of the protein (paper II), thus indicating that a mutation in position 876 should have the same effect on the three drugs. However, in addition to the catalytic cycle, residue 876 could be related to substrate specificity as well, as previously described for residues in NBDs (Benabdelhak *et al.*, 2003), which would explain the association with different genotypes. Alternatively position 876 could be associated with other polymorphisms that direct the association.

Decreased susceptibility to amodiaquine was associated with *pfmrp1* K1466. These results are not consistent with the results from paper II where no selection of K1466 was observed after treatment with artesunate-amodiaquine. However *in vivo* amodiaquine is rapidly metabolized to DEAQ from which most of the antimalarial activity (Li *et al.*, 2002), and thereby the main selection pressure is derived. Furthermore it has been suggested that amodiaquine and DEAQ may have different mechanisms of resistance (Echeverry *et al.*, 2007), which could explain the discrepancy between these results and paper II.

An association between *pfmrp1* F572, H785, I876, T1007 and wild type and double mutant haplotypes and decreased susceptibility to atovaquone was demonstrated. Resistance to atovaquone alone or in combination has previously been shown to occur through mutations in amino acid 268 in cytochrome *b* (Musset *et al.*, 2006, Sutherland *et al.*, 2008). Nevertheless treatment failures without cytochrome *b* mutations have

been observed, which suggests other mechanisms of resistance (Wichmann *et al.*, 2004) (Farnert *et al.*, 2003) and supports the herein demonstrated association. Maybe *pfmrp1* variation could explain why we observed a broad range of atovaquone susceptibilities, with many isolates with intermediate effective concentrations between the very low ($EC_{50} \leq 30$nM) and high ($EC_{50} > 1900$nM) values previously observed (Musset *et al.*, 2006).

5 OVERALL DISCUSSION AND REFLECTIONS

5.1 ROLE OF PFATP6 IN ART RESISTANCE

Investigations from S. Krishna's group in the University of London has provided convincing results suggesting that PfATP6 is a target of ART and that a residue in the M3 domain of PfATP6 may be involved in artemisinin binding to the protein, through heterologous expression of PfATP6 in *Xenopus* oocytes (Eckstein-Ludwig *et al*., 2003, Uhlemann *et al*., 2005). However the relevance of their findings needs to be confirmed in *P. falciparum* parasites *in vivo* and *in vitro*, in phenotypes with various sensitivities to ART.

The first indications of decreased susceptibility and even resistance to ART *in vivo* and *in vitro* have been reported. As decreased susceptibility or resistance *in vivo* have been demonstrated mainly after artesunate monotherapy (Menard *et al*., 2005, Noedl *et al*., 2008) and as WHO strongly recommends that ART should be used only in combination with other antimalarial drugs in treatment of uncomplicated *falciparum* malaria (WHO, 2005a), there are yet quite limited possibilities to study the influence of *P. falciparum* polymorphisms on treatment efficacy in clinical trials. In paper I we wanted to make a comprehensive report on *PfATP6* natural diversity to provide groundwork for following studies investigating the association of *PfATP6* polymorphisms when ART resistant or tolerant phenotypes emerge. Also, the difference in natural diversity between areas with different drug susceptibilities and drug pressure may provide an insight in the selection of drug related SNPs, as seen when comparing diversity in paper I and IV. The *PfATP6* diversity in Uganda, after years of ACT and maybe also ART pressure, was remarkably similar to the observed diversity in Tanzania and on Zanzibar before ACT was used. Either there is still little drug pressure on the study area in Uganda or the studied SNPs in *PfATP6* are of reduced importance in the response to ART.

The results of *in vitro* studies of artemisinin variability are yet difficult to interpret. Several studies have shown great variation in susceptibility to ART, with Jambou and colleagues even proposing to have identified artemisinin resistant fresh isolates (Jambou *et al*., 2005). However the clinical importance of this phenotype variability is difficult to determine as there is no established *in vitro* cut-off value for resistance to ART. The observed relatively high variability in artemisinin responses and higher mean effective concentrations in Uganda than in Thailand, in paper IV, may represent the first step towards ART resistance, although not having reached there yet in the majority of the parasite populations. This could explain the lack of association between PfATP6 SNPs and artemisinin response seen.

Mammalian SERCA are vital proteins and mutations in human SERCA can result in various diseases (Hovnanian, 2007). Therefore mutations in PfATP6 that affect both ART resistance and SERCA function may not be visible in a parasite population until the level of drug pressure creates a favorable cost-benefit relation between resistance and its associated loss of fitness. The fitness loss may be an explanation for why the mutated genotype of 769 does not seem to spread (Legrand *et al*., 2008). Consequently it is useful to follow up on functionally interesting SNPs, although their role in ART response in different areas is yet uncertain. From this point of view, another SNP in PfATP6 that could be worthwhile to further study is H747Y that was present in the most artemisinin tolerant/resistant isolate in Uganda, as reported in paper IV.

5.1.1 Other mechanisms of ART action and resistance

Although there are evidences supporting that PfATP6 might be a target of ART other mechanisms of action for ART cannot be excluded. Suggested alternatives include generation of free radicals and associated pleiotropic cytotoxic effects, alkylation of *Plasmodium* enzymes and targeting of cysteine proteases, proteins in the electron transport chain and the translationally controlled tumor protein (Krishna *et al.*, 2006). Besides mutations in the target, other mechanisms of resistance involving transport/efflux of ART may be central independent of ART target. Accordingly, gene amplifications and SNPs in *pfmdr1* can affect the parasite response to ART (Pickard *et al.*, 2003, Price *et al*., 2004, Sidhu *et al.*, 2005, paper IV). Furthermore new findings presented in this thesis suggest that polymorphisms in *pfmrp1* can also be of importance in the *P. falciparum* defense mechanisms against ART, supported by results from the disruption of *pfmrp1* (Raj *et al*., 2009). In conclusion, studies so far point for a potential role of PfATP6 in ART action and resistance, but also leaves the floor open for other possible targets and particularly for alternative mechanisms of resistance.

5.1.2 Future perspectives

The majority of *PfATP6* diversity studies, as paper I, have focused on analyzing polymorphisms in the transmembrane domain M3, including residue 263, the vicinity of residue 431, and/or the interspecies variable region, including residues 623 and 769. Other important genetic regions that may have been overlooked are M5 and M7, proposed to be involved in artemisinin binding (Jung *et al*., 2005, Uhlemann *et al*., 2005), which merits further investigation.

Further understanding of the proposed mechanism action by which artemisinin affect PfATP6 would clarify its role. A key issue is to understand the mechanism behind the rapid parasitocidal effect of ART. Is it due to induction of calcium release in the same way as demonstrated in *P. falciparum* by thapsigargin (Varotti *et al*., 2003)?

Furthermore transfection of proposed key functional mutations in *P.falciparum* is the next crucial step both to understand the function of the specific residues, but more importantly to confirm the role of PfATP6 as a target of ART since two of the key studies supporting its role (Jambou *et al*., 2005, Uhlemann *et al*., 2005) are based on particular amino acid changes. This crucial study is currently in progress (Fidock *et al.*, 2008).

5.2 ROLE OF *PF*MRP1 IN *P. FALCIPARUM* DRUG RESISTANCE

Recent advances provide the first promising steps describing an involvement of *pf*MRP1 in *P. falciparum* drug response. The disruption of pfMRP1 resulted in increased *in vitro* sensitivity to chloroquine, quinine, artemisinin, piperaquine and primaquine (Raj *et al*., 2009). In paper II, a comprehensive study of the natural diversity of *pf*MRP1 was performed and a number of SNPs were identified, most of which have not been described previously. These SNPs were further studied in papers II and III where I876 was shown to be selected in recurrent infections in patients treated with artemether-lumefantrine and K1466 was selected in recrudescent infections after SP treatment. Furthermore SNPs in *pf*MRP1 were associated with decreased *in vitro* susceptibility to all the studied drugs in paper IV, except for pyronaridine. These studies strongly suggest that *pf*MRP1 has a role in *P. falciparum* response to several structurally unrelated drugs, thus representing a putative mechanism of multidrug resistance, particularly suitable to the development of the parasite evasion strategies to the present ACT-based multidrug exposure.

5.2.1 *pf*MRP1 drug response mechanisms

Mammalian MRPs have been shown to confer resistance to a large range of xenobiotics through their efflux from the cell. Coherent with this mechanism, chloroquine and quinine were shown to be transported by *pf*MRP1 *in vitro* (Raj *et al*., 2009). It is probable that other drugs, whose sensitivity is altered by *pf*MRP1 polymorphisms, could also be transported by *pf*MRP1.

Since it was suggested that *pf*MRP1 is an exporter of GSSG (Bozdech & Ginsburg, 2004, Raj *et al*., 2009) an alternative/additional role of *pf*MRP1 could be to respond to oxidative stress generated by antimalarial drugs, as seen in human MRPs (Leier *et al*., 1996). *P. falciparum* is heavily exposed to oxidative stress originating from its metabolism of hemoglobin (Muller, 2004). Furthermore the parasite also needs to supply its host cell with GSSG since the infected RBC loses the ability to synthesise GSH. Therefore the parasite is dependent on an efficient antioxidant defence where intense de novo synthesis of GSH and export of GSSG plays an important role (Becker *et al.*, 2004). It has been demonstrated that chloroquine, amodiaquine and artemisinin

induce oxidative stress in the parasite. However it has been debated if they actually act by increasing the oxidative stress (Becker *et al.*, 2004, Golenser *et al.*, 2006, Parapini *et al.*, 2004). If that is the case, increased drug effect management of the parasite, through the action of MRPs, could be a general mechanism of evading the action of the drug.

There is yet another particular mechanism by which MRPs can affect drug resistance: by increasing the amount of a substance antagonizing the drug action. This can be important especially for drugs with one specific target, which is generally the case for antifolates. MRPs can transport folate out of the cell, as a normal physiological function. Repression of MRP genes impairs the export of folate, which may result in increased intracellular folate concentration and resistance to pyrimethamine, as demonstrated in mammalian cell lines selected for pyrimethamine resistance (Stark *et al*., 2003). Accordingly, we believe that the observed *in vivo* selection of K1466 in SP treated patients with recrudescent infections, is due to a poor folate transport by *pf*MRP1 with K1466, resulting in decreased pyrimethamine sensitivity contributing to treatment failures.

5.2.2 Substrate binding and interactions with the NBD

The mechanism of substrate binding and subsequent transport of P-gp is reasonably well understood. P-gp is "vacuum cleaning" the inner leaflet of the plasma membrane and hydrophobic substrates can enter the substrate binding sites in the core of the TMs of the transporter and be effluxed directly without having entered the cell. However, in MRPs it is more likely that the first interaction with the substrate occurs in the cytoplasm, as typical substrates including conjugated substrates, GSH and GSSG clearly originate from the cytoplasm where they are formed. Furthermore the typical MRP substrates are amphiphilic organic anions. The confirmed MRP substrate (for human MRP1) chloroquine is amphiphilic, containing a hydrophobic ring-structure and a hydrophilic side chain with a positively charged amine group. Substrates for conjugation in Phase II metabolism are often hydrophobic and the conjugated product is more hydrophilic or amphiphilic. In human MRP1 it has been proposed that the initial interaction with the substrate occurs in a basket formed by TMs 10, 11, 16 and 17 close to the cytoplasm (Deeley & Cole, 2006). To reach the basket, the substrates have to pass the NBDs. Maybe substrates could also interact with the NBDs as observed in the bacterial ABC transporter HlyB (Benabdelhak *et al*., 2003). This could provide an explanation for why *pfmrp1* I876 could be associated with decreased susceptibility to artemisinin, while 876V was associated with decreased susceptibility to chloroquine and quinine, although all three drugs may be transported by *pf*MRP1. Residue 876 in NBD1 could then be related to both the catalytic cycle, as suggested in paper II, and substrate specificity. The interaction could either be direct or residue 876

could be associated with another interacting amino acid. Interestingly, in South East Asian and Oceanian regions we identified a linkage between residue 876 and 1390, where 1390I is absolutely associated with 876V, but F1390 could be associated with both I876 and 876V (table). F1390 is an aromatic amino acid located in the cytoplasmic loop between TM 10 and 11 just upstream of TM 11 (figure 7). TM11 in *pf*MRP1 corresponds to TM16 in human MRP1 (figure 5) that is one of the domains in the "basket" in which aromatic residues seems to be crucial for substrate binding and potentially also for the overall protein function (Deeley & Cole, 2006). Also mutations in the loop connecting TM15 and 16 in MRP1 affect both substrate specificity and catalytic activity of MRP1 (Deeley & Cole, 2006). This suggests that residue 1390 in *pf*MRP1 may be important for the interaction between substrate binding sites and NBDs. Furthermore particular haplotypes involving residue 1390 and residues 572, 876, 785 and 1007 in NBD1 were observed in paper IV, supporting this hypothesis. Mutations in 1390 and 1007 are mutually exclusive. 1007 is found in the *pfmrp1* triple mutant haplotype including residues 572, 876, 1007 and sometimes 785, all located in the NBD1.

5.2.3 Importance of *pf*MRP1 polymorphisms for different drugs

While polymorphisms of *pfmdr1* are selected in different directions for chloroquine and mefloquine, the results from the *pf*MRP1 knock out suggest that a well functioning *pf*MRP1 is important for decreased susceptibility to chloroquine, quinine and artemisinin (Raj *et al*., 2009). Probably a good overall *pf*MRP1 function is important also for the *P. falciparum* response to all the drugs associated with *pf*MRP1 SNPs in paper II and IV. Accordingly, SNP changes that increase the overall protein function should be related to decreased susceptibility to all to the associated drugs and SNP changes that result in a less efficient transport protein should be related to increased drug susceptibility. An exception to this hypothesis is SP, that presumably selected for a less functional *pf*MRP1 (paper III), which could be supported by the observed antagonism between pyrimethamine and ART (Chawira *et al.*, 1987, Fivelman *et al.*, 1999) that possibly select for more MRP activity. There are three factors that explain why we still observed some differences in the association of SNPs with susceptibility to the different drugs although they may all potentially be transported by *pf*MRP1. A) An important factor is differences in drug specificity of *pf*MRP1. Even if MRP substrate binding sites are frequently overlapping, mutations of residues can be beneficial for binding of some substrates but not for all, resulting in drug specific SNP associations. B) Probably *pf*MRP1 is more important for those drugs with targets in the cytoplasm, as proposed for mefloquine and lumefantrine, than those with targets in the food vacuole, as chloroquine and amodiaquine/DEAQ. C) Furthermore for some drugs whose action is associated with induction of oxidative stress, *pf*MRP1 may have an

additional effect to transport GSSG and relieving the cell from the stress. Transport of GSSG may have different substrate binding sites than drugs.

5.2.4 Implications for antimalarial therapy

Results from paper II and IV and the published knock out study suggest that increased *pf*MRP1 activity could result in decreased susceptibility or even resistance to all of the key ACT partner drugs presently in use, including lumefantrine, mefloquine and amodiaquine. It is well established that although artemisinin derivatives in ACT protects against recrudescent infections, ACT partner drugs can select for more resistant reinfections, when the artemisinin derivative is eliminated. Selection of a more active *pf*MRP1 by one partner drug could then result in decreased susceptibility to all ACT partner drugs.

Alarmingly increased *pf*MRP1 activity may also result in decreased susceptibility to ART (paper IV) (Raj *et al*., 2009). Since ART are so rapidly eliminated, they may not select for resistance by themselves. However if the partner drugs select for the same mechanism that can confer resistance to ART, the risk of development of artemisinin resistance increases drastically. Development of resistance to ACTs could be devastating, as there is presently no further well established option for the treatment of *P. falciparum* malaria.

5.2.5 *pf*MRP1 in concert with PfMDR1 and PfCRT

*pf*MRP1 potentially contributes to drug resistance in concert with other *P. falciparum* membrane transporters. PfMDR1 is an importer in the food vacuole membrane (Rohrbach *et al*., 2006) that affects responses to drugs in opposite directions. For mefloquine, lumefantrine and ART, wildtype SNPs and gene amplification are associated with decreased susceptibility, while for chloroquine and amodiaquine/DEAQ these polymorphisms are associated with increased susceptibility. Mefloquine and lumefantrine have been proposed to have their targets outside the food vacuole(Famin & Ginsburg, 2002, Sisowath *et al*., 2009). Probably sensitivity to these drugs can be decreased by efficient PfMDR1-mediated transport into the food vacuole, decreasing the access to their respective targets. Conversely PfMDR1 with a less efficient transport capacity would decrease the sensitivity to chloroquine and amodiaquine, which may have their targets inside the food vacuole.

PfCRT is an exporter in the food vacuole membrane that is primarily known as the determinant for chloroquine resistance. PfCRT harbouring 76T is thought to have better transport capacities than the wildtype. As PfCRT effluxes drugs out of the FV, this will decrease the susceptibility of chloroquine and amodiaquine/DEAQ, with the target in

the food vacuole, while increasing the susceptibility to mefloquine and lumefantrine, since they returned to their sites of action.

*pf*MRP1 is likely to affect most drugs in a more similar way, by simply transporting them out of the parasite. The impact might however be more visible for drugs acting directly in the cytoplasm and plasma membrane, but it may also constitute an essential second step in the processing of food vacuole-targeting drugs which are expelled from the food vacuole through the action of PfCRT.

5.2.6 Interpretation and future perspectives

Decreased susceptibility to all the studied drugs, with the exception of SP, may be associated with increased effluxed capacity of *pf*MRP1. Therefore changes in the protein that induce markedly higher overall capacity may be selected for by one drug, while conferring cross-resistance to a range of drugs. It is of great importance to identify genetic factors that can increase the overall transport capacity. Analyses of *pf*MRP1 overexpression and copy number, which could increase the *pf*MRP1-mediated transport activity and has shown to be important in MRP-based resistance in cancers (Bates, 2003, Deeley *et al*., 2006), should be undertaken.

The SNPs associated with decreased drug susceptibility in paper II-IV represent a first sign of involvement of *pf*MRP1 in the *in vivo* and *in vitro* response to antimalarial drugs. The identified SNPs may include both those that have a role in the overall capacity of *pf*MRP1 and those that are drug specific. Important SNPs identified in these studies that especially merits further investigation include; I876V and K1466R in which there is variation in Africa and that are related to response to several drugs; F1390I proposed to be involved in substrate binding and interactions with NBD1; the haplotype SNPs located in NBD1 including 572, 785, 876 and 1007; and S1600F that was identified in the isolates with the highest effective concentrations to DBB and quinine in Thailand. As *pf*MRP1 can be knocked down with *P. falciparum* still being viable, the parasite can probably stand changes in its function when it is under drug pressure if they confer resistance to the drug.

When proceeding to study phenotypic associations with *pfmrp1* SNPs it is important to restrict the analysis to parasites of one origin, since the diversity of *pf*MRP1 is varying so much between different geographical regions. Otherwise the region can be a confounder in the relation between *pfmrp1* SNPs and parasite drug susceptibility.

6 CONCLUSIONS

6.1 PFATP6

Studies so far points for a role of PfATP6 in artemisinin action and resistance, but also leaves the floor open for other possible targets and particularly for alternative mechanisms of resistance.

- *PfATP6* harbor considerable sequence biodiversity which can be the basis for following studies investigating the association of *PfATP6* polymorphisms with artemisinin resistant or tolerant phenotypes when they emerge.

- We could not find an association between *PfATP6* polymorphisms and the relatively high variability in artemisinin responses observed in Uganda and Thailand.

6.2 *PF*MRP1

*pf*MRP1 has a role in *P. falciparum* response to several structurally unrelated drugs, thus representing a putative mechanism of multidrug resistance.

- *pf*MRP1 polymorphism are associated with decreased susceptibility to several antimalarial drugs *in vivo* and *in vitro*.

- Increased *pf*MRP1 activity may result in decreased susceptibility to nearly all the herein discussed drugs, probably through drug efflux and

- *pf*MRP1 polymorphisms may confer cross resistance to ART and all of the key ACT partner drugs presently in use.

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