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THE MOLECULAR BASIS OF *PLASMODIUM FALCIPARUM* RESISTANCE TO THE ANTIMALARIAL LUMEFANTRINE

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ABSTRACT

Malaria control is compromised by the development and spread of *Plasmodium falciparum* resistance to antimalarial drugs, which has caused an increase in malaria related morbidity and mortality. Artemisinin based combination therapy (ACT) has been implemented in almost all malaria endemic areas in an attempt to suppress drug resistance. The ACT artemether-lumefantrine (Coartem®; Novartis) is one of the most important and most widely used ACTs, especially in Africa.

The aim of this thesis was to investigate a possible molecular basis of *P. falciparum* resistance to the antimalarial drug lumefantrine, especially the role of the *P. falciparum chloroquine resistance transporter gene* (*pfcrt*) and the *P. falciparum multidrug resistance gene 1* (*pfmdr1*).

The results showed that treatment with artemether-lumefantrine selects parasites with *pfmdr1* N86, 184F, D1246 and *pfcrt* K76 alleles in high transmission Africa. The observed selection occurred in newly acquired infections after treatment (reinfections). Therefore these alleles were interpreted as markers of tolerance rather than resistance. Selection of *pfmdr1* N86 and *pfcrt* K76 carrying parasites was not observed after treatment with sulfadoxine-pyrimethamine (SP), indicating a drug specific selection. The involvement of *pfcrt* K76 in lumefantrine response was confirmed *in vitro* in isogenic *pfcrt*-modified parasite lines.

Pfmdr1 gene amplification has been suggested to increase the risk of artemether-lumefantrine treatment failure. In an *in vitro* study on field samples from Thailand, we observed that *pfmdr1* amplification was associated with a decreased susceptibility to lumefantrine as well as to its metabolite desbutylbenflumetol (DBB) and to artemisinin. *Pfmdr1* amplification was not detected in any of the African study sites in Zanzibar, Tanzania and Uganda, but may potentially represent a further development towards resistance to lumefantrine.

Overall, the results indicate that *pfmdr1* and *pfcrt* may be involved in the mechanism of resistance to lumefantrine.

Pfmdr1 N86 and *pfcrt* K76 as markers for tolerance may be of practical use, functioning as an early warning of emerging resistance. *Pfcrt* 76T, the molecular marker for chloroquine resistance, incurs lumefantrine sensitivity and may increase susceptibility to artemisinin. This suggests that artemether-lumefantrine is ideal in areas of chloroquine resistance. However, as some alleles are associated to both lumefantrine and artemisinin susceptibility, there is a risk for lumefantrine to accelerate the development of resistance to artemisinin derivatives. Although artemisinins may not select for these alleles themselves due to their fast elimination, lumefantrine pressure may provide the main selective force and drive a decrease in susceptibility to both drugs. This shows that an understanding of the molecular basis of drug resistance is crucial.

LIST OF PUBLICATIONS

I. **Christin Sisowath**, Johan Strömberg, Andreas Mårtensson, Mwinyi Msellem, Christine Obondo, Anders Björkman, José Pedro Gil. In vivo selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem®). *J Infect Dis*. 2005 Mar 15;191(6):1014-7.

II. **Christin Sisowath**, Pedro E Ferreira, Leyla Y Bustamante, Sabina Dahlström, Andreas Mårtensson, Anders Björkman, Sanjeev Krishna, José Pedro Gil. The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Trop Med Int Health*. 2007 Jun;12(6):736-42.

III. **Christin Sisowath**, Ines Petersen, M Isabel Veiga, Andreas Mårtensson, Zul Premji, Anders Björkman, David A Fidock, José Pedro Gil. In vivo selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible *pfcrt* K76 allele after treatment with artemether-lumefantrine in Africa. *J Infect Dis*. 2009 Mar 1;199(5):750-757.

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

*Shared authorship

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACT	Artemisinin based combination therapy
Ct	Cycle threshold
DBB	Desbutylbenflumetol
EC	Effective concentration
IC	Inhibitory concentration
IPTp	Intermittent preventive treatment for pregnant women
IRS	Indoor residual spraying
ITN	Insecticide treated net
MRP	Multidrug resistance protein
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PfATP6	Sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *Plasmodium falciparum*
PfATP6	*Sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of Plasmodium falciparum gene*
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
Pfcrt	*Plasmodium falciparum chloroquine resistance transporter gene*
Pfmdr1	*Plasmodium falciparum multidrug resistance gene 1*
PfMRP1	*Plasmodium falciparum* multidrug resistance protein 1
Pfmrp1	*Plasmodium falciparum multidrug resistance protein 1 gene*
Pfmsp2	*Plasmodium falciparum merozoite surface protein 2 gene*
Pgh1	P-glycoprotein homologue 1
Pgp	P-glycoprotein
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
WGA	Whole genome amplification

1 BACKGROUND

1.1 GLOBAL BURDEN OF MALARIA

It is over 100 years ago since the causative parasites of malaria were discovered. Nevertheless malaria still remains a major global health problem. Half of the world's population (~3.3 billion) live at risk of malaria in 109 countries or territories (WHO, 2008c) with about 250 million malaria cases registered, associated to almost 900 000 malaria deaths estimated in 2006. The majority of this burden falls on Africa with about 90% of all malaria cases and related deaths. Among these, children below 5 years are the most vulnerable age group, standing for 85% of the world's malaria deaths.

The burden of malaria extends well beyond morbidity and mortality, as malaria is closely correlated with the economic and social development (Malaney *et al.*, 2004). Poverty can cause malaria by a decreased ability of prevention, both at an individual and government level, as well as through hindrance of a general development. But malaria can also cause poverty. Medical costs, loss of income, negative impact on trade, tourism and foreign investment are some examples of indirect economic costs that can be ascribed to malaria. Social costs like absence from school, as well as reduced cognitive development and learning ability can also be attributed to malaria. Furthermore, high child mortality often leads to increased fertility rates, which may result in reduce investments in education per child. Overall, all these factors are mirrored in lower national economic growth rates, typical for high transmission areas (Sachs & Malaney, 2002).

1.2 *PLASMODIUM*

Malaria is caused by the protozoan parasites of the genus *Plasmodium*. This genus includes over one hundred *Plasmodium* species, able to infect reptiles, birds and mammals. Four species are traditionally considered human malaria parasites: *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale*. In addition, *P. knowlesi*, which normally infects the macaque monkeys, has been reported to infect humans as well, thus having been proposed as a fifth human malaria parasite (Cox-Singh & Singh, 2008, Singh *et al.*, 2004, Vythilingam *et al.*, 2008). *P. falciparum* and *P. vivax* are the

most prevalent human malaria parasites, although normally it is only the former that is fatal (WHO, 2009). This thesis will focus on *P. falciparum.*

1.3 LIFE CYCLE OF *P. FALCIPARUM*

Malaria parasites have a complex life cycle alternating between the female *Anopheles* mosquito and the human host. The life cycle of *P. falciparum* is illustrated in Figure 1. When an infected *Anopheles* mosquito bites, saliva containing sporozoites is injected from its salivary gland into the human host. After entering the human bloodstream, sporozoites reach the liver and invade the hepatocytes where they undergo asexual replication, which takes about 1-2 weeks. Each sporozoite may result in tens of thousands of merozoites. On release from the liver, the merozoites are delivered into the bloodstream, where they rapidly can infect erythrocytes. Inside the erythrocyte the asexual division starts. The merozoite matures from ring-stage trophozoite to pigmented trophozoite and multinuclear schizont. Finally the rupture of the erythrocyte releases several merozoites into the bloodstream to continue the erythrocytic cycle, which is about 48 hours. The erythrocytic cycle is followed by the malaria characteristic fever. Some merozoites in the erythocytes develop into micro- and macrogametocytes (male and female). These are necessary for carrying on the infection to a new host. When an anopheline mosquito takes a blood meal, the gametocytes may be taken up. These are transported to the mosquito midgut, where the sexual cycle starts. Macrogametes and microgametes fuse and develop into a zygote and then an ookinete, which goes through the wall of the midgut and develops into an oocyst. The rupture of the oocyst will result in many sporozoites that migrate to the salivary glands for transmission to a new host during the next blood meal of the mosquito. The development in the mosquito takes about 10-18 days, although the mosquito can be infective for another 1-2 months.

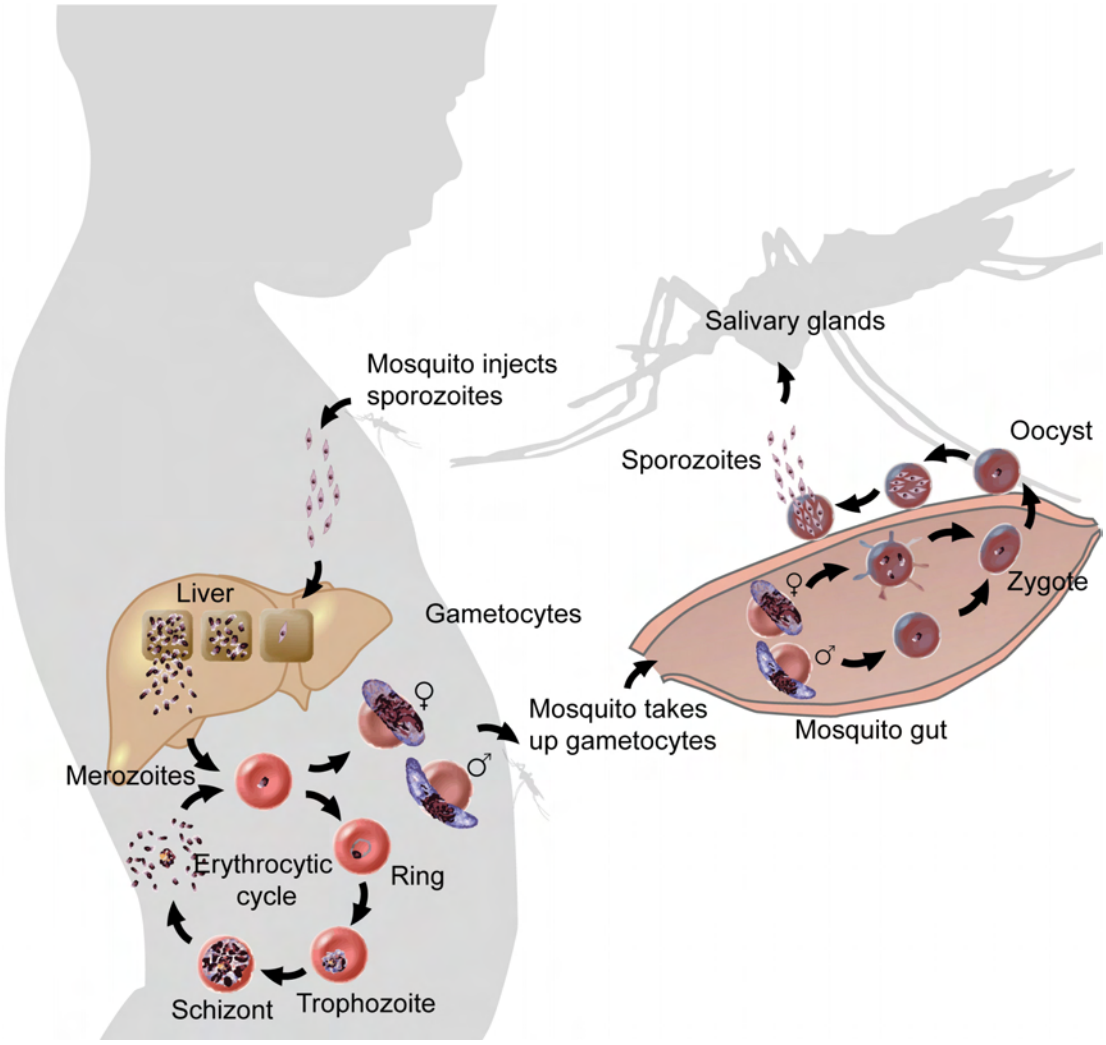

Figure 1. The life cycle of *P. falciparum*.

1.4 TRANSMISSION AND EPIDEMIOLOGY

The term malaria, from the Italian *mala aria* meaning *bad air*, originates from the common belief that malaria was caused by breathing in bad air from swamps and stagnant water, the breeding ground for mosquitoes. There are about 400 *Anopheles* mosquitoes, of which about 60 can transmit malaria. The major vector is *A. gambiae* in sub-Saharan Africa. Transmission and epidemiology of malaria is determined by the ecology and behavior of both humans and vectors. The human immunity is also a factor. Transmission can be stable or unstable. In stable areas, the transmission is rather constant from year to year, whereas in unstable areas, the transmission varies. Both are endemic, but in the unstable areas there is a high risk for epidemics, i.e. the number of malaria cases significantly exceeds what is normally expected. Stable transmission is more usual in areas where the vector prefers to feed on humans (anthropophilic) and

have high survival rate. People living in these areas acquire some immunity over time and mostly children, pregnant women and immigrants are vulnerable to the disease. Unstable transmission is more usual in areas where the vector bites animals and humans (zoophilic) and/or have a low survival rate (Reiter, 2008). Figure 2 shows the distribution of malaria endemic areas in the world.

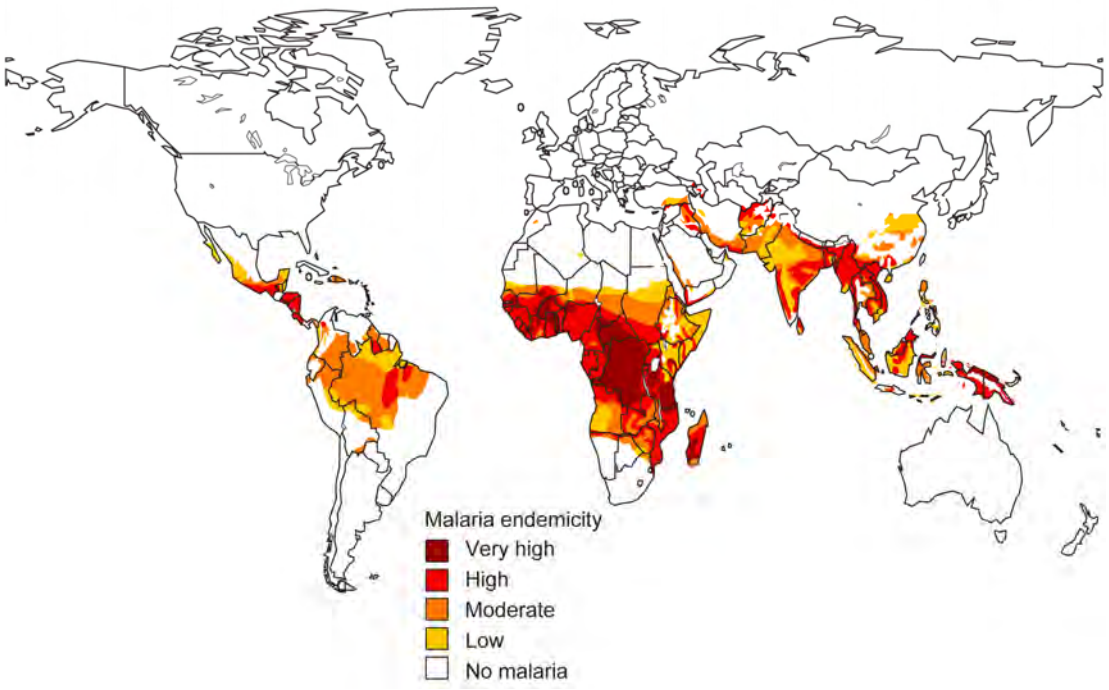

Figure 2. World map of malaria transmission. Adopted from (WHO, 2005).

1.5 CLINICAL MANIFESTATIONS OF *P. FALCIPARUM* MALARIA

The outcome of a malaria infection depends on the properties of the host and parasite. People with no previous exposure to malaria will become ill on the first exposure. In high transmission areas, young children (< 5 years old) are particularly susceptible, progressively developing immunity to the disease, upon repeated infections. This process of premunition is protective against the development of the clinical features of the disease, and particularly against the deadly severe malaria. The infections are kept suppressed, although frequently allowing asymptomatic carriage of parasites. This type of clinical immunity does not usually occur in areas of low transmission (Langhorne *et al.*, 2008). Pregnant women are also highly susceptible to infection, especially during first pregnancy, even if they previously have developed premunition (Hviid, 2004).

Malaria is asymptomatic during the liver stage of the infection, with the clinical symptoms only being associated with the erythrocytic stage. The first symptoms of malaria are non-specific, including features like headache, fatigue, abdominal discomfort muscle and joint aches, followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise. If the treatment for uncomplicated malaria is delayed the clinical picture may become worse very rapidly towards severe malaria. Severe malaria often includes one or more of the following symptoms: coma (cerebral malaria), metabolic acidosis, severe anaemia, hypoglycaemia and in adults, acute renal failure or acute pulmonary oedema. If untreated, severe malaria is almost always fatal (WHO, 2000, WHO, 2006).

Co-infections may affect the outcome of a malaria infection. HIV, having an overlapping geographical distribution with malaria-endemic areas, particularly in Eastern and Southern Africa, could potentially interfere with malaria immunity (Renia & Potter, 2006). Tuberculosis is also prevalent in many malaria-endemic areas. But the knowledge of the interactions between *Mycobacterium tuberculosis* and the malaria parasite is limited (Troye-Blomberg & Berzins, 2008).

1.6 MALARIA CONTROL

Malaria is both preventable and curable through prompt and effective chemotherapy. The number of antimalarial drugs under current clinical use is limited. These include the 4-aminoquinolines (chloroquine, amodiaquine), the arylaminoalcohols (mefloquine, halofantrine, lumefantrine), antifolates (sulfadoxine-pyrimethamine (SP)), atovaquone, artemisinin and its derivatives (artesunate, artemether) as well as antibiotics (WHO, 2008b). Quinine is used mainly for the treatment of severe malaria. The objective of antimalarial treatment in severe malaria is to prevent death. For uncomplicated malaria the objective is to cure, i.e. eradicate the infection to prevent progression to severe disease. Furthermore, from the public health perspective the objective is also to reduce transmission of the infection to others (WHO, 2006). Antimalarial drugs are also used as a preventing measurement, both as chemoprophylaxis for travellers to malaria endemic areas or for intermittent preventive treatment for pregnant women (IPTp). IPTp has been a recommended strategy by WHO in highly endemic areas since 1998, involving the administration of a full treatment dose of an antimalarial drug (generally SP, which has a long half life) at certain time points during pregnancy

independently of the presence of malaria parasites (WHO, 2007). Studies are also being done to explore the potential benefit of using intermittent preventive treatment for infants (WHO, 2008a).

Another key intervention to control malaria is the use of insecticide treated nets (ITNs). It has been shown that ITNs can have a major impact on malaria burden, decreasing both morbidity and mortality (Lengeler, 2004). Furthermore, indoor residual spraying (IRS), the use of long-acting chemical insecticides on the walls and roofs of houses in order to kill and repel the mosquitos, has been proven to be an effective way to reduce malaria transmission, especially in areas with low and variable/seasonal transmission (Mabaso *et al.*, 2004). In the future, other malaria control measures can become important, i.e. genetically modified mosquitos or vaccines.

1.7 ANTIMALARIAL DRUG RESISTANCE

1.7.1 Definition

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

Drug resistance may cause treatment failure, however treatment failure does not necessarily have to be caused by drug resistance. Other factors may bring about treatment failure: inadequate dosing or duration of treatment, fake drugs with no or little active ingredient, or significant changes in the drug exposure due to altered metabolism (e.g. caused by drug-drug interactions) or poor absorption.

1.7.2 Origins of antimalarial drug resistance

The emergence of resistance is a result of two events: the initiation event (the genetic change associated to the acquisition of the resistant parasite) and its selection. Resistance in nature occurs through spontaneous mutations, which are rare events. For some drugs a single mutation is enough while for others multiple mutations may be necessary to reach resistance. Once a mutation has occurred it may be subjected to selection. Selection can occur when parasites of a primary infection survive a treatment and recrudesce, being allowed to be transmitted, whereas the sensitive parasites are eliminated by the drug and hence not able to propagate their sensitive genome. Selection can also occur through new infections acquired after treatment (reinfections) when subtherapeutic levels of a slowly eliminating drug are present in the blood. As before, but now at lower levels of resistance, this remaining drug is still able to eliminate the fully sensitive parasites but not the less sensitive or, evidently, the resistant parasites. The genetic configurations associated with this low resistance (or tolerance) will give the parasite better chances to proceed to be transmitted.

How fast and how intense resistance emerges depends on several factors. The single most important reason is nevertheless the drug pressure. Appropriate drug treatment means maintaining an adequate drug level for a sufficient length of time for the complete elimination of all potential subpopulations present in the infection. Inadequate drug concentrations and/or during insufficient amount of time will inevitably increase the risk of selecting less susceptible parasites. The conditions for the development and spread of resistance are difficult to define, as it is highly multifactorial and can occur due to many different circumstances. In simplistic terms, we can consider two extreme types of settings: high transmission and low transmission. In a low transmission area with good health care coverage, essentially every parasite will be submitted to drug pressure, as all situations of *P. falciparum* infection will be associated to clinical disease. By covering all the parasite population, even very rare subpopulations of resistant parasites will have their opportunity of being selected. If this situation is composed by the fact that the parasite population is prone to accumulate genetic diversity (e.g. Southeast Asia where the mutation rate of the local parasites seems to be higher than in most other locations), this can create a focus of resistance. This might be the case for the borders between Thailand and Myanmar or Cambodia. In such a scenario the inadequate relation between the parasite and the drug occurs because the

parasite is already sufficiently resistant to evade drug concentrations that are expected to be adequate. In a high transmission setting, due to the development of premunition, there are significant fractions of the human population that carry asymptomatic infections. This means that at any point in time there are large parasite populations usually not exposed to antimalarials. In these populations, a fitter, sensitive parasite will thrive, while the rare resistant ones will not have the right environment (i.e. drug pressure) to take advantage of. An expected way of resistance to develop in such an environment is through the exposure of parasites to subtherapeutic levels of drugs. While this will be rare in low transmission areas (i.e. Thailand) where all infections will be exposed to high levels of drug, in high transmission settings, reinfection is likely to occur shortly after treatment, at a moment when the concentrations of drug in the blood are below the therapeutic levels. Here resistance will develop more gradually, with successive populations of parasites being selected because they are fractionally more resistant, although not enough to withstand a full course of drug treatment. In contrast, the low transmission site will create full resistant parasites in a sudden event, generating a clear focus (e.g. as witnessed with chloroquine resistance). In a high transmission setting, the development of resistance will be more gradual, but likely to be more spread (i.e. multifoci). As previously stated, this is a relatively simplistic view. The reality lies between these two situations, in a more complex way.

1.7.3 The impact of drug resistance

Resistance to antimalarial drugs compromises the control of the disease. Today resistance has emerged to virtually all antimalarials. Even artemisinin derivatives, the only drugs that *P. falciparum* has not developed resistance to seem to be loosing their effect in some areas (Jambou *et al.*, 2005, Noedl *et al.*, 2008). Resistance can be manifested in different ways depending on the area. In high transmission areas, where people have developed a degree of immunity, the clinical effects can be prolonged infections and increased risk of severe disease, and hence, mortality. In low transmission areas, resistance may be more apparent, as these areas are more prone to epidemics (Bjorkman & Bhattarai, 2005). The most devastating effect may have been that of chloroquine resistance, since chloroquine was such a widely used drug. Chloroquine resistance has caused both increased mortality and morbidity (Trape, 2001).

1.8 ARTEMISININ BASED COMBINATION THERAPY (ACT)

Combination therapy has been used for some time in the treatment of tuberculosis, cancer and HIV for reducing the risk of development and spread of drug resistance. For antimalarials, the idea of combination therapy was in the beginning to take advantage of the synergy between different drugs, such as SP (White, 1999). This type of combination is however not seen as a "real" combination therapy since their drug targets are evidently linked (WHO, 2006). The rationale for combining two antimalarial drugs with different mechanisms of action is that the probability of a parasite developing resistance to both drugs simultaneously is significantly reduced compared to developing resistance to one drug. The probability of a parasite developing resistance to both drugs simultaneously is the product of the individual parasite mutation rate for each drug, multiplied by the number of parasites in the infection exposed to the drugs. This means that if the probability of resistance to arise to one drug is one in 10^6 and to another drug one in 10^7, the probability of a parasite to develop resistance to both drugs in a combination is one in 10^{13}, providing that the genetic mutations that confer resistance are not linked. In this way, the risk of selecting drug resistant parasites is consequently reduced. Furthermore, the spread of established resistance may also be slowed down to some extent, since resistant parasites to one of the drugs in a combination will be killed by the other (White, 1999).

Artemisinin based combination therapy (ACT), the combination of an artemisinin derivative with another structurally unrelated and more slowly eliminated antimalarial, is highly advocated. Artemisinin and its derivatives have a very high parasite reduction ratio (PRR), the ratio of the parasitemia at the time of treatment to 48 hours later (one asexual cycle), being able to reduce the number of parasites faster than any other antimalarial. This is also the reason for their ability to provide clinical relief fast (WHO, 2006). In an ACT, the artemisinin derivative will reduce the majority of the parasites, leaving its partner drug with only a few parasites left to eliminate. These parasites are exposed to a higher drug concentration of the partner drug than if the drug would have been used as a monotherapy. The drug concentration may even be high enough to kill parasites that are partially resistant to the drug (Figure 3). In this way the artemisinin derivative protects its partner drug. If the treatment is successful, the slowly acting partner drug also protects the artemisinin

derivative by removing all residual parasites originally exposed to the artemisinin (Nosten & White, 2007).

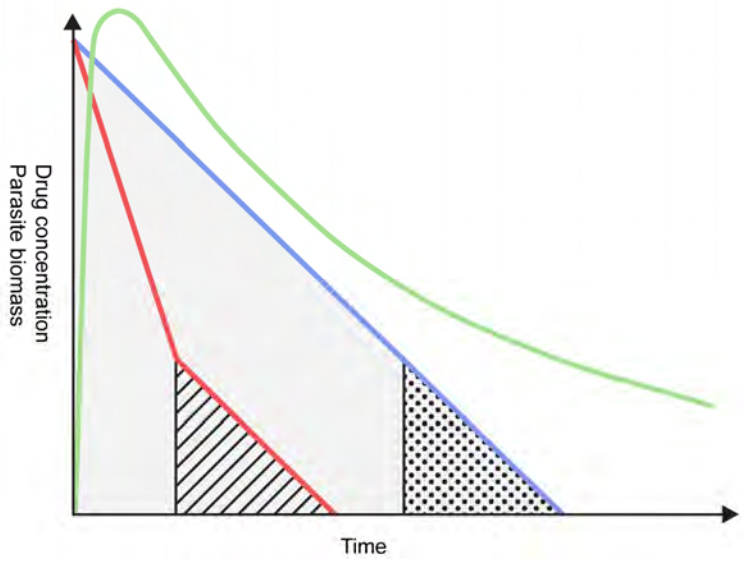

Figure 3. The large shaded triangle under the blue line represents the total parasite biomass exposed to a drug (green) if used alone in a monotherapy. If an artemisinin derivative is added, the parasite biomass is reduced significantly (red line) and only a few parasites are exposed to the partner drug alone (small striped triangle). These parasites are exposed to a higher concentration of the long acting partner drug compared to a monotherapy (small dotted triangle). In this way, the artemisinin derivative protects the partner drug. Adapted from (White, 1999).

Most drugs are schizonticidal but artemisinin and its derivatives can also reduce gametocytes. The consequence of this gametocytocidal effect may result in a reduction in transmission (White, 1999). Considering that artemisinin derivatives can kill gametocyte stages of parasites resistant to other antimalarials, they can reduce the spread of resistance. Furthermore the result of a reduced transmission can, since it may lead to reduced infections among people, decrease the need for treatment and thus reducing the drug pressure (White, 1999).

Thailand was the first country to introduce ACT in 1994, due to the reduced efficacy of mefloquine at the border areas to Myanmar. Artesunate-mefloquine is now widely used in many areas in Southeast Asia. While artesunate-amodiaquine and artemether-lumefantrine are the most used ACTs in Africa (WHO, 2008b).

Because of the fast elimination of the artemisinin component, the partner drug with the longer half life is left unprotected shortly after treatment. At this point, as previously pointed out, there is a risk of selection of less susceptible or resistant

newly acquired parasites, reinfections. In low transmission areas, this does not constitute a major threat. Using partner drugs with very long half lives, such as mefloquine is therefore not of particular concern. However, in high transmission areas this may constitute a problem.

1.8.1 Artemether-lumefantrine (Coartem®)

Artemether-lumefantrine, also known as Coartem® or Riamet®, is a fixed formulation developed by the Academy of Military Medical Sciences (Beijing, China) and Novartis Pharma (Basel, Switzerland). Artemether-lumefantrine is a well tolerated drug and is highly effective when given as a 6 dose treatment, with a 4 dose regimen having been observed to be inferior, especially in areas where the immunity to malaria is low and multidrug resistance is high (Wernsdorfer, 2004).

Lumefantrine (Figure 4), formerly known as benflumetol, is a schizontocidal drug that belongs to the arylaminoalcohol group of antimalarials, which also includes mefloquine and halofantrine. It was first synthesised in China and was first mentioned in scientific literature outside China in 1990 (Wernsdorfer, 2004). The half life of lumefantrine is relatively short, 4-5 days (Ezzet *et al.*, 1998) making it suitable for high transmission areas. Lumefantrine is a highly lipophilic substance and its absorption may vary between individuals. Its bioavailability increases substantially if the drug is administered with fat (Ashley *et al.*, 2007, Ezzet *et al.*, 2000).

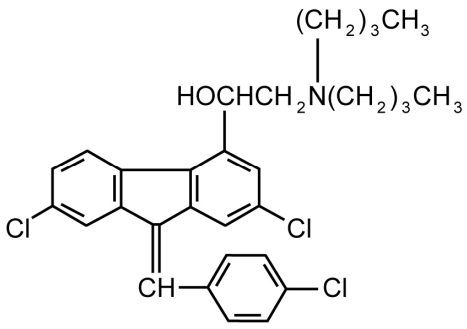

Figure 4. The chemical structure of lumefantrine.

Artemether is a semisynthetic drug and a derivative of artemisinin. It is like lumefantrine a lipophilic compound. The main active metabolite, dihydroartemisinin, has a correlated effect with artemether, but has been observed to be more potent. (Wernsdorfer, 2004). Synergy between artemether and lumefantrine has been observed within the mol:mol ratio 100:1 to 1:100 in two chloroquine resistant and mefloquine sensitive laboratory strains from India and Thailand. The synergy was most significant at the higher effective concentrations (ECs) of EC90 and EC99 (concentration where 90% and 99% respectively of the parasite growth is inhibited) (Hassan Alin *et al.*, 1999). One tablet of Coartem® contains 20 mg artemether and 120 mg lumefantrine, which corresponds to a artemether:lumefantrine ration of ~2:7.

Desbutylbenflumetol (DBB), a main active metabolite of lumefantrine (Ntale *et al.*, 2008) shows a certain degree of cross resistance *in vitro* with both artemether and lumefantrine, especially at EC50 levels (Noedl *et al.*, 2001). These observations indicate a risk for a common mechanism of reduced susceptibility (Wernsdorfer, 2004).

1.9 *P. FALCIPARUM IN VITRO* AND *IN VIVO* DRUG RESISTANCE

The clinical failure upon appropriate administration of a drug can be considered as the golden standard in terms of drug resistance, as it is the “real world” phenotype. Hence clinical trials where malaria patients are treated under supervision and followed up for a pertinent period of time probably best reflect the actual situation in an area. However, a disadvantage with *in vivo* trials, besides their logistic demands, is that when combination treatments like ACT are evaluated, parasites resistant to only one of the drugs may not be detected. Complementary, *in vitro* studies can be used to assess susceptibility to single drugs. Furthermore, multiple drugs can be tested which can supply information of a possible correlation/cross resistance between different drugs. The *in vitro* approach provides an opportunity to study the intrinsic parasite susceptibility to a drug without patient related confounding effects like immunity or pharmacokinetic factors like bioavailability and drug metabolism. These factors may also be why *in vitro* studies do not always correlate with treatment failures (Aubouy *et al.*, 2004). Moreover, not all parasites will adapt to culture conditions, which can bias the results. Additionally, resistant minority parasite strains may have an important effect on treatment failure but may not contribute significantly to the *in vitro* phenotype. Finally, sequestered parasites, absent from the peripheral circulation

may be missed when sampling for *in vitro* tests but may cause recrudescence in a patient after treatment. In summary, when researching the biological basis of parasite drug susceptibility, it is important to have access to both *in vivo* and *in vitro* approaches, for a better understanding of the phenomena.

1.10 MECHANISMS OF DRUG RESISTANCE

In most systems, drug resistance is based on the following different mechanisms: (a) The loss of the drug's pharmacological capacity, either by drug metabolism or target change, the later typically through genetic mutations (e.g. atovaquone resistance through cytb mutations); (b) The access of the drug to the target is decreased, typically by its displacement from the relevant cellular compartment through the action of transporter proteins, which themselves are mutated.

A mutation can most frequently be either a gene amplification or a single nucleotide polymorphism (SNP). Two genes may act epistatically, in combination, to produce a specific phenotype (Duraisingh & Refour, 2005). One gene may also have the ability to produce a multidrug resistant phenotype.

1.10.1 Genes influencing artemether-lumefantrine susceptibility

1.10.1.1 Pfmdr1

In the 1980s the first molecular data on *P. falciparum* chloroquine resistance emerged. Resistant parasites were demonstrated to have decreased drug accumulation, which was also documented to be associated with drug resistance in cancer cells (Krogstad *et al.*, 1987, Martin *et al.*, 1987). In these, multidrug resistance had been observed to be mediated by a drug efflux transporter, the P-glycoprotein (Pgp). This triggered the search for an equivalent protein in *P. falciparum.* The *P. falciparum* homologue, P-glycoprotein homologue 1 (Pgh1), was subsequently found through the cloning of its coding gene, named *P. falciparum multidrug resistance gene 1* (*pfmdr1*) (Foote *et al.*, 1989). Similarly to Pgp, Pgh1 belongs to the ATP-binding cassette (ABC) transporter superfamily. Pgh1 is comprised by two domains of six transmembrane regions, two nucleotide binding domains (NBDs) and Walker A and B conserved sequences, which are typical for ABC transporters (Peel, 2001). The protein is expressed during the

asexual erythrocytic stages and is essentially located in the membrane of the digestive vacuole (Cowman *et al.*, 1991).

Sequencing of *pfmdr1* on laboratory adapted strains from different geographical areas resulted in the identification of its main SNPs N86Y, Y184F, S1034C, N1042D and D1246Y (Foote *et al.*, 1990) (Figure 5). Although these SNPs were initially clearly associated with chloroquine resistance *in vitro* (Foote *et al*., 1990), the association has been confirmed in some later *in vitro* and *in vivo* studies (Foote *et al*., 1990, Basco *et al.*, 1995, Duraisingh *et al.*, 1997) but not in all (Awad-el-Kariem *et al.*, 1992, Haruki *et al.*, 1994, Basco & Ringwald, 1998, Wilson *et al.*, 1993, McCutcheon *et al.*, 1999). Subsequent research by genetic crossing ruled out *pfmdr1* as a central factor in chloroquine resistance (Wellems *et al.*, 1990). Nevertheless, a certain consensus has been reached on *pfmdr1* having a secondary role to enhance the chloroquine resistance (Djimde *et al.*, 2001, Babiker *et al.*, 2001), and/or to compensate for any fitness loss derived from the mechanism of chloroquine resistance (Wellems & Plowe, 2001, Mita *et al.*, 2006).

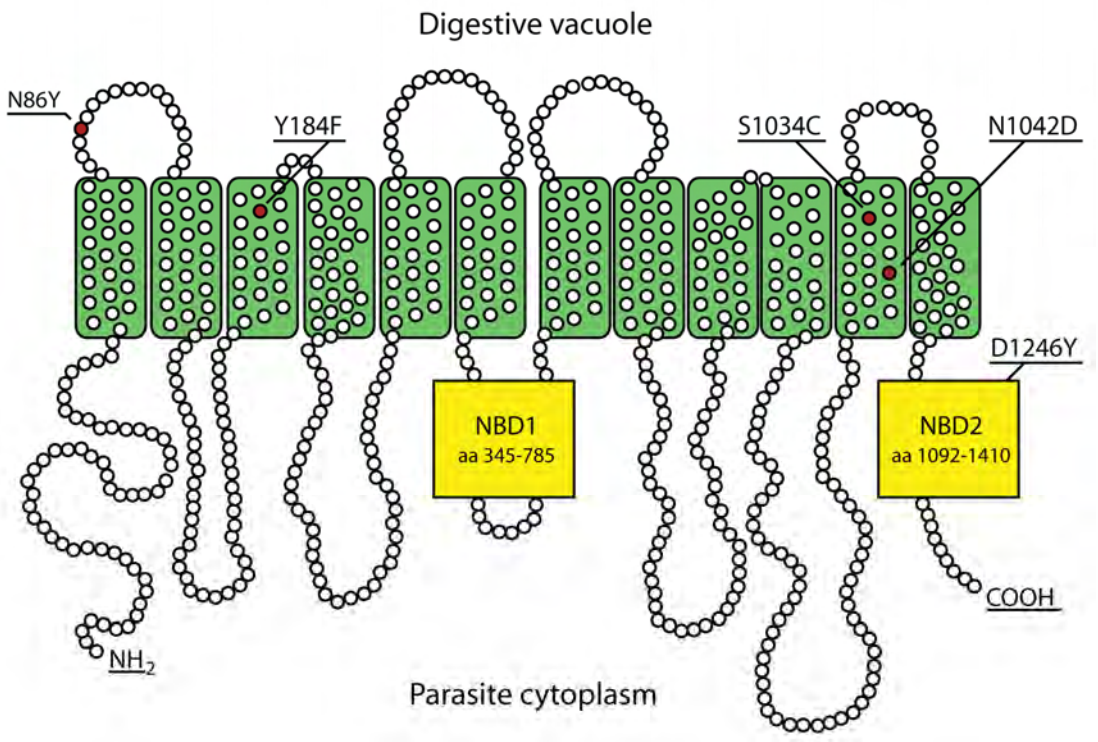

Figure 5. The Pgh1 protein, encoded by *pfmdr1*, has two homologues domains, each consisting of six transmembrane regions. The two nucleotide binding domains (NBD1 and NBD2) are indicated as boxes. The N86Y, Y184F, S1034C, N1042D and D1246Y SNPs are marked at their respective amino acid position. Adapted from (Valderramos & Fidock, 2006).

The first evidence for the transport function of Pgh1 was the reported ability of *pfmdr1* heterologous expression to complement the transporter STE6 in yeast cells. *Pfmdr1* expressing S1034C and N1042D did not have this ability however (Volkman *et al.*, 1995). More recently, *pfmdr1* gene amplification was observed to be associated with increased Fluo-4 accumulation in the digestive vacuole hence Pgh1 was suggested to import solutes into the digestive vacuole (Rohrbach *et al.*, 2006). These results were further supported by studies of *pfmdr1* expressed in *Xenopus laevis* oocytes, where analysis of the Pgh1 capacity of transporting antimalarials showed that these SNPs were able to alter the transporter substrate specificity (Sanchez *et al.*, 2008).

Field isolates, transfection experiments and genetic crosses have shown an association between *pfmdr1* SNPs and susceptibility to the arylaminoalcohols mefloquine, lumefantrine and halofantrine, as well as to quinine and artemisinins (Price *et al.*, 1999, Duraisingh *et al.*, 2000a, Reed *et al.*, 2000, Sidhu *et al.*, 2005, Anderson *et al.*, 2005, Price *et al.*, 2006, Duraisingh *et al.*, 2000b). Furthermore, gene amplification of *pfmdr1* may have an important role in the mechanism of resistance to several antimalarials. Increased *pfmdr1* copy number has been associated with decreased *in vitro* susceptibility to mefloquine, halofantrine and quinine in field isolates (Wilson *et al*., 1993, Price *et al*., 1999). Moreover the development of *in vitro* mefloquine resistance has been shown to be closely associated with increased *pfmdr1* copy number (Wilson *et al*., 1989, Cowman *et al*., 1994, Peel *et al*., 1994, Preechapornkul *et al*., 2009). Finally, the knock down of one of the two copies in the clone FCB has been shown to consequently increase the sensitivity of the parasites to mefloquine, halofantrine, lumefantrine, quinine and artemisinin (Sidhu *et al.*, 2006). Supporting the available *in vitro* data, *pfmdr1* amplification has been associated with treatment failures after mefloquine, artesunate-mefloquine and 4 dose artemether-lumefantrine treatment in Southeast Asia (Price *et al.*, 2004, Alker *et al.*, 2007, Lim *et al.*, 2009, Rogers *et al.*, 2009, Price *et al*., 2006).

1.10.1.2 Pfcrt

The *P. falciparum chloroquine resistance transporter* (*pfcrt*) gene is the main determinant of chloroquine resistance, with K76T as the key mutation (Djimde *et al*., 2001, Sidhu *et al.*, 2002, Lakshmanan *et al.*, 2005, Fidock *et al.*, 2000) (Figure 6). Chloroquine has its mode of action inside the digestive vacuole where it binds to hematin, preventing the detoxification process of the byproducts produced during

haemoglobin digestion (Bray *et al.*, 1998). The PfCRT protein is exclusively located in the digestive vacuole membrane. The mutated form is believed to function as an exporter, transporting chloroquine out from the digestive vacuole (Sanchez *et al.*, 2007, Valderramos & Fidock, 2006).

In addition to its nowadays very well documented involvement in chloroquine resistance**,** PfCRT has also been seen to affect the response to arylaminoalcohols and artemisinins *in vitro*. In a transfection experiment, it was shown that acquisition of the *pfcrt* K76T mutation increased the susceptibility to mefloquine and artemisinin (Sidhu *et al*., 2002). Accordingly, when 76T was exchanged to K76, the wild type form had a decreased susceptibility to mefloquine and to some extent also to artemisinin (Lakshmanan *et al*., 2005). In a different approach, selection experiments where the Sudanese 106/1 clone, which harbours some *pfcrt* mutations but lacks the K76T, was pressured with chloroquine, new *pfcrt* SNPs, K76I and K76N, were observed. These resulted in an increased susceptibility to both mefloquine and halofantrine, as witnessed for K76T (Cooper *et al.*, 2002). In another selection experiment, halofantrine pressure produced halofantrine and mefloquine resistant parasites, which had acquired novel *pfcrt* mutations, a S163R change being the central factor in the altered parasite response to these arylaminoalcohols (Johnson *et al.*, 2004).

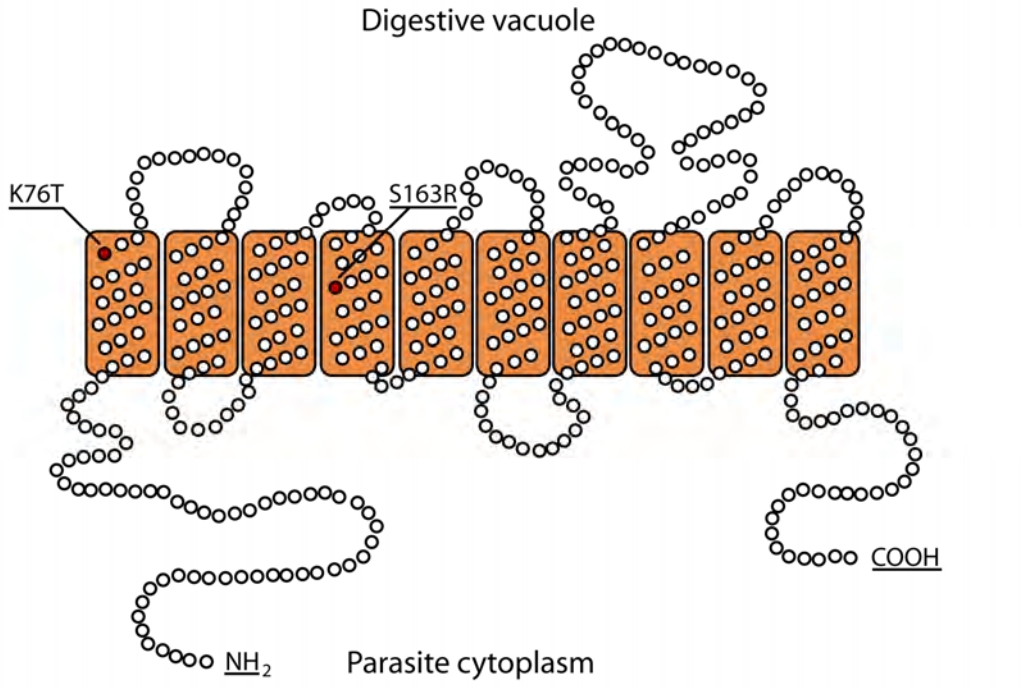

Figure 6. The PfCRT protein, predicted to have ten transmembrane domains. The K76T and S163R SNPs are marked at their respective amino acid position. Adapted from (Valderramos & Fidock, 2006).

1.10.1.3 Pfmrp1

Multidrug resistance proteins (MRPs) are, like Pgh1, ABC transporters. They are well documented to be able to efflux a large range of xenobiotics in many biological systems, contributing in this way to clinically relevant drug resistance. This has been observed in different organisms like mammalian tumour cells (Haimeur *et al.*, 2004), bacteria (Poelarends *et al.*, 2003) and parasites (Ouellette & Légaré, 2003).

Two MRP homologues have been identified in *P. falciparum*. So far, limited research has been done on the involvement of MRP in *P. falciparum* drug resistance. Recently, knockout experiments have suggested that *P. falciparum* Multidrug Resistance Protein 1 (PfMRP1) (PFA0590w) is involved in the mechanism of resistance to chloroquine, quinine and artemisinin, possibly through drug efflux (Raj *et al.*, 2009). Moreover, *in vitro* studies have shown an association between *pfmrp1* Y191H and A437S with chloroquine and quinine susceptibility (Mu *et al.*, 2003). Although this association has not been confirmed (Anderson *et al*., 2005).

1.10.1.4 PfATP6

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) orthologue of *P. falciparum* (PfATP6) has been suggested to be a target for artemisinins (Eckstein-Ludwig *et al.*, 2003). *In vitro* experiments have shown that the *PfATP6* L263E SNP reduces artemisinin inhibition of PfATP6, suggesting this amino acid position to be involved in the binding of artemisinin to PfATP6 (Uhlemann *et al.*, 2005a). Furthermore, it has been observed in field isolates from French Guiana that another *PfATP6* SNP (S769N) is associated with decreased *in vitro* susceptibility to artemether (Jambou *et al*., 2005).

2 AIMS OF THE THESIS

2.1 OVERALL OBJECTIVE

The objective of this thesis was to investigate a possible molecular basis of *P. falciparum* resistance to the antimalarial drug lumefantrine, especially the role of the *pfmdr1* and *pfcrt* genes.

2.2 SPECIFIC AIMS

The specific aims were as follows:

1. To investigate if polymorphisms in the *pfmdr1* and *pfcrt* genes would be selected by artemether-lumefantrine treatment, indicative of their relation with resistance mechanisms against these drugs. Paper I, II and III aimed at investigating two 42-day follow-up clinical trials in East Africa to determine if a possible selection occurred in recrudescences and/or reinfections (Paper I-III).

2. To determine if the observed selection of polymorphisms after artemether-lumefantrine treatment was drug specific or fitness related (Paper III).

3. To study the association of *pfmdr1* and *pfcrt* polymorphisms with *in vitro* drug susceptibility to the most important antimalarial drugs, including lumefantrine and its metabolite DBB, in Africa and Southeast Asia, two areas with different *P. falciparum* populations and drug usage (Paper IV). This was put in context with the second aim of Paper IV, to study the association of polymorphisms in *pfmrp1* and *PfATP6* with *in vitro* drug susceptibility to a large range of antimalarial drugs, such as lumefantrine, DBB and artemisinin.

3 MATERIAL AND METHODS

3.1 STUDY SITES

The studies that are included in this thesis have been performed in East Africa and Southeast Asia. Two clinical trials, where children were treated with artemether-lumefantrine and followed up, were performed in Zanzibar and mainland Tanzania respectively. In Uganda and Thailand, fresh parasite isolates were collected from patients before treatment for *in vitro* drug susceptibility testing.

3.1.1 Zanzibar and mainland Tanzania

Transmission occurs all year round, with seasonal peaks in Zanzibar and mainland Tanzania. The clinical trial under molecular analysis in Paper I and II was performed between October 2002 and February 2003, at a time when *P. falciparum* transmission was holoendemic. Two study sites were included in Zanzibar: Kivunge Hospital on Unguja Island and Micheweni Hospital on Pemba Island. Both hospitals are located in densely populated rural areas. At the time of the study, the local government supplied chloroquine and SP to the sites. In the private sector, other antimalarial drugs were also available, but no ACT. Therefore the analysed parasites were expected to be naive to both lumefantrine and artemether. Zanzibar was, shortly after the trial in 2004, one of the first regions in Africa to change their official drug policy to ACT, with artesunate-amodiaquine as first line and artemether-lumefantrine as second line treatment for uncomplicated *P. falciparum* malaria.

The clinical trial, analysed in Paper III, was conducted in Fukayosi Primary Health Care Center, located in a rural holoendemic area of Bagamoyo District, mainland Tanzania. At the time of the study (2004), SP and amodiaquine were used as first and second line treatments, respectively. Tanzania changed its drug policy to artemether-lumefantrine as one ACT line only, in 2006.

3.1.2 Uganda

Transmission occurs all year round in most parts of the country. The study was conducted in a hyperendemic area in Gulu, Amuru district. Uganda changed drug policy to artemether-lumefantrine in 2004, before the clinical trial of Paper IV was performed (2007). Artesunate-amodiaquine is used as an alternative treatment when artemether-lumefantrine is not available. The official second line treatment is quinine. Before the policy change, a combination of chloroquine and SP was the first line treatment for uncomplicated malaria.

3.1.3 Thailand

Malaria is endemic in some regions of Thailand, especially in the forest, forest fringes and border areas. Mefloquine has been used in Thailand since 1985, first in combination with SP and then as a monotherapy. Because of rising mefloquine resistance, artesunate-mefloquine was introduced in 1995, in high-level mefloquine resistant areas, mainly around Mae Sot on the border to Myanmar. Artesunate-mefloquine is now the first line treatment for all uncomplicated *P. falciparum* malaria in Thailand (Na-Bangchang & Congpuong, 2007).

3.2 ETHICAL CONSIDERATIONS

Ethical clearance has been obtained from the respective country where the clinical trial was performed. Furthermore, the studies have also been approved by the Ethical committee of Karolinska Institutet in Sweden. Informed consent was obtained from all enrolled patients or their parents/legal guardians.

3.3 *IN VIVO* FOLLOW-UP TRIALS

The two clinical trials performed in Zanzibar and mainland Tanzania, included children with microscopically confirmed uncomplicated *P. falciparum* malaria. All enrolled children had a parasitemia of 2000-200 000 asexual parasites/µl blood and an axillary temperature of 37.5°C or a history of fever the last 24 hours. Children with severe malaria were not included in the trial. Follow-up visits after initiation of treatment were conducted on days 1, 2, 3, 7, 14, 21, 28, 35, 42 and on any day of recurrent illness occurring during the 42 day follow-up period. On every visit blood smear was collected

for the assessment of parasitemia through microscopy. Parasites were counted against 200 white blood cells and parasitemia was calculated with the assumption that one µl of peripheral blood contains 8000 white blood cells. Finger prick blood was collected on filter paper for molecular analysis.

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

On mainland Tanzania, the clinical trial was performed between April and July 2004 and included 106 children ≥ 6 months of age and weighing ≥ 6 kg. The children were randomly allocated to receive either SP (Fansidar®; Roche) or artemether-lumefantrine. Children who were allocated to receive SP and who had body weights of 5 – 10 kg, 11 – 20 kg, and 21 – 30 kg were given a single dose of sulfadoxine/pyrimethamine of 250 mg/12.5 mg, 500 mg/25 mg, and 750 mg/37.5 mg, respectively, whereas children who were allocated to receive artemether-lumefantrine and who had body weights of 5 – 14 kg, 15 – 24 kg, and 25 – 34 kg were given a dose of artemether/lumefantrine of 20 mg/120 mg, 40 mg/240 mg, and 60 mg/360 mg, respectively, twice a day for 3 days. For logistic reasons, only the first of the two daily doses of artemether-lumefantrine was administered under supervision.

Children developing severe malaria during follow up were treated with quinine, according to the national malaria treatment guidelines, and withdrawn from the study.

3.4 *IN VITRO* DRUG SUSCEPTIBILITY TESTING

In Paper III the *in vitro* analysis was performed on genetically modified laboratory strains using the 72-hours ^{3}H-hypoxanthine assays with serial drug dilutions as described (Fidock *et al.*, 1998). Lumefantrine was tested in duplicate. ^{3}H-hypoxanthine was added after 48 hours, and cells were harvested after another 24 hours. The percentage reduction in ^{3}H-hypoxanthine incorporation, a standard marker of parasite growth inhibition, was equal to [100 x (geometric mean cpm of wells without drug) -

(mean cpm of test wells)] / (geometric mean cpm of wells without drug). Inhibitory concentrations (ICs) for two points only, IC50 and IC90, were determined by linear extrapolation.

In paper IV, the *in vitro* testing was performed in the field in Gulu, Uganda and Mae Sot, Thailand respectively, with fresh field isolates. The isolates were grown on plates with serial diluted drugs for 24 hours using the candle jar method (Trager & Jensen, 1976). Lumefantrine, DBB, artemisinin, quinine and chloroquine were tested in both Uganda and Thailand. Additionally, mefloquine, pyronaridine and atovaquone were tested in Thailand and amodiaquine was tested in Uganda. The *in vitro* drug susceptibility tests were performed as described (Knauer *et al.*, 2003) with minor modifications. Immediately after incubation, thick blood smears from each well were prepared. These were stained with Giemsa and schizont maturation was assessed by microscopy from thick smears, counting all schizonts detected/200 trophozoites. Calculations of the parasite isolates' drug susceptibilities were performed using the WHO log dose response software (WHO 1983). Effective concentrations were assessed by linear regression. Probits representing the inhibited proportion of parasites were plotted against the logarithmic concentration of the analysed drug (log-response-probit model). It is common that only EC50 or EC90 is analysed for *in vitro* studies. We extended the analysis of ECs, to include a range of effective concentrations (EC10-EC90). The reason for this was to better understand the role of the analysed genotype in the parasite response to different levels of drug exposure. When analysing an effective concentration at a single point there is a risk to miss or overestimate the importance of the studied genotype. We hypothesised that an association throughout the whole range is due to a significant contribution of the genotype to the drug susceptibility. If association was only seen in a part of the EC range, we interpreted the analysed genotype not to be the only influence on the drug response. Isolates with both the analysed alleles present (mixed genotype) were excluded from the analysis of association between susceptibility and that particular genotype.

The threshold, i.e. the EC50 where parasites with a higher value are considered resistant, has been suggested to be 150 nM for lumefantrine (Kaddouri *et al.*, 2008). Since DBB is a metabolite of lumefantrine and not used as drug treatment it has no suggested threshold value. Artemisinin also lacks a suggested threshold value.

3.5 GENOTYPING

3.5.1 Blood sampling and storage

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

3.5.2 DNA extraction

In Paper I, DNA extraction for analysis of *P. falciparum merozoite surface protein 2 gene* (*pfmsp2*), *pfcrt* K76 and *pfmdr1* N86Y, was performed using methanol based methods. Briefly, a piece of filter paper with dried blood was incubated in methanol at room temperature. The methanol was then discarded and the sample was dried before adding sterile water. The filter paper was mashed using a pipette tip and then incubated at 97°C for 15 minutes.

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

3.5.3 Whole genome amplification

For Paper IV whole genome amplification (WGA) was performed following DNA extraction to save DNA. The Illustra GenomiPhi V2 DNA amplification kit was used according to the protocol of the manufacturer (GE Healthcare). The amplified DNA was only used for SNP analysis. However, for confirmation of the presence of new SNPs when they only were present in one or two samples, extracted DNA was used without WGA. Furthermore, if the samples were PCR negative, the PCR was repeated with extracted DNA.

3.5.4 *Pfmsp1* and *pfmsp2*

For categorisation of recurrent infections as recrudescences or reinfections, the *pfmsp2* was analysed as described (Snounou *et al.*, 1999). *Pfmsp2* is a single copy gene that is highly polymorphic both in sequence and size. First the outer conserved region of polymorphic repetitive block 3 was amplified followed by two separate nested reactions. One pair of oligonucleotide primers specific for the FC27 allelic type was used in one of the nested reactions while one pair specific for the IC/3D7 type was used in the other. The PCR products were separated by gel electrophoresis and visualized by UV transillumination, after staining with ethidium bromide. Samples with at least one matching band from day 0 and day of recurrent infection were interpreted as recrudescences, while no matching bands were interpreted as reinfections.

Studies have shown that two markers should be used for the discrimination between recrudescences and reinfections (Mugittu *et al.*, 2006). Therefore in Paper II additional analysis of *pfmsp1* was made. This method of analysing *pfmsp2* and *pfmsp1* was also used in Paper III. Similar to the analysis of *pfmsp2*, the outer conserved region of the polymorphic repetitive block 2 was amplified, followed by 3 separate nest amplifications. The nest amplifications were specific for the allelic type of MAD20, K1 and RO33. Only samples that were recrudescences according to *pfmsp2* genotyping were analysed for *pfmsp1*.

3.5.5 PCR-RFLP

PCR-Restriction Fragment Length Polymorphism (RFLP) is, equipment wise, a simple way to analyse SNPs and is therefore a suitable method to use in the field. All analyses were based on nested PCR, except for the analysis of *PfATP6* where a seminest was used. The nested PCR products were incubated over night at the conditions described by the restriction endonucleases manufacturer, New England Biolabs (NEB) or Fermentas. The restriction enzymes that were used are listed in Table 1. After incubation, restriction fragments were analysed on 2%–2.5% agarose gels with ethidium bromide and were visualized by UV transillumination in a BioRad GelDoc 2000. In Paper III, the analysis of *pfmdr1* N86Y and *pfcrt* K76T were performed in a multiplex PCR as described in Veiga *et al.*, 2006.

Papers where the enzymes were used	SNPs cleaved allele marked in **bold**	Restriction enzymes (manufacturer)
I and II	*Pfcrt* **K**76T	*Apo*I (NEB)
II and III	*Pfcrt* S163**R**	*Hinf*I (NEB)
I and II	*Pfmdr1* **N**86Y	*Apo*I (NEB)
II	*Pfmdr1* Y184**F**	*Tsp*509I (NEB)/TasI (Fermentas)
II	*Pfmdr1* **S**1034C	*DdeI* (NEB)
II	*Pfmdr1* **N**1042D	*AseI* (NEB)/VspI (Fermentas)
II and III	*Pfmdr1* D1246**Y**	*Eco*RV (NEB)
II	*PfATP6* **S**769N	*Rsa*I (NEB)

Table 1. Restriction enzymes used for RFLP.

3.5.6 Pyrosequencing

In Paper III, the analysis of *pfmdr1* Y184F was performed by pyrosequencing. This technology is based on sequencing by synthesis and is especially suitable for shorter DNA fragments. Pyrosequencing is advantageous because it is able to provide quantitative allele data, meaning it can give a more truthful ratio of mixed genotypes compared to RFLP. The technology is based on an enzymatic cascade: a nucleotide is added to the reaction and is, if complementary to the base in the template strand, incorporated into the DNA strand. This releases pyrophosphate, which is converted to ATP by sulfurylase. The ATP drives the luciferase-mediated oxidation of luciferin and generates visible light. The light is detected by a camera and seen as a peak in a pyrogram. Apyrase degrades unincorporated nucleotides and excess ATP before another nucleotide is added. In this way the complementary DNA strand is built up and the nucleotide sequence can be determined from the pyrogram. Briefly, samples are prepared for pyrosequencing as followed: DNA is amplified in a nested PCR with a biotinylated primer in the nest amplification. The biotinylated PCR products are incubated with streptavidin-coated Sepharose™ beads and then aspirated with a Vacuum Prep Workstation. The template is then washed with ethanol to remove salts and PCR byproducts and NaOH to make the DNA single stranded. The DNA is then hybridized with a sequencing primer and run in pyrosequencing.

For the analysis of *pfmdr1* Y184F the Pyro Gold Reagents PSQ™ 96MA and PyroMark™ MD System (Biotage AB) were used. Data analysis was performed using the PSQ™ Assay Design software. The results were adjusted after a standard curve based on mixed clones: 3D7 (Y184) and 7G8 (184F).

Unfortunately, this method is not suitable for fragments that are diverse for several consecutive amino acid positions, i.e. for *pfcrt* amino acid positions 72-76.

3.5.7 Sequencing

Sequencing is an effective way to find new mutations. It can cover large DNA fragments and is also advantageous to use for analysis of adjacent positioned SNPs, since they can be analysed in one run, i.e. *pfcrt* amino acid positions 72-76 or *pfmdr1* 1034 and 1042.

Analysis of *pfmdr1* S1034C and N1042D in Paper III as well as all genotyping in Paper IV were performed by high throughput sequencing by Macrogen. Moreover, the *PfATP6* S769N was confirmed by sequencing using the Megabace 1000 (Amersham Biosciences). It is to note that the sensitivity of sequencing in detecting alleles carried by minority parasite populations in mixed infections is limited. It is therefore possible that mixed genotypes sometimes are interpreted as a single genotype.

3.5.8 Real-time PCR

Assessment of *pfmdr1* copy number in Paper II, III and IV, was performed with TaqMan® probe based real-time PCR as described in Price *et al*., 2004 with minor modifications. The analysis for Paper II was performed using an ABI PRISM 7700 Sequence Detection System, while the other analyses were made on an ABI PRISM 7000 Sequence Detection System. The machines, TaqMan® buffer and probes were from Applied Biosystems.

Briefly, this technology is based on oligonucleotide probes with a reporter dye covalently ligated at the 5´-end and a quencher dye at the 3´-end. The proximity between them suppresses the emission of fluorescence by the reporter dye. During PCR, the primers and probes specifically hybridise to their complimentary DNA

sequence and as the DNA polymerase extends the primers, the hybridised probe is cleaved. This separates the reporter dye and the quencher dye, resulting in increased fluorescence from the reporter dye. For analysis of *pfmdr1* copy number, TAMRA® probes were used. The *pfmdr1* probe was labelled with FAM® and the probe for the endogenous control, *β-tubulin*, was labelled with VIC®. The *P. falciparum* clone 3D7 was used as a one copy calibrator for *pfmdr1* and Dd2 was used as a multi-copy control. All samples were run in triplicate. The comparative ΔΔCt (cycle threshold) method was used. Ct is the cycle number where the fluorescent crosses a set threshold. The amount of target (*pfmdr1*) is $2^{-\Delta\Delta Ct}$. Where $\Delta Ct = Ct_{pfmdr1} - Ct_{\beta\text{-}tubulin}$ and $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{3D7}$. Results of triple replicate samples were excluded if: (1) more than one replicate exhibited a Ct > 35; (2) the triple replicate samples had Ct SD > 0.5 and the Ct difference between the two remaining replicates was > 0.7 after the removal of any outlier. The parasites were considered to have an amplified *pfmdr1* gene if copy number was > 1.5.

3.6 STATISTICS

3.6.1 *In vivo*

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

In paper I and II, proportions were compared using Yates's corrected χ^2 testing (Microstat ®software, release 4; Ecosoft) and confidence intervals were calculated with the Confidence Interval Analysis (CIA) program (version 1.1). In Paper III, proportions were compared using Fisher's exact test (GraphPad QuickCalcs software; GraphPad Software). Statistical significance was defined as $P \leq 0.05$.

3.6.2 *In vitro*

For comparisons of IC/EC values, obtained from *in vitro* drug susceptibility testing, Mann-Whitney tests were used in both Paper III and IV. In the latter, the SPSS software was used, which provides two *P*-values: asymtotic and exact. The output asymtotic *P*-value was used if the size of each genotype group were > 10. Otherwise the exact *P*-value was used (Motulsky, 1995). Statistical significance was defined as $P \leq 0.05$.

4 RESULTS

4.1 PAPER I

The aim of this study was to investigate if the two main SNPs in *pfcrt* and *pfmdr1* would be selected by artemether-lumefantrine treatment in a 42 day follow-up efficacy trial performed in Zanzibar.

Pfmdr1 N86Y and *pfcrt* K76T were analysed by PCR-RFLP, for all day 0 (prior to drug administration) and all recurrent infections recorded during the follow-up period. Mixed infections were considered to contribute equally to each allele. Recurrent infections were classified as recrudescences or reinfections based on *pfmsp2* genotyping.

The *pfcrt* 76T allele was present in such high frequency, both before (97.4%) and after treatment (97.5%), that any meaningful analysis of selection after artemether-lumefantrine exposure could not be made at this stage.

A statistically significant selection of the *pfmdr1* N86 allele was observed after artemether-lumefantrine treatment. Taking into account the contribution of the mixed infections, the frequency of N86 increased from 23.4% to 45.2% (P = 0.002) after treatment. Excluding the mixed infections, the frequency of N86 increased 2.7-fold, from 15.3% to 41.0% (P = 0.00075). The majority of the parasites carrying *pfmdr1* N86 were identified between 20 and 30 days after treatment (Figure 7).

Based on *pfmsp2* analysis, 11 recrudescences were observed, but 3 were reclassified as uncertain when SNP analysis did not support the results. *Pfmsp2* analysis showed that the selection of *pfmdr1* N86 mainly occurred in reinfections (Figure 8).

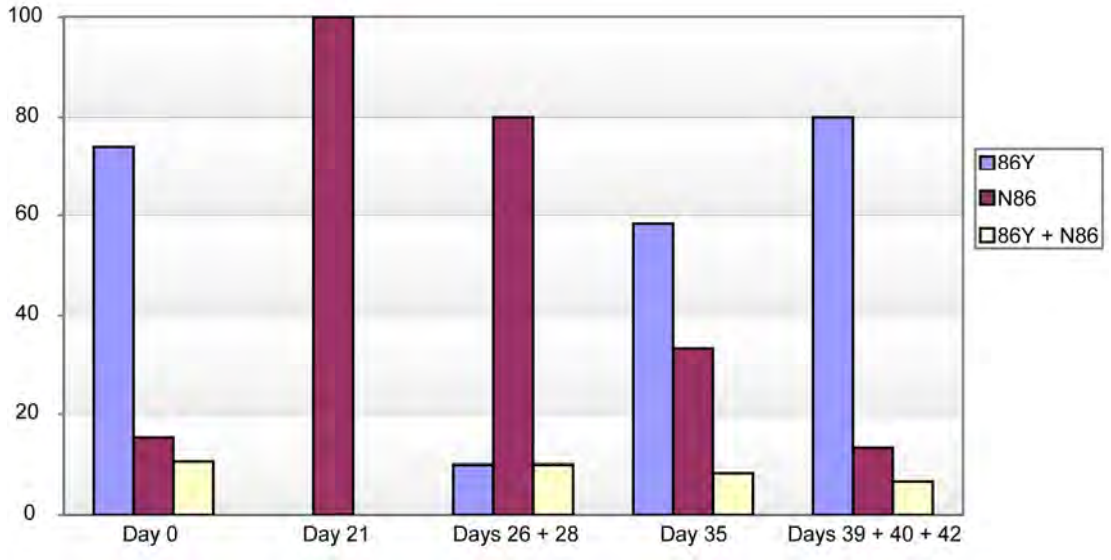

Figure 7. *Pfmdr1* N86Y SNP frequencies in infections before artemether-lumefantrine treatment (day 0) and in recurrent infections at different time points after treatment.

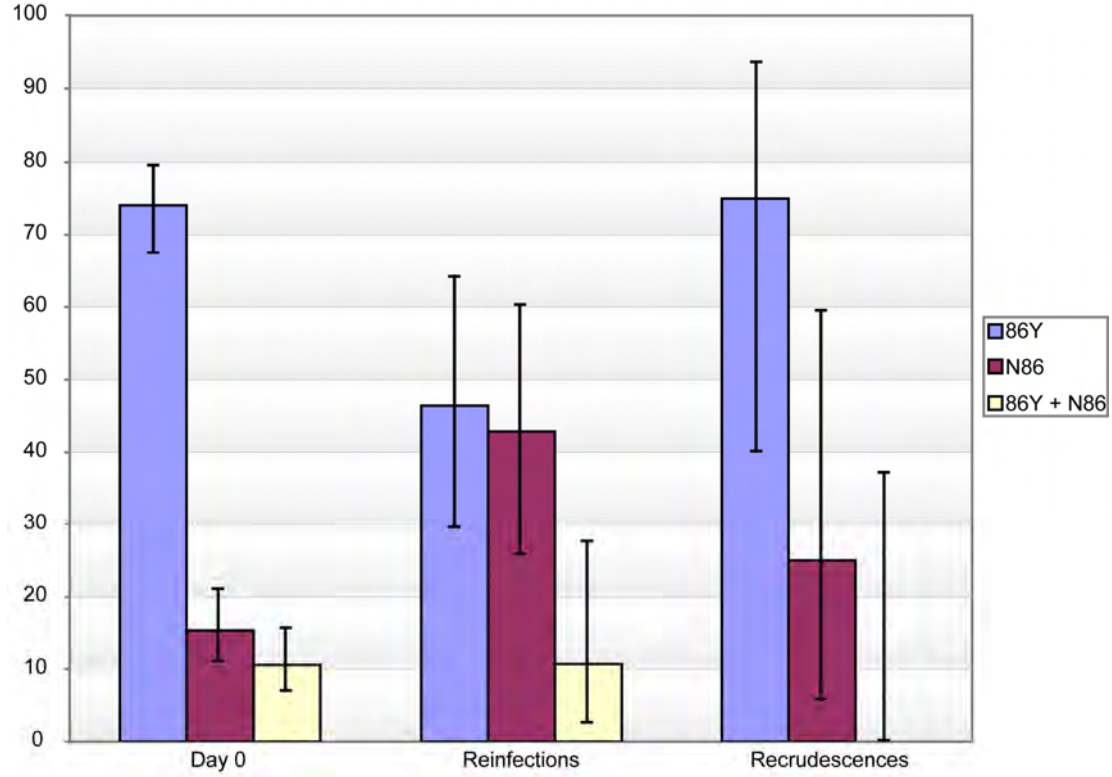

Figure 8. *Pfmdr1* N86Y SNP frequencies, with 95% confidence interval, for infections before artemether-lumefantrine treatment (day 0), reinfections and recrudescences.

4.2 PAPER II

The aim of this study was to follow up on the *in vivo* study investigated in Paper I to further explore the possible selection of other known mutations in *pfmdr1* and *pfcrt* following artemether-lumefantrine treatment. In addition, *PfATP6* S769N, suggested to be associated with decreased susceptibility to artemether (Jambou *et al*., 2005), was also investigated.

Pfmdr1 Y184F, S1034C, N1042D, D1246Y, *pfcrt* S163R and *PfATP6* S769N were analysed by PCR-RFLP while *pfmdr1* gene amplification was analysed by TaqMan® probe based real-time PCR. All day 0 and all recurrent infections recorded during the 42 day follow-up period were analysed, except for *PfATP6* S769N, where only *pfmsp2* defined recrudescent infections were analysed.

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

The *pfmdr1* 184F and D1246 alleles were observed to be selected after artemether-lumefantrine treatment. When calculating mixed infections to contribute equally to each allele, the frequency of *pfmdr1* 184F showed a statistically significant increase, from 16.6% at day 0 to 35.5% among reinfections ($P = 0.027$). Likewise, the *pfmdr1* D1246 allele increased from 66.8% to 83.9% ($P = 0.086$).

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

Similar to *pfmdr1* N86, 184F allele carrying parasites were observed to be selected mainly in the early reinfections. Furthermore the 1246Y allele was absent in the early reinfecting parasites (Figure 9).

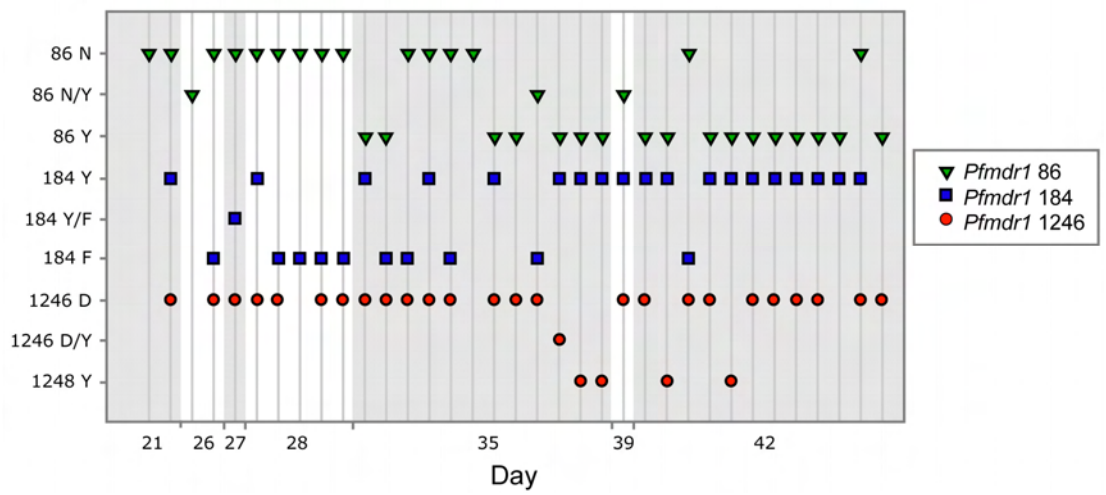

Figure 9. Distribution of *pfmdr1* N86Y, Y184F and D1246Y SNPs among the 28 *pfmsp2* adjusted reinfections during the 42-day follow-up period. Infections with *pfmdr1* N86 alleles are being selected particularly in the early reinfections. A similar selection was seen for *pfmdr1* 184F. The 1246Y allele was not present in any of the early reinfections.

The two recrudescences detected had the *pfmdr1* haplotypes N86/Y184/D1246 and Y86/Y184/1246Y respectively.

No *pfmdr1* gene amplification was recorded. *Pfmdr1* S1034C, N1042D, *pfcrt* S163R and *PfATP6* S769N were only prevalent as wild type.

4.3 PAPER III

The aim of this study was to further investigate the role of *pfmdr1* and *pfcrt* mutations in the resistance to lumefantrine, by searching for a selection of these mutations in a 42 day follow-up clinical trial performed in Fukayosi, Tanzania. In this trial children with uncomplicated *P.falciparum* malaria were randomly assigned to be treated with artemether-lumefantrine or SP. SP inhibits the synthesis of folate by targeting the dihydropteroate synthetase and dihydrofolate reductase enzymes. Accordingly, mutations in these genes are known to be responsible for the mechanism of resistance to these particular drugs, with no contribution of *pfmdr1* and *pfcrt*. Therefore we could test if any selection of *pfcrt* or *pfmdr1* mutations after artemether-lumefantrine treatment was due specifically to this drug, or a result of fitness associated with the parasite response to the pressure of any antimalarial drug treatment, in which case we would see a selection also following SP treatment. Furthermore, the aim of this study

was also to evaluate the specific influence of mutant *pfcrt* alleles, particularly the K76T mutation, on lumefantrine susceptibility by testing isogenic parasites resulting from allelic exchange experiments.

All day 0 infections as well as all recurrent infections after artemether-lumefantrine treatment were analysed. The *pfmdr1* N86Y, D1246Y, *pfcrt* K76T and S163R SNPs were analyzed by PCR-RFLP. The *pfmdr1* Y184F SNP was analysed by pyrosequencing and the *pfmdr1* S1034C and N1042D SNPs were analysed by direct PCR amplicon sequencing. The *pfmdr1* gene copy number was assessed through TaqMan® probe based real-time PCR. Mixed infections were evaluated by comparing carriers of the selected allele (including pure allele carriers and mixed infections) with parasites carrying the alternative allele in pure form.

In total, 50 children were treated with artemether-lumefantrine and 38 of these experienced recurrent infections during the 42 day follow-up. Of the 56 children treated with SP, 39 had recurrent infections. Stepwise genotyping of *pfmsp2* and *pfmsp1* revealed 2 recrudescences after artemether-lumefantrine treatment and 16 recrudescences after SP treatment.

Pfcrt K76 and *pfmdr1* N86 alleles were found to be selected by artemether-lumefantrine treatment but not by SP. The frequency of parasites carrying the *pfcrt* K76 allele, increased from 48.0% day 0 to 86.5% ($P < 0.0001$) among all recurrent infections recorded after artemether-lumefantrine treatment. Among reinfections the frequency was 85.7% ($P = 0.0001$). The limited number of recrudescences recorded after artemether-lumefantrine treatment, precluded any proper analysis of selection. *Pfmdr1* N86 carriers increased from 55.9% day 0 to 75.7% among all recurrent infections after artemether-lumefantrine treatment ($P = 0.048$). Among reinfections the frequency was 74.3% ($P = 0.071$). Only the *pfmdr1* N86Y and *pfcrt* K76T SNPs were analysed for infections occurring after SP treatment. There were no changes in the frequency of *pfcrt* K76T or *pfmdr1* N86Y in recurrent infections recorded after SP treatment (Figure 10 and 11).

In contrast to the previous study, the frequencies of the *pfmdr1* 184F and D1246 alleles did not change significantly in infections observed after artemether-lumefantrine treatment compared to day 0 (Figure 10 and 11). The *pfmdr1* S1034C

and N1042D SNPs, as well as the *pfcrt* S163R SNPs were all wild type and *pfmdr1* gene amplification was not detected. Our sequencing procedures revealed a novel *pfmdr1* W1031R SNP, located in a predicted transmembrane domain, in one day 0 infection.

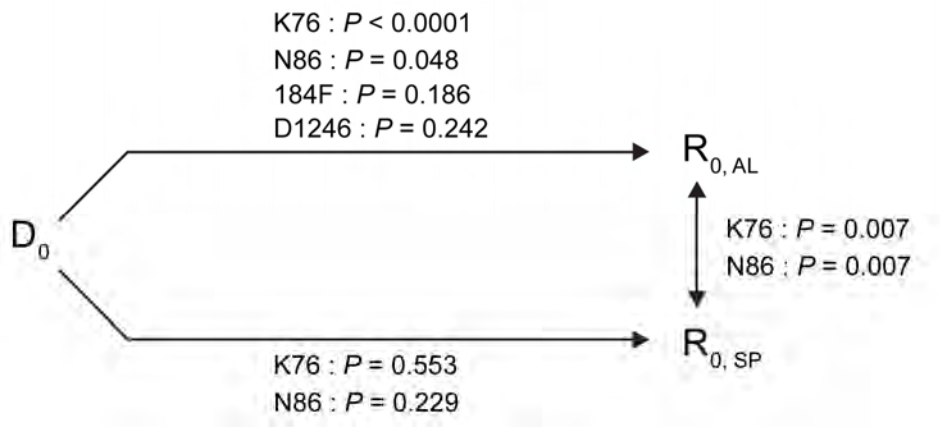

Figure 10. Comparisons between all infections before treatment (D_0) and all recurrent infections recorded after artemether-lumefantrine treatment ($R_{0,AL}$) and SP treatment ($R_{0, SP}$) respectively, for the alleles *pfcrt* K76, *pfmdr1* N86, 184F, and D1246.

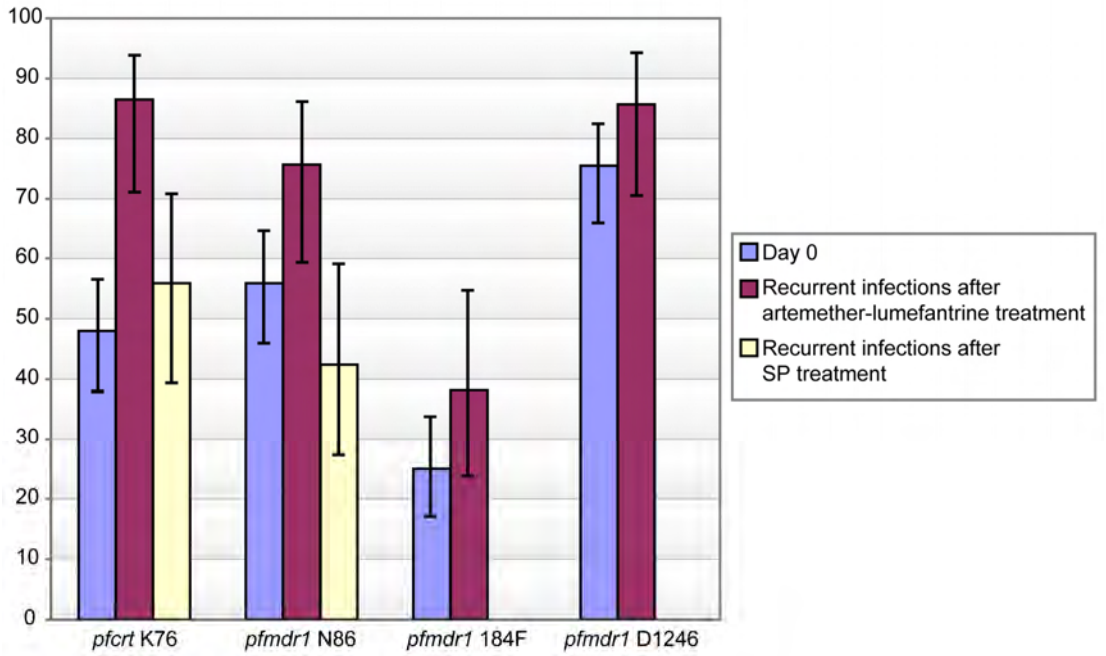

Figure 11. Frequencies of *pfcrt* K76, *pfmdr1* N86, 184F and D1246, with 95% confidence interval, in infections before treatment and after artemether-lumefantrine or SP treatment. Fisher's exact test was used to compare the frequency between day 0 and recurrent infection.

P. falciparum lines, genetically engineered to express distinct *pfcrt* alleles, were tested for their susceptibility to lumefantrine using 72-hours ^{3}H-hypoxanthine assays

with serial drug dilutions. The clones tested were GC03 (parental line), $C2^{GC03}$ (expressing the chloroquine sensitive *pfcrt* allele), $C4^{Dd2}$ (expressing the chloroquine resistant Dd2 allele typical in Asia and Africa) and $C6^{7G8}$ (expressing the chloroquine resistant 7G8 allele typical in South America, the Oceanic region and India) (Sidhu *et al.* 2002). Furthermore, clones originated from the Dd2 and 7G8 lines, expressing either the parental allele ($C\text{-}1^{Dd2}$ and $C\text{-}1^{7G8}$) or the wild type K76 allele ($T76K\text{-}1^{Dd2}$ and $T76K\text{-}1^{7G8}$), were also tested for lumefantrine susceptibility. The results showed that clones with mutant *pfcrt* alleles were consistently more susceptible to lumefantrine. The $C4^{Dd2}$ and $C6^{7G8}$ clones had about 35% lower IC50 values for lumefantrine, ($P < 0.05$ and $P < 0.01$, respectively) compared to $C2^{GC03}$. The K76T mutation itself was seen to be a large mediator of lumefantrine susceptibility. Both the $T76K\text{-}1^{Dd2}$ and $T76K\text{-}1^{7G8}$ clones had a 2-fold reduced lumefantrine susceptibility compared to their recombinant counterparts ($P < 0.01$). In the parental lines, we observed substantially lower lumefantrine IC50 values in Dd2 and 7G8, compared to GC03 (Figure 12).

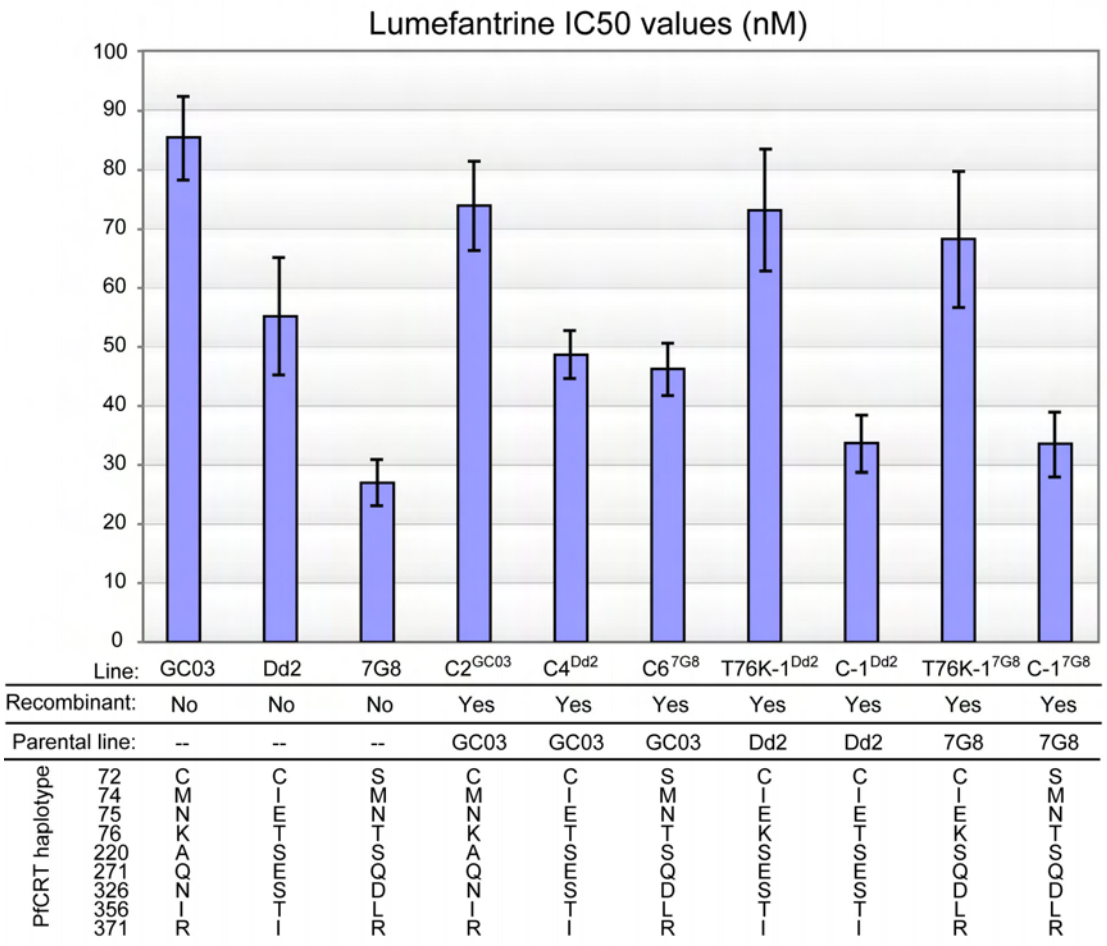

Figure 12. Lumefantrine susceptibility levels in *pfcrt* modified and parental lines. IC50 values are shown as mean values ± SE.

4.4 PAPER IV

The objective of this study was to investigate the association of *pfmdr1*, *pfcrt*, *pfmrp1* and *PfATP6* polymorphisms with *in vitro* drug susceptibility to lumefantrine and other important antimalarial drugs used in Southeast Asia and Africa. The studies were conducted in two areas with different *P. falciparum* populations, drug management and levels of transmission: Gulu in Uganda and Mae Sot in Thailand.

48 fresh isolates from Thailand and 30 from Uganda were tested for their susceptibility to lumefantrine, DBB, artemisinin, quinine and chloroquine. Furthermore the isolates from Thailand were also tested for mefloquine, pyronaridine and atovaquone and the isolates from Uganda were tested for amodiaquine. Schizont maturation was assessed by microscopy. A log-concentration-response-probit model was used to calculate the range of effective concentrations from EC10 to EC90. SNPs were analysed by sequencing and *pfmdr1* gene copy number was analysed by TaqMan® probe based real-time PCR.

In Uganda the susceptibility to lumefantrine was observed to be very diverse, as opposed to Thailand. However, all except one isolate, had an EC50 below the suggested threshold of 150 nM and were therefore considered sensitive. The lumefantrine metabolite DBB was observed to be a more potent antimalarial than its parent drug. The individual isolate EC50 values for artemisinin were observed to be highly variable in both areas (Table 2).

	Mae Sot, Thailand		**Gulu, Uganda**	
drugs	**mean EC50 (95% CI)**	**range**	**mean EC50 (95% CI)**	**range**
lumefantrine	17.9 (10.4-30.7)	6.8-56.0	32.9 (19.2-56.2)	3.2-151.2
DBB	5.4 (3.3-8.9)	0.8-17.8	11.4 (7.4-17.4)	3.4-47.3
artemisinin	12.3 (7.9-19.0)	1.5-40.5	30.7 (18.2-51.9)	5.0-175.9

Table 2. Mean and range of EC50s for lumefantrine, DBB and artemisinin in Thailand and Uganda.

The associations between drug susceptibility and mutations are presented in Table 3. We observed an association between lumefantrine susceptibility and *pfmdr1* copy number, *pfmdr1* E130K, N1042D and *pfmrp1* F1390I in Thailand. However, we did not see any association between the SNPs that we previously observed to be selected among reinfections after artemether-lumefantrine treatment and lumefantrine susceptibility, in Uganda or Thailand. Although in Thailand, the presence of *pfmdr1* N86, D1246 and *pfcrt* 76T seemed fixed.

					P-values								
Study site	**Drug**	***Gene***	**allele associated with decreased susceptibility**	***P****	**EC10**	**EC20**	**EC30**	**EC40**	**EC50**	**EC60**	**EC70**	**EC80**	**EC90**
Mae Sot	mefloquine	*pfmdr1*	N1042	e							0.043	0.021	
			copy number ≥ 2	a						0.022	0.01	0.009	0.009
		pfmrp1	H191	e			0.048	0.036	0.031	0.031	0.026	0.036	
			S437	e			0.048	0.036	0.031	0.031	0.026	0.036	
			H785	a		0.032	0.035	0.032	0.032	0.049			
			T1007	a		0.028	0.033	0.028	0.028	0.042			
			haplotype wt + double	a		0.033	0.037	0.027	0.025	0.033	0.04		
	lumefantrine	*pfmdr1*	130K	e						0.037	0.045		
			N1042	e									0.04
			copy number ≥ 2	a								0.028	0.009
		pfmrp1	1390I	e								0.041	
	DBB	*pfmdr1*	Y184	e	0.017	0.017	0.015	0.021	0.028	0.033	0.044		
			N1042	e				0.02	0.02	0.013	0.013	0.007	0.007
			copy number ≥ 2	a	0.024	0.028	0.028	0.042	0.042				
		pfmrp1	H785	a	0.008	0.008	0.013	0.013	0.013	0.013	0.014	0.014	
			T1007	a	0.013	0.012	0.017	0.016	0.017	0.019	0.019	0.016	
			haplotype wt + double	a	0.004	0.004	0.007	0.007	0.007	0.009	0.009	0.012	
	artemisinin	*pfmdr1*	copy number ≥ 2	a									0.042
		pfmrp1	F572	a				0.031	0.016	0.024	0.021	0.041	
			H785	a					0.033	0.042	0.03	0.03	
			I876	e					0.027	0.033			
			T1007	a					0.024	0.031	0.02	0.02	0.04
			haplotype wt + double	a					0.029	0.045	0.035	0.032	
Gulu	DBB	*pfcrt*	M74	e	0.048	0.048							

Table 3. Polymorphisms associated with decreased susceptibility to mefloquine, lumefantrine, DBB and artemisinin *in vitro*. *Pfmrp1* haplotype could be wild type (wt) H785/I876/T1007/F1390, double mutant H785/876V/T1007/1390I or triple mutant 785N/876V/1007M/I1390

* *P*-value is marked as asymptotic (a) or exact (e)

5 DISCUSSION

Artemether-lumefantrine, a fixed formulation known as Coartem® is currently one of the most widely implemented ACTs (WHO, 2008b) and has demonstrated high efficacy (Martensson *et al.*, 2007, Martensson *et al.*, 2005, Zongo *et al.*, 2007, Adjei *et al.*, 2008). Nevertheless high parasite transmission, common in sub-Saharan Africa, increases the probability of reinfections to occur after treatment. As a consequence, the risk is high for selection of reinfecting parasites that are tolerant or even resistant to lumefantrine, at subtherapeutic concentrations after treatment.

5.1 *PFMDR1*

We observed a selection of *pfmdr1* N86 allele carrying parasites, subsequent to artemether-lumefantrine treatment in Zanzibar (Paper I). To our knowledge, this was the first time that a statistically significant selection of a SNP, previously associated with quinoline antimalarial drug resistance, was observed following ACT treatment. However, this phenomena had already been suggested to occur previously (Duraisingh, 1999). Furthermore, the N86 allele had been observed to be associated with decreased susceptibility to lumefantrine in laboratory strains (Duraisingh *et al*., 2000a, Duraisingh *et al*., 2000b). Our *in vivo* finding has also been confirmed by others (Dokomajilar *et al.*, 2006b, Happi *et al.*, 2009, Humphreys *et al.*, 2007). We extended our first report to additional *pfmdr1* polymorphisms, including gene amplification. In Paper II we found that parasites carrying *pfmdr1* 184F and D1246, were also selected by artemether-lumefantrine.

We observed that the use of a single marker, *pfmsp2*, for determining recurrent infections as recrudescences or reinfections was not adequate. Of the 39 recurrent PCR positive samples recorded in the study from Zanzibar, 11 were classified as recrudescences based on *pfmsp2* alone. Additional analysis of *pfmsp1* showed that only 3 of these 11 were actual recrudescences. Distinguishing recrudescences and reinfections apart through stepwise genotyping of *pfmsp2* and *pfmsp1* has been recommended by others (Mugittu *et al*., 2006). In paper III we therefore used this stepwise method of *pfmsp2* and *pfmsp1* analysis for the classification of recurrent infections. The results, for both the Zanzibar and Tanzania studies, showed that the

observed allele selections occurred in reinfections, mainly during the elimination phase of lumefantrine, 20 – 30 days after the initiation of treatment. So parasites carrying *pfmdr1* N86, 184F and/or D1246 are killed by artemether-lumefantrine treatment, but are able to successfully reinfect a person earlier after treatment, compared to parasites carrying 86Y, Y184 and 1246Y.

In Zanzibar, the N86/184F haplotype was especially selected by lumefantrine followed by a similar de-selection of 86Y/184Y, while the other two haplotypes (86Y/184F and N86/Y184) were not affected. The N86/D1246 haplotype selection was also significant but not at the cost of any specific allele. The reason behind this co-selection is unclear. Possibly, the alleles act together to increase the specificity of lumefantrine. Sanchez *et al*., 2008 showed that SNPs in *pfmdr1* can change the specificity for drugs such as quinine, chloroquine and halofantrine. It is likely that this also extends to lumefantrine. Nevertheless that does not exclude that mutations in *pfmdr1* may also compensate for a possible fitness loss caused by other, yet unknown resistance mechanisms. (See section 5.3 for further discussion about fitness.)

As pointed out by Hastings & Ward, 2005, since the *pfmdr1* N86 allele carrying parasites are killed by artemether-lumefantrine treatment, but can reinfect a person earlier after treatment than 86Y carriers, the N86 form is a marker for increased tolerance to lumefantrine (Figure 13). Furthermore, they argued that mutations may increase tolerance progressively until lumefantrine resistance is reached. Alternatively, resistance may be determined by only one additional high impact mutation. What path the parasite will or has taken is still to be discovered but our observations as well as others (Dokomajilar *et al*., 2006b, Humphreys *et al*., 2007, Price *et al*., 2006, Dahlström *et al.*), have correlated many different polymorphisms with lumefantrine susceptibility suggesting that the first alternative is more likely.

Additionally we also saw an association between the *pfmdr1* SNPs E130K and N1042D and lumefantrine response in the *in vitro* study from Thailand (Paper IV). *Pfmdr1* E130K has to our knowledge only been observed in one previous report in Southeast Asia and was not associated with drug response to any of the tested drugs, mefloquine, quinine, artesunate and chloroquine (Khim *et al.*, 2005).

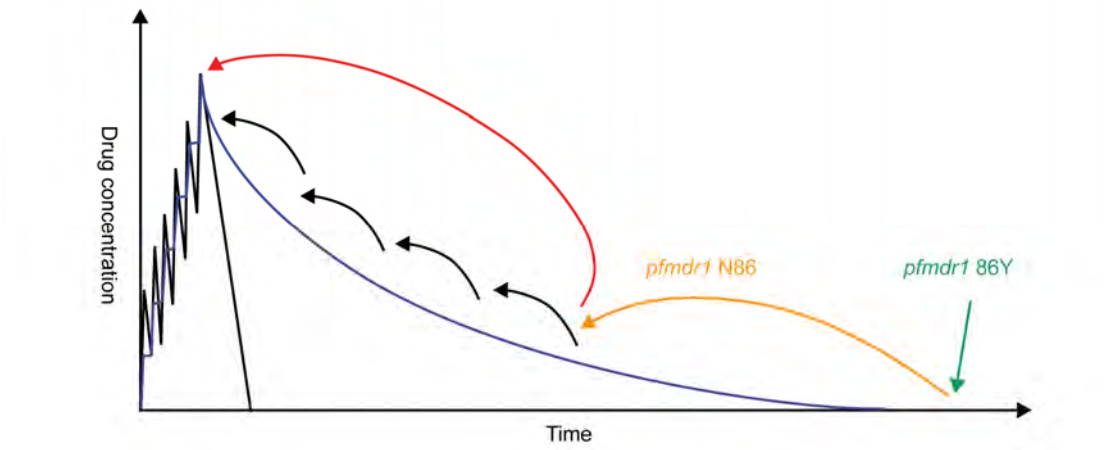

Figure 13. Shortly after a full treatment of artemether-lumefantrine, the artemether levels (shown in black) in the serum of the patient disappears leaving lumefantrine (shown in blue) unprotected. At a certain point of subtherapeutic lumefantrine levels, tolerant parasites carrying the *pfmdr* N86 allele are able to infect a person when fully sensitive 86Y carriers cannot. Hence, parasites with N86 are able to infect a person earlier after treatment. The next step in the development of resistance to lumefantrine may be gradual with small increases in tolerance. It is also possible that the next step may increase the tolerance to such a degree that the parasite will be fully resistant to lumefantrine. Adapted from (Hastings & Ward, 2005).

Pfmdr1 gene amplification has been established as a contributor to altered parasite response to arylaminoalcohols *in vitro* and *in vivo* (Price *et al*., 2004, Price *et al*., 2006, Cowman *et al*., 1994, Wilson *et al*., 1993, Price *et al*., 1999, Wilson *et al*., 1989, Peel *et al*., 1994, Preechapornkul *et al*., 2009, Alker *et al*., 2007, Lim *et al*., 2009, Rogers *et al*., 2009). The results from the study in Thailand in Paper IV, further supports these observations. Although we did not see an association between *pfmdr1* amplification and decreased susceptibility to lumefantrine throughout the whole analysed EC range, only in EC80 and EC90. For mefloquine this association was observed in EC60 to EC90. Interestingly, for the lumefantrine metabolite DBB association was seen between EC10 to EC50. According to our interpretation, that suggests that *pfmdr1* amplification is not to the only influence to the drug response, implying that the mechanism of resistance to these drugs is multifactorial or that there are alternative mechanisms of resistance. Analysis from the African study sites (Paper II-IV) did not detect any *pfmdr1* amplification, which supports its observed rarity in Africa (Holmgren *et al.*, 2006a, Dokomajilar *et al*., 2006b, Ursing *et al.*, 2006a). This may be due to a counter selection by the extensive use of chloroquine, as it has been observed that parasites selected for high levels of chloroquine resistance deamplify *pfmdr1* (Barnes *et al.*, 1992, Peel *et al*., 1994). In Gabon, low frequencies of *pfmdr1* amplification were found after treatment with low doses of mefloquine (Uhlemann *et al.*, 2005b), suggesting that selection of amplification may occur in Africa. Anyway,

the recrudescent infections found after artemether-lumefantrine treatment in Zanzibar and Tanzania cannot be explained by *pfmdr1* amplification. It is important to note that in these studies the treatment failures could have been a result of insufficient drug bioavailability since the treatment was not necessarily taken with fatty food as recommended. Unfortunately, lumefantrine plasma concentrations were not measured.

The impact of *pfmdr1* gene amplification in the mechanism of resistance to lumefantrine should be further explored, as a wide range of susceptibility levels to lumefantrine and other arylaminoalcohols has been observed without the presence of *pfmdr1* amplification (Basco *et al*., 1995, Ritchie *et al.*, 1996, Chaiyaroj *et al.*, 1999, Johnson *et al*., 2004, Price *et al*., 2004), which suggests that *pfmdr1* amplification alone is not the determinant of resistance.

5.2 *PFCRT*

In the Zanzibar study (Paper I) the observed prevalence of the *pfcrt* 76T allele was too high, both before and after treatment, to allow any conclusions to be made concerning the possible involvement of this SNP in artemether-lumefantrine response. The high prevalence of 76T is most probably a result of the selection pressure by chloroquine, which was used as first line treatment in Zanzibar for a long time and was still the first line chemotherapy when the study was performed. Compared to Zanzibar, the prevalence of 76T was markedly lower in Tanzania, which enabled us to study if *pfcrt* K76T could be involved in the tolerance/resistance to lumefantrine, i.e. if any of the alleles would be selected by artemether-lumefantrine treatment. We observed a significant selection of the *pfcrt* K76 allele following artemether-lumefantrine treatment. The recurrent infections were almost exclusively reinfections. Thus we considered the K76 allele carrying parasites, as with *pfmdr1* N86, to be tolerant to lumefantrine, not resistant. To our knowledge, this was the first report of a significant *in vivo* association between *pfcrt* and an ACT with an arylaminoalcohol.

Allelic exchange experiments of *pfcrt* have shown that K76T increases the susceptibility to mefloquine and halofantrine (Lakshmanan *et al*., 2005, Sidhu *et al*., 2002). Similarly we observed that this was also true for lumefantrine. *Pfcrt* 76T carriers were more susceptible than their K76 counterpart, irrespective of if their

background was Dd2, representing Asia and Africa, or 7G8, representing South America, the Oceanic region and India. This indicates the *pfcrt* K76T SNP as a determinant of lumefantrine susceptibility.

In Paper IV, *pfcrt* K76T was not associated with lumefantrine response. However, the frequency of 76T seemed fixed in Thailand, despite the selection pressure of mefloquine in the area. In Uganda, the K76 allele was present, but at low frequency (11% of pure K76 isolates). The observed lack of association may be due to a combination of the low frequency of K76 and the low number of isolates studied. Only one isolate in Paper IV, in Uganda, had a lumefantrine EC50 value that was higher than the suggested threshold value of 150 nM. This resistant isolate had however a 76T allele, suggesting that even though *pfcrt* K76T SNP can influence the parasite response to lumefantrine, it is not necessary for lumefantrine resistance.

The *pfcrt* S163R SNP, which was observed following *in vitro* selection experiments with halofantrine and also reduced susceptibility to mefloquine (Johnson *et al.*, 2004), was not found in any of the four study sites included in this thesis. Other studies have also failed to detect this mutation (Ursing *et al.*, 2006b, Holmgren *et al.*, 2006b), indicating that the role of this mutation in the field may not be significant for the moment.

5.3 FITNESS RELATED SELECTION

In Paper III, we had the opportunity to test if the selection of *pfmdr1* N86 and *pfcrt* K76 observed after artemether-lumefantrine treatment would also occur after treatment with SP. Resistance to SP is established to be unrelated to *pfmdr1* and *pfcrt* (Myrick *et al.*, 2003). A selection of *pfmdr1* N86 or *pfcrt* K76 by SP could be plausible since these alleles are considered wild type, implicating those parasites to be fitter than their mutant alternatives. This view is supported by *in vitro* parasite population competition experiments, using clones with allelic exchanges at the *pfmdr1* codons 1034, 1042 and 1246. These experiments showed that *pfmdr1* mutations decreased the fitness of the parasite by reduced viability of its merozoites, not being able to produce as many ring stage parasites in the subsequent generation (Hayward *et al.*, 2005). Since no selection was observed for the *pfmdr1* N86Y or *pfcrt*

K76T SNPs after SP treatment, it implies that *pfmdr1* N86 and *pfcrt* K76 are specifically associated with a decreased susceptibility to lumefantrine.

Our findings are in contrast to reports from Malawi where it was observed that the 76T allele was replaced by K76 after the introduction of SP in 1993 (Kublin *et al.*, 2003, Mita *et al.*, 2003). A possible explanation for this observation is that *pfcrt* K76 may give a relatively small fitness advantage, compared to 76T and the effect of the absence of chloroquine pressure on the parasite population might only be detectable after many years (Kublin *et al*., 2003, Laufer *et al.*, 2006, Mita *et al*., 2003, Mita *et al.*, 2004). Other observations support the role of *pfcrt* K76T in fitness. It has been observed in high transmission areas that the prevalence of *pfcrt* 76T parasites fluctuates depending on season. When the transmission is high, the prevalence of 76T and 86Y is high as a result of chloroquine use. During low transmission season when drug pressure is low, the prevalence of 76T decreases at the benefit of K76 (Abdel-Muhsin *et al.*, 2004, Ord *et al.*, 2007). In contrast to the observations in Malawi, many areas in Southeast Asia and South America, where chloroquine pressure has produced a 76T parasite rich population, re-emergence of K76 parasites have not been observed despite cessation of chloroquine treatment for *P. falciparum* malaria (Cooper *et al.*, 2005, Rungsihirunrat *et al.*, 2009, Congpuong *et al.*, 2005). In fact it has been suggested that chloroquine resistant parasites are actually fitter, having a better *in vitro* schizont maturation (Wernsdorfer *et al.*, 1995). If K76T incurs fitness cost remains unclear. Nevertheless, our results suggest that *pfcrt* 76T as well as *pfmdr1* 86Y carriers do not have a significantly decreased fitness compared to their wild type counterparts, in terms of their ability to successfully reinfect a host at the presence of SP and that the observed selection of *pfmdr1* N86 and *pfcrt* K76 is specifically associated with a tolerance to lumefantrine.

If *pfcrt* K76, *pfmdr1* N86, 130K, 184F, N1042, D1246 and gene amplification are actually involved in the mechanism of tolerance/resistance to lumefantrine or if they are indeed compensating mechanisms to maintain or increase parasite fitness gained by other mutations, similar to *pfmdr1* N86Y in chloroquine resistance, should be further explored.

5.4 THE LUMEFANTRINE METABOLITE DBB

DBB is a metabolite of lumefantrine (Ntale *et al*., 2008) and as previously reported by others (Noedl *et al*., 2001), we observed it to be more potent than lumefantrine (Paper IV). Considering this, DBB can be clinically relevant even though the plasma concentrations may be low (Ntale *et al*., 2008). In retrospect, it is reasonable to hypothesise that the observed selection patterns by artemether-lumefantrine treatment, was not driven solely by lumefantrine but also by the presence of DBB.

In Thailand we observed some similarities between parasite response to DBB and lumefantrine. *Pfmdr1* amplification and the N1042 allele were associated with decreased susceptibility to both drugs. Furthermore in Paper IV, similar to our observation of *pfcrt* K76 selection following artemether-lumefantrine treatment in Paper III, we observed an association between another wild type allele of *pfcrt* (M74) and decreased susceptibility to DBB. Therefore it is conceivable that an association of DBB and *pfcrt* K76T would be detected if the sample size would have been bigger. Overall, these similarities indicate that it is possible that DBB may contribute to the selection of lumefantrine tolerance/resistance.

Interestingly, decreased susceptibility to DBB was associated with *pfmdr1* Y184 in Paper IV in Thailand. This is in contrast to the observed selection of 184F among reinfections after artemether-lumefantrine treatment in Zanzibar. A different genetic background in Thailand compared to Africa and/or a multigenetic mechanism of resistance could be an explanation for this discrepancy.

5.5 LUMEFANTRINE MODE OF ACTION

The mode of action for lumefantrine is still not known. What we do know now is that the parasite through the action of transporter proteins survives lumefantrine better, presumably through reduction of the drug concentration at the target site. It has been suggested that Pgh1 is localized in an apical position in the membrane of the digestive vacuole and imports compounds into this organelle. In fact, experiments have shown that when *pfmdr1* is expressed in yeast it can functionally complement a plasma membrane exporter (Volkman *et al*., 1995), which has its nucleotide binding-domains facing the cytoplasm (Michaelis, 1993). *P. falciparum* Pgh1 has its nucleotide binding-

domains in the cytoplasm (Duraisingh & Cowman, 2005, Karcz *et al.*, 1993), making it a probable importer. Transport experiments showed an association between *pfmdr1* copy number and increased Fluo-4 accumulation in the digestive vacuole, which further supports this hypothesis (Rohrbach *et al*., 2006). Previous observed association between treatment failures after 4 -dose treatment with artemether-lumefantrine (Price *et al*., 2006), and our own observation of correlation between *pfmdr1* gene amplification and decreased lumefantrine susceptibility imply that the target of lumefantrine is outside the digestive vacule. PfCRT on the other hand may function as an exporter. Mutant PfCRT is believed to transport compounds out of the parasite digestive vacuole, into the cytoplasm (Valderramos & Fidock, 2006). We observed that parasites carrying *pfcrt* 76T are more susceptible to lumefantrine, compared to K76 carriers. This suggests that in parasites carrying 76T, lumefantrine might be transported out of the digestive vacuole, by PfCRT, and into the cytoplasm, consequently making the parasites susceptible to the drug action. Hence, the target of lumefantrine may lie in the cytoplasm. While PfCRT K76, is less able (or maybe unable) to transport lumefantrine, maintaining the drug inside the organelle, protecting the parasite (Figure 14).

5.6 ALTERNATIVE MECHANISMS OF RESISTANCE

Pfcrt and *pfmdr1* may not be the only genes involved in the mechanism of tolerance or resistance to lumefantrine. Other genes, such as the *pfmrp1* gene may also influence the lumefantrine response. Experiments show that disruption of *pfmrp1* results in increased accumulation of chloroquine and quinine (Raj *et al*., 2009). Possibly, PfMRP1 may have the same function when it comes to lumefantrine. In Paper IV we observed that *pfmrp1* F1390I was correlated with lumefantrine susceptibillity. Furthermore, in a follow-up study on the trials from Zanzibar and Tanzania it was observed that *pfmrp1* 876I carrying parasites were selected among recurrent infections after artemether-lumefantrine treatment (Dahlström *et al*.).

Our results in Paper IV showed a correlation between SNPs in *pfmrp1* and susceptibility to various unrelated antimalarial drugs, suggesting that *pfmrp1* may be involved in a broad range multidrug resistance mechanism.

As with *pfmrp1*, other yet unstudied genes may also have a function in the *P. falciparum* mechanism of resistance. Overall, the parasite response to lumefantrine is a result of a multigenic effect, where at least the *pfcrt*, *pfmdr1* and *pfmrp1* gene coded proteins seem to produce the phenotype.

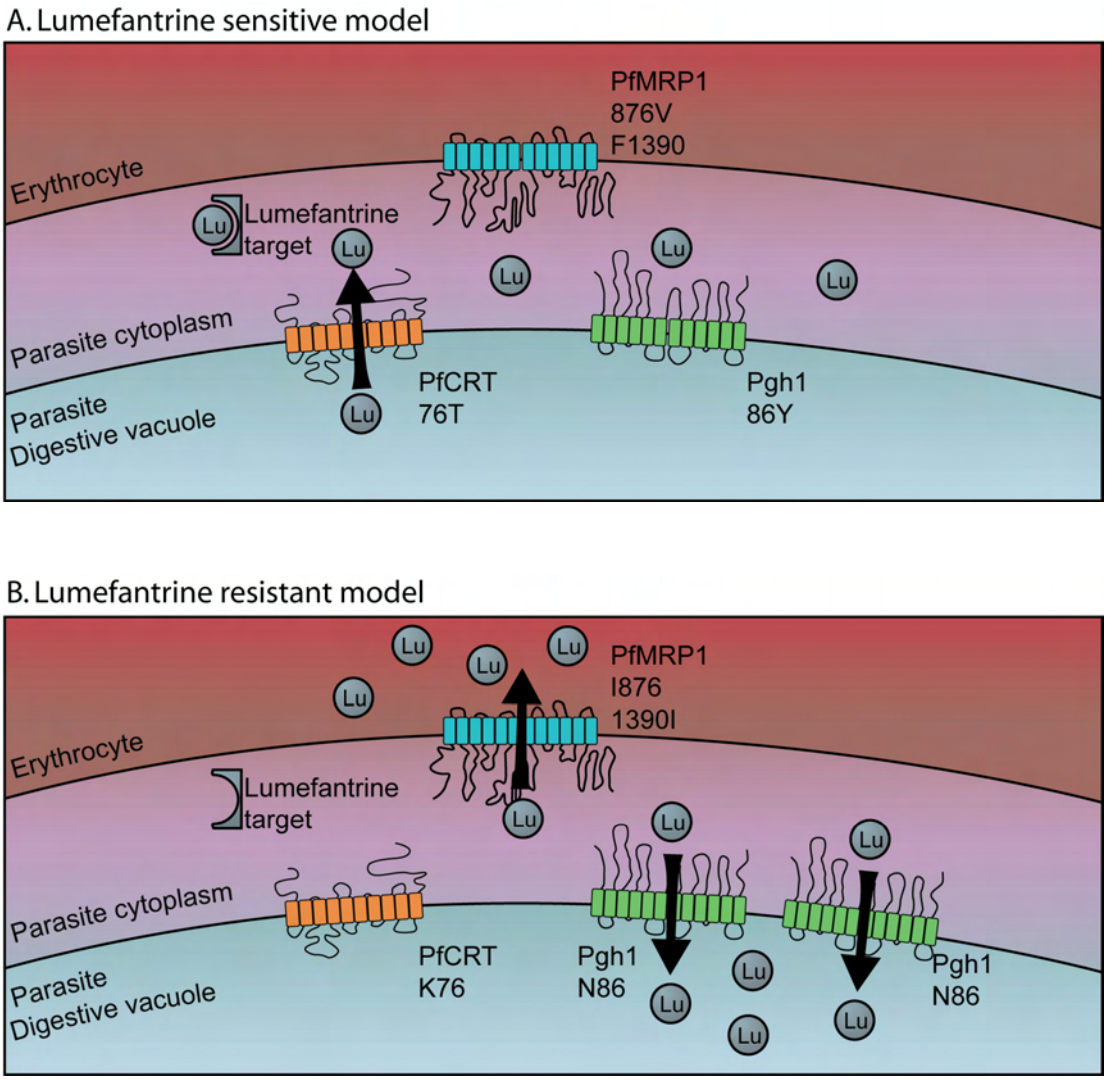

Figure 14. Possible model of PfCRT, Pgh1 and PfMRP1 mediated lumefantrine resistance.
A) In lumefantrine sensitive parasites, mutant PfCRT (76T) transports lumefantrine out of the digestive vacuole and into the cytoplasm, where lumefantrine may have its target. Mutant Pgh1 (86Y) and PfMRP1 with 876V and/or F1390 on the other hand are not able to transport lumefantrine (at all or insufficiently).
B) In lumefantrine resistant parasites, wild type Pgh1 (N86) transports lumefantrine into the digestive vacuole away from its supposed target in the parasite cytoplasm. Increased number of Pgh1 further increases the transportation. PfMRP1 with I876 and/or 1390I transports lumefantrine out from the parasite, while PfCRT K76 is not/less able to transport lumefantrine, leaving the drug inside the digestive vacuole.

6 CONCLUSIONS

6.1 OVERALL CONCLUSION

The overall conclusion of this thesis is that *pfmdr1* and *pfcrt* may be involved in the mechanism of resistance to lumefantrine.

6.2 SPECIFIC CONCLUSIONS

- Resistance to lumefantrine may be multigenic, including *pfmdr1*, *pfcrt* and other genes such as *pfmrp1*.

- Selection of *pfmdr1* N86 and *pfcrt* K76 alleles by artemether-lumefantrine treatment is drug specific and not fitness related.

- The *pfmdr1* N86, 184F and D1246 alleles alone, or in combination as haplotypes (N86/184F and N86/D1246), may be used as markers for *in vivo* lumefantrine tolerance, especially in Africa.

- *Pfcrt* K76 may be used as a marker for artemether-lumefantrine tolerance in areas of different genetic backgrounds.

- *Pfmdr1* gene amplifications are rare in East Africa but may potentially represent a further development towards resistance to lumefantrine.

7 PERSONAL REFLECTIONS AND FUTURE PERSPECTIVES

As a response to our findings of *pfmdr1* N86 being selected by artemether-lumefantrine treatment, concerns about the concept of combination therapy with drugs of unmatched pharmacokinetics were raised (Hastings & Ward, 2005). Hastings and Ward recognised the weakness of ACT, that parasites may evolve resistance sequentially as the partner drug with the longer half life remains alone after treatment, a weakness that may undermine the benefits of combination therapy. Our results are indeed a reflection of that. Artemether-lumefantrine tolerance or resistance could emerge in Africa. This would have major public health implications for artemether-lumfantrine as a malaria treatment, in terms of its ability to reduce malaria associated morbidity and mortality. It is therefore important to understand the progression of resistance to artemether-lumefantrine and to determine molecular markers for surveillance of both tolerance and resistance. In this context, markers for tolerance will most probably have a more practical use, since it will function as an early warning of emerging resistance, thereby offering the policy makers more time to change to a more effective drug before the impact of resistance results in clinical failures.

The potentially multigenic mechanism of resistance to lumefantrine offers hope for a slower development of resistance. To our knowledge, no treatment failures due to true resistance have so far been unquestionably reported. However, it is to note that one isolate in the study from Uganda (Paper IV) had a lumefantrine EC50 above the suggested threshold of resistance to this drug. If this threshold value is of clinical relevance is yet to be determined. Unfortunately this patient was not followed up.

The mean EC50 and EC90 values for lumefantrine were marginally higher in Uganda compared to Thailand. The lumefantrine susceptibility in Uganda was also remarkably diverse, going from very sensitive to resistant. The high effective concentrations in Uganda could be due to uncontrolled usage of antimalarials. In the study area, about 80% of malaria patients are only clinically diagnosed, i.e. all fever cases are treated with artemether-lumefantrine. Artemether-lumefantrine is also accessible in the local pharmacies, without prescription. Consequently, the overuse may have selected for

parasite populations with decreased susceptibility to both artemether and lumefantrine. In contrast, in Thailand all malaria cases are laboratory-confirmed prior to treatment and antimalarial drugs are not available outside the health facilities. Improper drug use will certainly add to the spread of resistance unnecessarily. Proper diagnosis and proper treatment is fundamental. Otherwise, our present hope, ACT, will be lost. Artemisinin derivatives are the only antimalarial drug that *P. falciparum* has not managed to overcome yet. Alarmingly, this could soon change, since artesunate resistance has recently been reported (Noedl *et al*., 2008). Moreover, in Paper IV, we observed a great deal of variation for artemisinin.

Our results from Paper III showed that *pfcrt* 76T, the molecular marker for chloroquine resistance, incurs lumefantrine sensitivity. Mutant *pfcrt* alleles has also been observed to increase susceptibility to artemisinin (Sidhu *et al*., 2002), suggesting that artemether-lumefantrine is ideal in areas of high prevalence of mutant *pfcrt.* The downside is however that as some alleles are associated to both lumefantrine and artemisinin susceptibility there is a risk for ACT to accelerate the development of resistance to artemisinin derivatives. Although artemisinin or its derivatives may not select for these alleles themselves due to their fast elimination, lumefantrine pressure may provide the main selective force and drive a decrease in susceptibility to both drugs. This shows that an understanding of the molecular basis of drug resistance is crucial, both when it comes to combining drugs and implementing them. Zanzibar chose to implement artesunate-amodiaquine as first line treatment and artemether-lumefantrine as second line. This has proven to be an excellent choice since amodiaquine selects *pfmdr1* 86Y and *pfcrt* 76T (Holmgren *et al*., 2006b, Happi *et al*., 2006, Dokomajilar *et al*., 2006a), the opposite of lumefantrine. It is likely that this counter selection by the two drug combinations will delay an emergence of resistance in the area.

Malaria is a major public health problem which is further enhanced by *P. falciparum* drug resistance. The understanding of the mechanism of resistance to antimalarial drugs is crucial. This thesis has shed some light on the complex issue of *P. falciparum* resistance to lumefantrine. Much further work is needed to fully understand its molecular basis.

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