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PLASMODIUM FALCIPARUM RESISTANCE TO AMODIAQUINE IN MONOTHERAPY AND IN COMBINATION THERAPY WITH ARTESUNATE

Gabrielle Holmgren

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ABSTRACT

In response to the increasing resistance to antimalarial monotherapies, artemisinin based combination therapy (ACT) is now recommended as first line therapy against uncomplicated *Plasmodium falciparum* malaria. However, the choice of partner drug to artemisinin (ART) is critical for ACT efficacy to endure. One main partner drug option for ACT is amodiaquine (AQ), with its active long half-life metabolite desethyl-amodiaquine (DEAQ). AQ is related to chloroquine (CQ) and has been widely used in Africa for decades, but despite widespread CQ resistance it has remained relatively effective. CQ resistance has been associated with mutations in the *P. falciparum* CQ resistance transporter (*pfcrt*) gene and the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene. Possible mutations associated with AQ/DEAQ resistance have remained unclear. In this thesis we explore whether mutations, especially in the *pfcrt* and *pfmdr1* genes, are associated with tolerance/resistance to AQ/DEAQ in monotherapy and in ACT, as well as a possible associated parasite fitness cost.

The thesis is based on (a) in vivo clinical trials in East-Africa with AQ monotherapy or ART plus AQ (ASAQ) combination therapy and (b) in vitro studies on isolates from Colombia and reference clones in which the *pfmdr1* gene has been modified by allelic exchange. Mutation analyses were done by PCR followed by either RFLP and/or DNA pyrosequencing or full sequencing and gene amplification analysis was done by TaqMan probe based Real-Time PCR. The genetic results were related to drug susceptibilities determined by an HRP2-ELISA assay and parasite growth in competition experiments in vitro and/or the clinical outcome in vivo.

The treatment failure rate after AQ therapy was relatively high (20%), while after ASAQ therapy it fulfilled the efficacy criteria of WHO (<10%). AQ/DEAQ tolerance/resistance was found to be associated with *pfmdr1* 1246Y in addition to *pfcrt* (a.a. 72 – 76) CVIET/SVMNT and possibly in a synergistic or compensatory relation with *pfmdr1* 86Y, 184Y, 1034C and 1042D. Possibly *pfcrt* 326S/D and 356T/L, as well as a newly identified mutation *pfcrt* 334N, are also involved. Treatment failure after AQ monotherapy was not found to be associated with rare findings of *pfmdr1* amplifications or variable DEAQ blood concentrations. No new mutations could be verified in the *pfcrt* and *pfmdr1* genes in recrudescent parasites after AQ or ASAQ therapy. The partial cross-resistance with CQ is probably conferred to mainly DEAQ through the *pfcrt* gene, while resistance to AQ may be more dependent on the *pfmdr1* gene. *Pfmdr1* 1246Y was associated with a substantial fitness cost to the parasite. The relative growth for parasites with a particular mutation represents a concentration dependent balance between the fitness cost and the specific drug selection benefit. The in vitro estimated cost-benefit from *pfmdr1* 1246Y correlated with the allele dynamics after AQ and ASAQ therapy in vivo. The added effect of ART will potentially prevent against a selection of *pfmdr1* 1246Y by a more effective reduction of parasite biomass and an opposite *pfmdr1* allele selection and in the absence of AQ exposure *pfmdr1* 1246Y will possibly incur too substantial fitness cost to sustain in competition with wild type parasites. We conclude that several factors counteract a selection of AQ resistance, which support sustained efficacy of ASAQ. Thus, we estimate that AQ represents a valuable partner drug option in ACT in East Africa.

LIST OF PUBLICATIONS

I. **Gabrielle Holmgren**, José P. Gil, Pedro E. Ferreira, Isabel M. Veiga, Charles O. Obonyo and Anders Björkman. Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with a selection of *pfcrt* 76T and *pfmdr1* 86Y. Infect Genet Evol. 2006 Jul;6(4):309-14.

II. **Gabrielle Holmgren**, Anders Björkman and José P. Gil. Amodiaquine resistance is not related to rare findings of *pfmdr1* gene amplifications in Kenya. Trop Med Int Health. 2006 Dec;11(12):1808-12.

III. **Gabrielle Holmgren**, Johan Hamrin, Jenny Svärd, Andreas Mårtensson, José P. Gil and Anders Björkman. Selection of *pfmdr1* mutations after amodiaquine monotherapy and amodiaquine plus artemisinine combination therapy in East-Africa. Infect Genet Evol. 2007 Sep; 7(5):562-9.

IV. Diego F Echeverry, **Gabrielle Holmgren**, Claribel Murillo, Juan C Higuita, Anders Björkman, Jose P Gil and Lyda Osorio. Polymorphisms in the *pfcrt* and *pfmdr1* genes and in vitro susceptibility to amodiaquine and desethylamodiaquine.. Am J Trop Med. Hyg. 2007; 77(6): 1034-1038.

V. **Gabrielle Holmgren**, Pedro E. Ferreira, Anders Björkman and José P. Gil. The cost-benefit of *Plasmodium falciparum mdr1* mutations associated with resistance to amodiaquine and desethylamodiaquine. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACT	Artemisinin-based combination therapy
ART	Artemisinin
AS	Artesunate
ASAQ	Artesunate plus amodiaquine
AQ	Amodiaquine
CI	Confidence interval
Ct	Cycle threshold
CQ	Chloroquine
DEAQ	Desethylamodiaquine
DNDi	Drugs for Neglected Diseases Initiative
EIR	Entomological inoculation rate
g	Relative growth excl fitness cost
g´	Relative growth incl fitness cost
IC	Inhibitory concentration
IPTp	Intermittent preventive treatment for pregnant women
IRS	Indoor residual spraying
ITN	Insecticide treated net
PCR	Polymerase chain reaction
PfATP6	*P. falciparum* SERCA orthologue 6 protein
PfATP6	*P. falciparum* SERCA orthologue 6 gene
PfCRT	*P. falciparum* chloroquine resistance transporter protein
Pfcrt	*P. falciparum* chloroquine resistance transporter gene
PfMDR1	*P. falciparum* multidrug resistance 1 protein
Pfmdr1	*P. falciparum* multidrug resistance 1 gene
PfMRP1	*P. falciparum* multidrug resistance protein 1 protein
Pfmrp1	*P. falciparum* multidrug resistance protein 1 gene
Pfmsp2	*P. faciparum* merozoite surface protein 2 gene
Pgh1	P-glycoprotein homologue 1
qPCR	Quantitative PCR (Real-time PCR)
RDT	Rapid diagnostic test
RFLP	Restriction fragment length polymorphism
SERCA	Sarco/endoplasmatic reticulum Ca^{2+}-ATPase
SNP	Single nucleotide polymorphism
w	Relative fitness
WHO	World Health Organisation
*	p-value < 0.05 as compared with base-line
**	p-value < 0.001 as compared with base-line

1 INTRODUCTION

1.1 THE GLOBAL BURDEN OF MALARIA

Malaria remains as one of the leading causes of death from a single infectious pathogen. Approximately 5% of the world's population is infected and almost 250 million cases of malaria and 900 000 associated deaths worldwide were estimated in 2008. The African region accounts for about 90% of this burden, out of which children below five years are the most vulnerable group accounting for 85% of malaria deaths (White, 2003; WHO, 2009c).
The burden of malaria morbidity and mortality also has a devastating impact on social and economic development. The global distribution of per-capita gross domestic product (GDP) shows a remarkable correlation between poverty and malaria. Poverty may cause increased malaria transmission by hampered malaria prevention and treatment. But most probably causality runs in both directions. Malaria affects the micro economy by increased medical costs, absenteeism from work and school, impaired cognitive development, increased fertility rate, as well as the macro economy by negative effects on tourism, investments and trade (Sachs and Malaney, 2002).

1.2 THE *PLASMODIUM* PARASITE

Malaria is a parasitic disease caused by a protozoan of the genus *Plasmodium.* The name malaria, from the Italian *mal aria* meaning *bad air*, derives from the belief that malaria was caused by the malodorous air surrounding marshy areas, which are the breeding places for the *Plasmodium* vector i.e. the *Anopheles* mosquitoes.
Plasmodium includes hundreds of species able to infect birds, reptiles and mammals. Traditionally four species are able to infect humans i.e. *P. falciparum, P. vivax, P. ovale* and *P. malariae. P. falciparum* is the most common parasite and is found in the tropics worldwide, as well as the uncommon *P. malariae. P. vivax* is the most widespread, but uncommon in Africa. *P. ovale,* on the other hand, exists mainly in Africa. Main features of these species are summarized in Table 1. Recently a fifth species, *P. knowlesi,* normally infecting macaque monkeys on Borneo, has been reported to also infect humans (White, 2008).
Archeological findings suggest that the parasite has been infecting human since prehistoric times. *P. falciparum* appear to be acquired most recently within perhaps 10 000 years ago, where recent research suggest *P. falciparum* malaria as the most likely cause of death in Tutankhamun in Egypt (Hawass et al., 2010). References to seasonal fever dates back thousands of years in the ancient Assyrian, Chinese and Indian religious and medical texts. Hippocrates was the first by describing the recurrent fever and enlarged spleen typical for malaria and correlated these findings to season and where the patients lived. The parasite was first discovered in human blood by the French military doctor Alphonse Laveran in Algeria 1880. Eighteen years later in 1898 the British army surgeon Ronald Ross stationed in India observed the parasite within the mosquito gut and could describe the complete life cycle. In 1917 the German psychiatrist Julius Wagner-Jauregg was the first to give a patient with neurosyphilis "fever therapy" by inoculating malaria infected blood, which became the standard practice until the discovery of penicillin thirty years later. Laveran, Ross and Wagner-Jauregg were all rewarded with the Nobel Prize for their respective discoveries (White, 2003).

This thesis will concentrate on the most fatal *P. falciparum*, which cause about 90% of all malaria cases worldwide. At the same time it is the species most associated with drug resistance and thus responsible for the majority of the global burden of malaria.

	P. falciparum	***P. vivax***	***P. ovale***	***P. malariae***
Pre-erythrocytic period (days)	7	7[a]	9[a]	15
Incubation period	9 - 14	12 – 16[a]	16 – 18[a]	18 - 40
Hypnozoites	No	Yes	Yes	No
Duration of erythrocytic cycle/fever periodicity	48 h	48 h	48 h	72 h
Erythrocytes parasitized	All	Reticulocytes	Reticulocytes	Mature
Multiple parasites per erythrocyte	Yes	No	No	No
Merozoites per schizont	8 – 32	12 – 24	4 – 16	6 - 12
Sequestration	Yes	No	No	No
Parasitaemia (average and maximum)	0.4 – 10%[b] 40%	0.4% 1%	0.2% 0.6%	0.1% 0.4%
Clinical severity	+++[b]	++	+	+
Duration of untreated infection (years)	1 – 2	2 – 5	2 – 5	3 – 50
Drug resistance	+++	+	-	-

Table 1. Main features of the human *Plasmodium* species (Warrell and Gilles, 2002). [a]The pre-erythrocytic period and incubation period can be prolonged with hypnozoite development. [b]The parasitaemia and clinical severity are very much dependent on immunity.

1.3 THE LIFE CYCLE OF *PLASMODIUM FALCIPARUM*

P. falciparum has an intricate life cycle rotating between the female *Anopheline* mosquito (vector) and the human (host) (Figure 1). In summary, the pre-erythrocytic stage starts by the infected *Anopheline* mosquito injecting saliva containing sporozoites into the human host whilst having a blood meal. The sporozoites promptly find their way in the bloodstream to the liver, where they invade the hepatocytes within 30-60 minutes. During the following 7-14 days they undergo asexual replication where each mature schizont contains up to 30 000 merozoites (tissue schizogony).

The erythrocytic stage, which is associated with the clinical disease, starts by the merozoites being released from the liver and invade the erythrocytes within 30 seconds. During the following 48 hours the parasites undergo asexual replication again (erythrocytic schizogony). The merozoites mature from early trophozoites (ring-stage) to late trophozoites to schizonts containing 8 - 32 merozoites. The erythrocytes finally burst and release the merozoites into the blood stream, which is associated with a fever episode. The merozoites readily invade new erythrocytes and start a new replication cycle. Thus, the parasite biomass is approximately ten folded with every replication cycle.

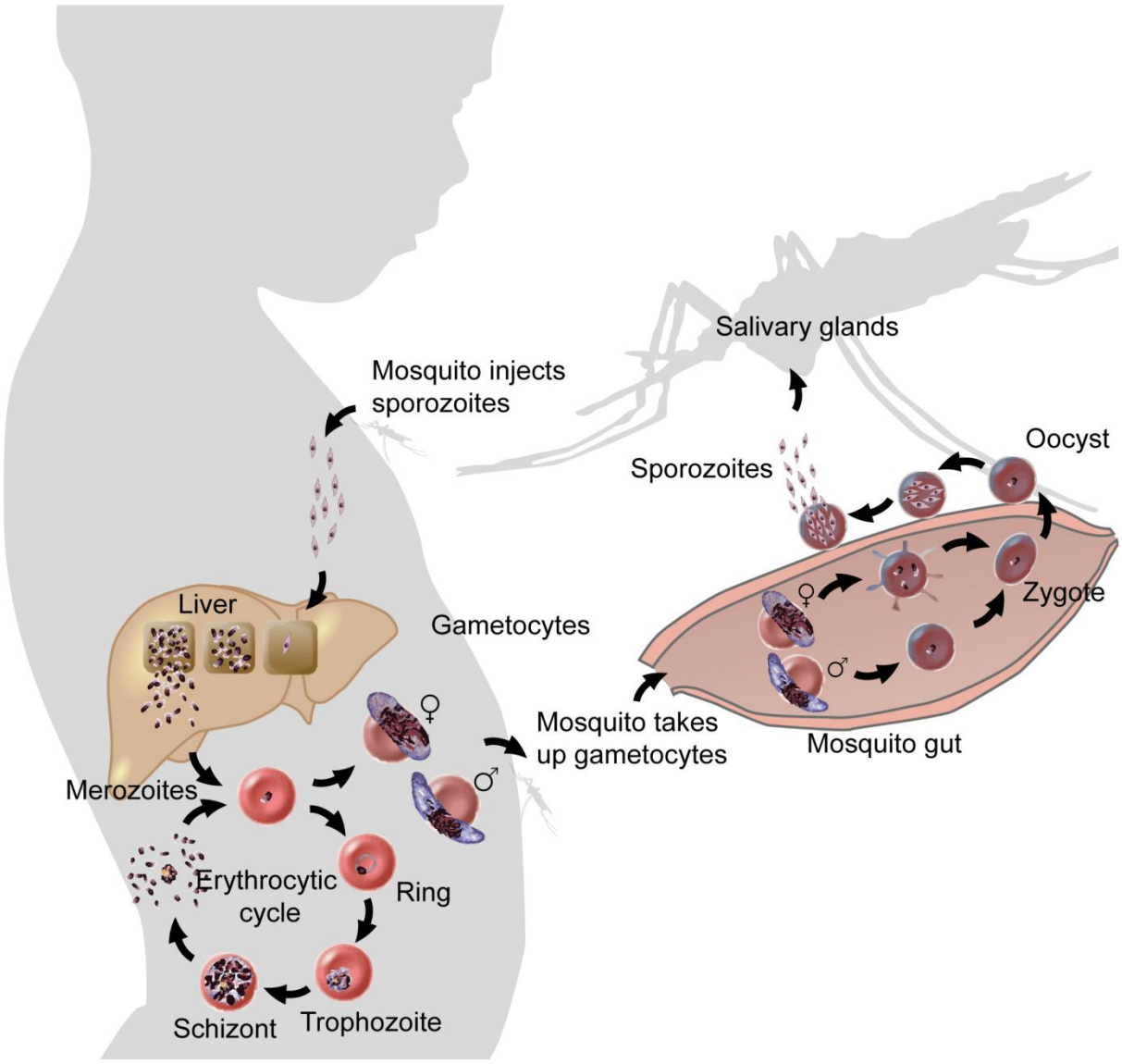


Figure 1. The life cycle of *P. falciparum.* (Published with kind permission from Leopold Roos.)

After approximately 12 hours of erythrocytic schizogony *P. falciparum* starts to exhibit an erythrocyte membrane protein (*Pf*EMP1) that mediates adherence to capillary endothelium in the deep vascular system i.e. sequestration, which might cause vital organ dysfunction. Therefore only trophozoites, but not schizonts, can be observed in peripheral blood taken from patients infected with this species.

After a number of asexual cycles a small proportion of parasites develop over 7-10 days into gametocytes, the sexual forms of the parasite (gametocytogenesis), with a 1:4 male-female ratio. If an *Anopheline* mosquito happens to ingest gametocytes whilst having a blood meal, the parasites are activated and during the following 8-16 days the parasites undergo formation into sporozoites (sporogony). First the male parasites develop a flagellum (microgametes) and seek and fuse with the female parasites (macrogametes) to form diploid zygotes. After sexual recombination (meiosis) is completed motile ookinetes are formed and penetrate the mosquito gut where they encyst. Within this oocysts the parasites undergo asexual replication to form thousands of haploid sporozoites. Before the mature oocysts burst, they reach a diameter of

approximately 0.5 mm and are visible to the naked eye. The liberated sporozoites migrate to the salivary glands awaiting an inoculation into the next human host whilst the mosquito is having a blood meal.

1.4 EPIDEMIOLOGY

Half of the world's population lives at risk of malaria (~3.3 billion) in 108 countries in tropical and sub-tropical Africa, Asia, Oceania and Latin Americas (WHO, 2009b) (Figure 2). Malaria was endemic also in Europe, North America and Russia until the nineteenth century, when it was eliminated, probably due to improved housing, farming and infrastructure.
The epidemiology of malaria is complex and is dependent on an interaction between the *Plasmodium* parasite, the *Anopheles* vector and the human host, which has to be taken into account in the efforts of malaria control (Warrell and Gilles, 2002). *Plasmodium* sporogony is optimal in humid air and depending on species in a temperature between 20 - 30ºC and cannot occur below 16ºC or above 33ºC and at altitudes above 2000 m. There are about 430 *Anopheles* species, out of which about 40 are able to transmit malaria. The *Anopheles* mosquitoes are found almost worldwide, but are most common in tropical and sub-tropical areas. Thus, the malaria vector still exists in Europe with a potential risk of reintroduction of malaria. Normally the mosquitoes feed between dusk and dawn, but in densely forest some species can feed during daytime. Depending on species the mosquitoes prefer to feed on humans (*anthropophilic*) or cattle (*zoophilic*) and outdoor (*exophagic*) or indoor (*endophagic*). *A. gambiae* which predominates in sub-Saharan Africa is mainly *anthropophilic* and *endophagic*. Seasonal fluctuations in temperature, humidity and rainfall affect the breeding, with an explosive increase during and after the rainy seasons in the tropics.

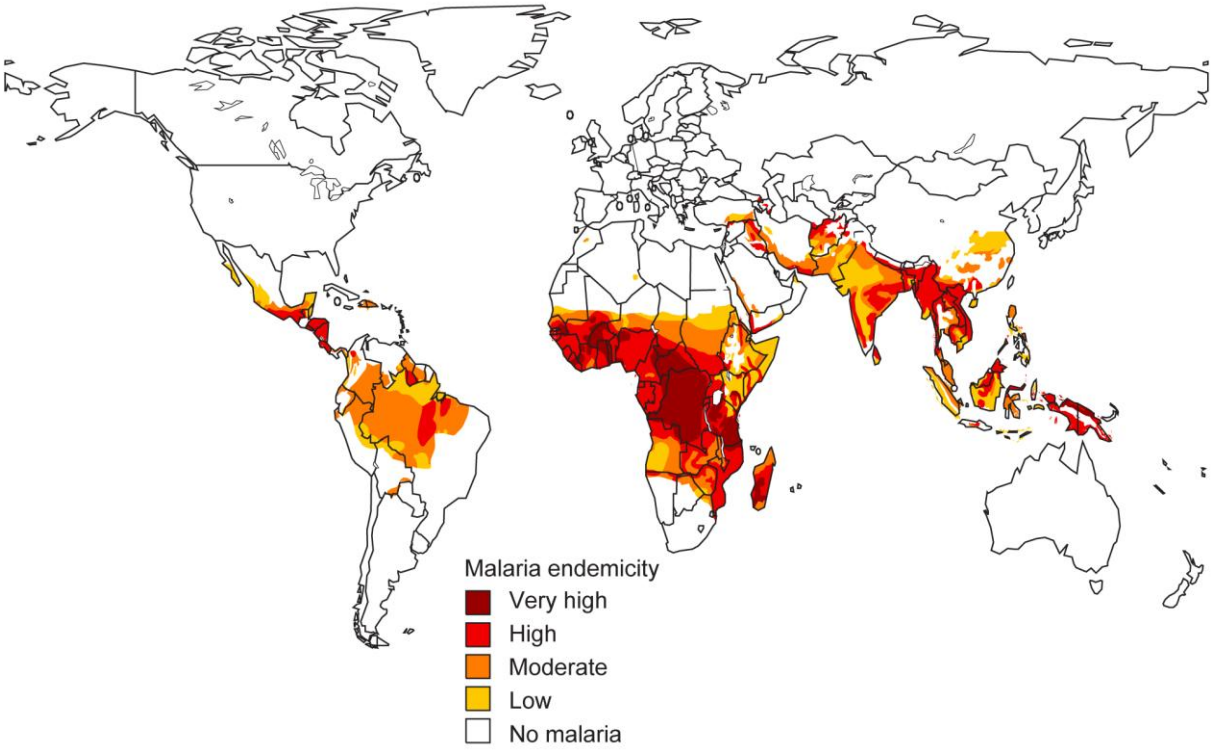


Figure 2. Global distribution of malaria transmission (WHO, 2005a).

Malaria transmission can either be continuous (endemic) or intermittent (epidemic) and regular (stable) or irregular (unstable). Transmission intensity i.e. the number of infective mosquito bites received per person per year, is defined by the entomological inoculation rate (EIR). In most of sub-Saharan Africa the transmission is endemic and stable with high EIR (>100) or in the most intense areas very high EIR (>500), while in most of Asia and Latin America the transmission is unstable with low EIR (<10) and more prone to epidemics.
A rare way of malaria transmission that does not include the mosquito vector is from mother to child during pregnancy or delivery (vertical transmission) or via blood transfusions (iatrogenic transmission).

1.5 MALARIA CONTROL

Malaria control can be defined as reducing the disease burden to a level at which it is no longer a public health problem (WHO, 2009c). The Millenium Development Goals established by The Roll Back Malaria (RBM) Partnership at WHO are to reduce the numbers of malaria cases and deaths recorded in 2000 by at least 50% by 2010 and at least 75% by 2015 (UN, 2000). To further define and accelerate this achievement, RBM launched the Global Malaria Action Plan in 2008 (WHO, 2008). The plan includes prompt parasitological confirmation by microscopy or with a rapid diagnostic test (RDT), modern and efficacious artemisinin based combination therapy (ACT), intermittent preventive treatment for pregnant women (IPTp), improved vector control with long lasting insecticide treated nets (ITN) and indoor residual spraying (IRS). Convincing proof of concept, such as marked reduction in overall child morbidity and mortality in endemic areas of Africa (Bhattarai et al., 2007), has encouraged the debate on a potential elimination of malaria (WHO, 2008). However, parasite resistance to antimalarial drugs and mosquito resistance to insecticides are major threats to achieving global malaria control (WHO, 2009c).

1.6 THE CLINICAL DISEASE OF *PLASMODIUM FALCIPARUM*

The clinical disease is associated with the erythrocytic stage of the infection, with an incubation time of 9 – 14 days. Clinical malaria can be classified into uncomplicated and complicated (severe) disease. The cardinal symptoms are fever and chills, often associated with malaise, headache, muscle and joint pain, anorexia, nausea, vomiting, abdominal discomfort and cough. The clinical disease is also often associated with anaemia and enlarged spleen. Pregnant women are more prone to anaemia and are also at risk for placental malaria with increased risk of abortion, low birth weight and infant mortality (Rogerson et al., 2007).
If untreated the disease might proceed within hours to days to the severe disease with vital organ dysfunction. Specific diagnostic criteria include prostration, generalized convulsions, impaired consciousness (cerebral malaria), shock, respiratory distress with pulmonary oedema (ARDS), renal failure, jaundice, severe anaemia (Hb<50g/L), hypoglycaemia (<2.2 mmol/L), metabolic acidosis, abnormal bleedings or hyperparasitaemia (>5% infected erythrocytes) (WHO, 2000).
Even with good standard treatment the mortality risk associated with uncomplicated malaria is approximately 0.1% and with severe malaria 10 – 20% (WHO, 2006). If untreated severe malaria is almost always fatal (Warrell, 1999). In high transmission areas the predominant cause of death from *P. falciparum* malaria is severe anaemia in children below 2 years old, while

death from cerebral malaria is more common among children 2 – 5 years old and among persons living in low transmission areas. About 10% of African children who survive cerebral malaria are left with neurological sequelae, such as cognitive impairment, blindness, ataxia and hemiplegia (Warrell and Gilles, 2002).

1.7 IMMUNITY

In high transmission endemic areas children, persons with impaired immunity e.g. pregnant women and to some extent HIV infected persons, as well as immigrants are the most vulnerable to severe malaria (Hviid, 2004; Renia and Potter, 2006). However, if they survive the repeated infections, a more or less area specific partial immunity (semi-immunity) to malaria is acquired over the years. Consequently, most adults are somewhat resistant to clinical disease even though fluctuating parasitaemia is still prevalent. In low transmission areas, on the other hand, all age groups are more or less vulnerable to clinical malaria, as they do not acquire semi-immunity.

1.8 MALARIA DIAGNOSIS

In rural areas malaria diagnosis is most often based on symptoms and signs, which is sensitive but has a low specificity. This results in an over diagnosis and treatment of malaria, with an increased risk of development of drug resistance, while maybe an under diagnosis and treatment of other febrile diseases (Ngasala et al., 2008).

Light microscopy of giemsa stained thick and thin blood smear is the standard diagnostic method for malaria. With a skilled microscopist and a good microscope the method is sensitive with a detection limit of approximately 50 parasites/µL blood (0.001% infected erythrocytes) and it also allows determination of parasite density and species. However, adequate microscopy facilities are often difficult to uphold in rural settings (Ngasala et al., 2008).

RDTs, based on malaria antigen detection, have recently been introduced as an alternative to light microscopy. The test is easily performed on site within 20-30 min. Depending on which test is used it is also sensitive and can determine species. However, it cannot quantify parasite density and it is more expensive than light microscopy.

1.9 MALARIA TREATMENT

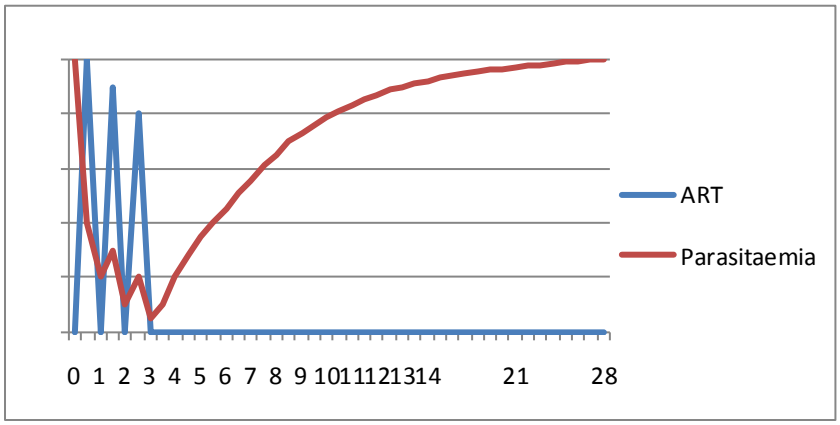
The history of fever treatment dates back to ancient times. In the seventeenth century the Jesuit missionary Juan Lopez recorded the use of the "fever tree bark" by Peruvian Indians and in 1735 the Swede Carl von Linnaeus gave it the name *Cinchona*. In 1820 the French scientists Pierre-Joseph Pelletier and Joseph Caventou finally isolated the antimalarial alkaloid quinine, allowing missionaries, explorers, colonialists and military troops to survive in malaria endemic areas with the immense changes of the world map during the 1900th century. During the World War I the devastating effect of malaria on the military troops was eminent, while the supply of quinine was uncertain. This forced German scientists to develop the first synthetic antimalarial drugs e.g. pamaquine (1924), mepacrine (1930) and chloroquine (CQ) (1934) and the insecticide dichlorodiphenyltrichloroethane (DDT) (1936). During World War II the French, British and Americans followed up on these discoveries with proguanil (1944), amodiaquine (AQ) (1946), primaquine (1950) and pyrimethamine (1952). In 1955 a global eradication program was launched by the 14th World Health Assembly, which included mass deployment of CQ and DDT. In 1961 - 65 *P. falciparum* resistance to CQ was reported simultaneously from

South America and South East Asia. Consequently, the value of sulfonamides as antimalarial compounds was rediscovered (sulphadoxine-pyrimethamine) (1960-66) and during the Viet Nam War the US Army developed a couple of new antimalarial drugs e.g. mefloquine (1971) and halofantrine (1980). Since then there has been sparse development of new antimalarial drugs, except for lumefantrine (1990) and atovaquone (1991), which are only used in combination therapies.

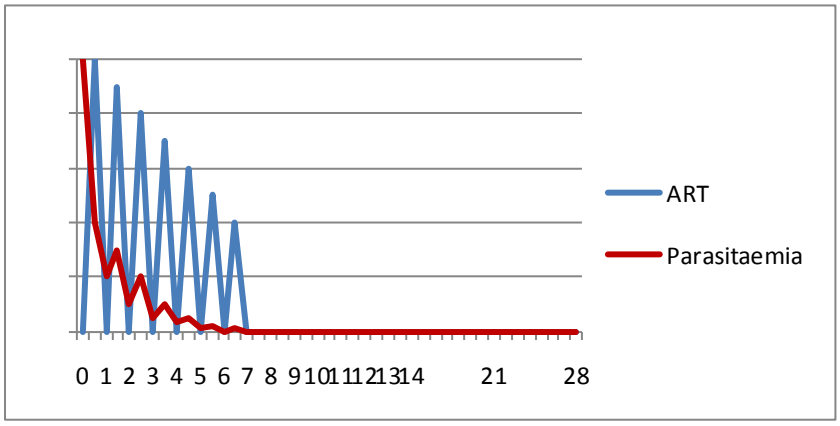
During the 1980s the CQ resistance spread to Africa and in the 1990s most countries changed their first line therapy from CQ to sulphadoxine-pyrimethamine. However, in contrast to CQ which remained efficacious in decades, resistance to sulphadoxine-pyrimethamine was reported already after a few years. The development and expansion of resistance to these mainstay antimalarials caused a major increase in morbidity and mortality of *P. falciparum* malaria in Africa (Bjorkman and Bhattarai, 2005) and there was an urgent need for a new treatment policy. Knowledge from combination therapy against cancer, HIV and tuberculosis, encouraged the suggestion on antimalarial combination therapy (White, 1999; White and Olliaro, 1996). The rationale for combination of two drugs is that the probability of a parasite to develop resistance to both drugs is the product of the probability to develop resistance to each of the drugs. This is provided that the two drugs have different modes of action, as compared with e.g. sulphadoxine-pyrimethamine. With the rediscovery of the Chinese traditional remedy artemisinin (*Qinghaosu*) (ART) from the sweet wormwood (*Artemisia annua sv. malört*) the concept of ACT was launched, which is now recommended as first line therapy against uncomplicated *P. falciparum* malaria (White, 1998; WHO, 2009c). The fast acting and efficacious ART will leave only a fraction of the parasite biomass to a relatively high remaining concentration of the partner drug. This will achieve effective clinical and parasitological cure and an optimized protection against development of drug resistance (Figure 3). Finally, ART is also gametocytocidal which might reduce the overall transmission of malaria (White, 1998).

In 1979 – 82 a number of ART derivatives were developed by the Qinghaosu Antimalaria Coordinating Group e.g. artesunate (AS), artemether and dihydroartemisinin (Hien and White, 1993) Five ACTs are now recommended (WHO, 2009c). Thailand was first by implementing AS plus mefloquine in 1994 due to reduced efficacy against mefloquine monotherapy in the border areas to Myanmar. AS plus AQ (ASAQ) and artemether plus lumefantrine are the main ACTs in Africa, while AS plus sulphadoxine-pyrimethamine is implemented in parts of Latin America (WHO, 2009a). Dihydroartemisinin plus piperaquine is just added to the list of recommended ACTs last year. The choice of ACT should be based on the efficacy of the partner drugs in the country (Olliaro and Taylor, 2003). In 2009 all but four *P. falciparum* endemic countries had implemented an ACT in their national drug policies. However, in a recent survey only 15% of children below five years old with fever in Africa had received an ACT (WHO, 2009c).

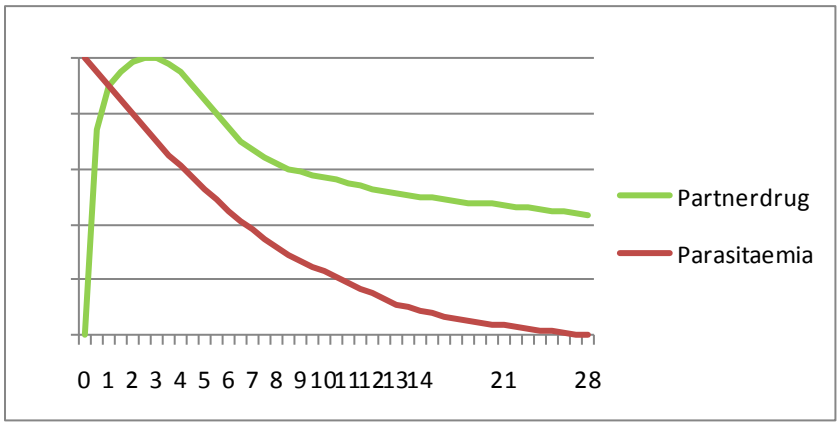
The recommended treatment for severe malaria is a parental ART derivative or quinine, followed by a full oral course of an ACT.



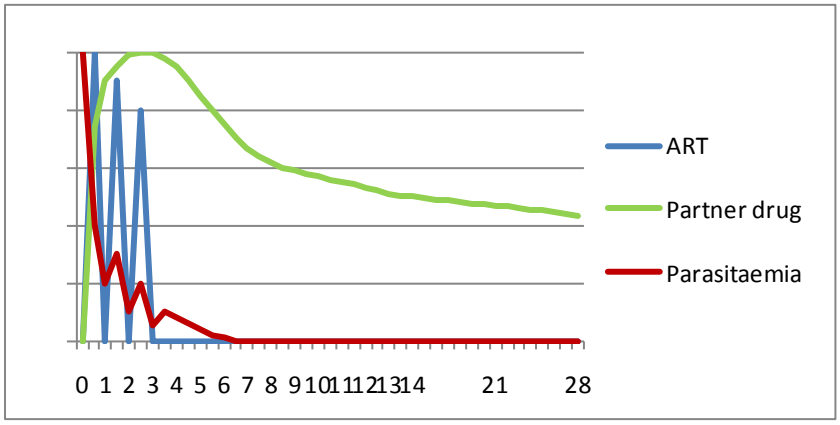
Recrudescence of parasitaemia after 3 days ART monotherapy.



Parasitological cure after 7 days ART monotherapy, which might compromise compliance and cost effectiveness.



Parasitological cure after 3 days monotherapy with a long acting drug. Parasites remaining during lower drug concentrations might be more prone to development of resistance.



Parasitological cure after 3 days ACT. Only a fraction of the parasite biomass is left to a relatively high partner drug concentration, optimizing a protection against development of resistance.

Figure 3. Pharmacodynamics of monotherapy vs ACT.

1.10 ANTIMALARIAL DRUGS

Malaria is both preventable and curable through prompt and effective chemotherapy. However, the arsenal of antimalarial drugs is limited. These include the aminoquinolines (CQ, AQ, primaquine), the arylaminoalcohols (mefloquine, halofantrine, lumefantrine, quinine), the antifolates (sulfadoxine-pyrimethamine, chlorproguanil-dapsone), atovaquone, the ART derivatives (AS, artemether, dihydroartemisinin) and antibiotics (tetracycline, clindamycin). Drugs explored in this thesis are here presented in more detail (Figure 4).

1.10.1 Chloroquine

CQ is a 4-aminoquinoline readily absorbed after oral intake and with a peak plasma concentration within 2 hours. CQ is extensively distributed and bound to tissues such as the liver, spleen, kidney and connective tissue. The total volume of distribution is therefore enormous (>100L/kg). The concentration in plasma is only 15% of that in the whole blood, out of which 50 - 60% is protein bound. Plasma concentration after parental administration is about 10 times higher than after oral administration. Elimination of CQ is slow and complex due to its multi compartment distribution with a terminal plasma half-life of 1-2 months. Half of the absorbed drug is eliminated via the kidney, while the rest is metabolized in the liver to the main metabolite desethylchloroquine with approximately equivalent antimalarial activity (Abdi et al., 1995; Krishna and White, 1996; Warrell and Gilles, 2002).

The mode of action by CQ is by interfering with the parasite detoxification of haem. During the erythrocytic cell cycle the parasite endocytoses cytoplasm from its host cell. The haemoglobin is concentrated in the parasite digestive vacuole where it is degraded to peptides and the toxic haem. The peptides are transported back into the cytoplasm and used as composite, while haem is detoxified by dimerization into inert haemozoin (Egan et al., 2002). CQ is a weak base and accumulates inside the digestive vacuole where it is protonated and binds to haem, inhibiting the detoxification into haemozoin, which is lethal for the parasite (Bray et al., 2005).

Standard dose is 10 mg/kg on day 0 and 1 and 5 mg/kg on day 2 (WHO, 2006). However, double standard dose divided in two daily doses have been given successfully to patients with CQ resistant malaria in Guinea Bissau (Ursing et al., Submitted).

The drug is generally well tolerated, although it has a bitter taste. The most common adverse events are pruritus, headache, visual disturbances, nausea and gastrointestinal discomfort. Severe adverse events have rarely been reported e.g. neuromyopathy and retinopathy. CQ has low safety margin and doses as low as 30 mg/kg have caused fatal hypotension and cardiac arrhythmias. This is of special concern when CQ is given parentally (Taylor and White, 2004; WHO, 2006). CQ is considered safe to give in pregnancy (Nosten et al., 2006).

1.10.2 Amodiaquine

AQ is a 4-aminoquinoline structurally related to CQ and with a similar mode of action. It is readily absorbed after oral intake with a peak plasma concentration within 2 hours and a terminal plasma half-life of approximately 4 - 8 hours. AQ is rapidly and extensively metabolized by the hepatic enzyme system CYP2C8 to the active main metabolite desethylamodiaquine (DEAQ) with peak plasma concentration within 4 - 6 hours. DEAQ has a total volume of distribution of about 20 – 30 L/kg, more than 90% is protein bound in plasma and the terminal plasma half-life is 10 – 14 days (Krishna and White, 1996; Pussard and

Verdier, 1994). However, the AQ/DEAQ pharmacokinetics appear to vary significantly (Lindegårdh et al., 2002). Due to the longer half-life, it is probable that DEAQ is mainly responsible for the antimalarial activity (Churchill et al., 1985; Li et al., 2002). The recommended dose is 10 mg/kg once daily for 3 days (WHO, 2001).
The adverse events are similar with those of CQ, although it lacks the bitter taste and pruritus is less of a problem. In the 1980s severe adverse events, such as agranulocytosis in 1/2000 and toxic hepatitis in 1/16000, were reported in travelers taking it as prophylaxis, which is no longer recommended (Hatton et al., 1986; Larrey et al., 1986; Neftel et al., 1986). The mechanism seems to be a rare auto immunological reaction against a second minor extra-hepatic metabolite, quinoneimine (Clarke et al., 1990; Maggs et al., 1988). A slower conversion of AQ to DEAQ in CYP2C8 poor metabolizer individuals might predispose to form this highly reactive metabolite and in doing so with an increased risk of these severe adverse events (Kerb et al., 2009). Consequently, screening of CYP2C8 genotypes before a wide scale introduction of an AQ compound in an area might be a promising approach to reduce this risk. However, these severe adverse events have not been reported with AQ treatment, even though asymptomatic neutropenia and elevated liver enzymes have been reported in low frequency (Olliaro and Mussano, 2003; Olliaro et al., 1996; Olliaro and Taylor, 2003; Sirima et al., 2009; Taylor and White, 2004). AQ appears to lack the serious cardiac toxicity of CQ, but large doses have been reported to cause syncope and convulsions (Jaeger et al., 1987).
Available data suggest that AQ in standard dosage is not teratogenic and that the adverse events frequency is not elevated in pregnancy. Thus, AQ in combination with other antimalarial drugs may be useful for malaria treatment in pregnancy, but inadequate data on its safety and pharmacokinetics in pregnancy limit its deployment for intermittent preventive treatment in pregnancy (Nosten et al., 2006; Tagbor et al., 2007).

1.10.3 Artesunate

AS is a sesquiterpene lactone semi-derivative from the natural remedy ART (Hien and White, 1993). ART itself has poor bioavailability limiting its efficacy, while AS is rapidly absorbed after oral intake reaching peak plasma concentrations within minutes and with a plasma half-life of about 0.5 hours. AS is extensively metabolized in the liver to dihydroartemisinin with a plasma half-life of about 1 hour and accounts for much of the antimalarial activity. They both have a plasma protein binding of 40 - 80% (Gautam et al., 2009; Medhi et al., 2009; WHO, 2006). Dihydroartemisinin is eliminated by the liver, but it appears that it auto induces its own metabolism, possibly reducing the plasma concentration with repeated dosing (Ashton et al., 1998).
The ART derivatives are the most potent antimalarials known, with a parasite reduction ratio of 10 000 per parasite cycle. In addition, it has an unusual broad effect on all erythrocytic stages, as well as gametocytes (White, 1997). ART possibly inhibits the essential calcium adenosine triphosphatase 6 (PfATPase 6) in the parasite (Eckstein-Ludwig et al., 2003). Recommended dose in combination with a partner drug is 4mg/kg/day for 3 days (WHO, 2001). ART and its derivatives are safe and remarkably well tolerated. The most common adverse events are mild dizziness and gastrointestinal discomfort. (Ashley and White, 2005; Price et al., 1999a). Severe adverse events, such as neurotoxicity and teratogenicity in early

pregnancy, have been reported in animal studies, but not verified in humans (Nosten et al., 2006; Taylor and White, 2004).

1.10.4 Artesunate + amodiaquine combination therapy

AQ with its active long half-life metabolite DEAQ and in vitro reports on a synergistic relationship with ART (Gupta et al., 2002; Mariga et al., 2005), have proven to be very efficacious in combination with AS either for first or second line ACT (Martensson et al., 2005; Zwang et al., 2009). Since 2007 a fixed-dose combination is available (Coarsucam® AS 25 mg/AQ 67.5 mg) and is now registered in 24 African countries and India (DNDi, 2009). Recommended dose is 2 tablets once daily for 3 days, which has proven to be safe and just as efficacious as given twice daily (Ndiaye et al., 2009; Sirima et al., 2009).

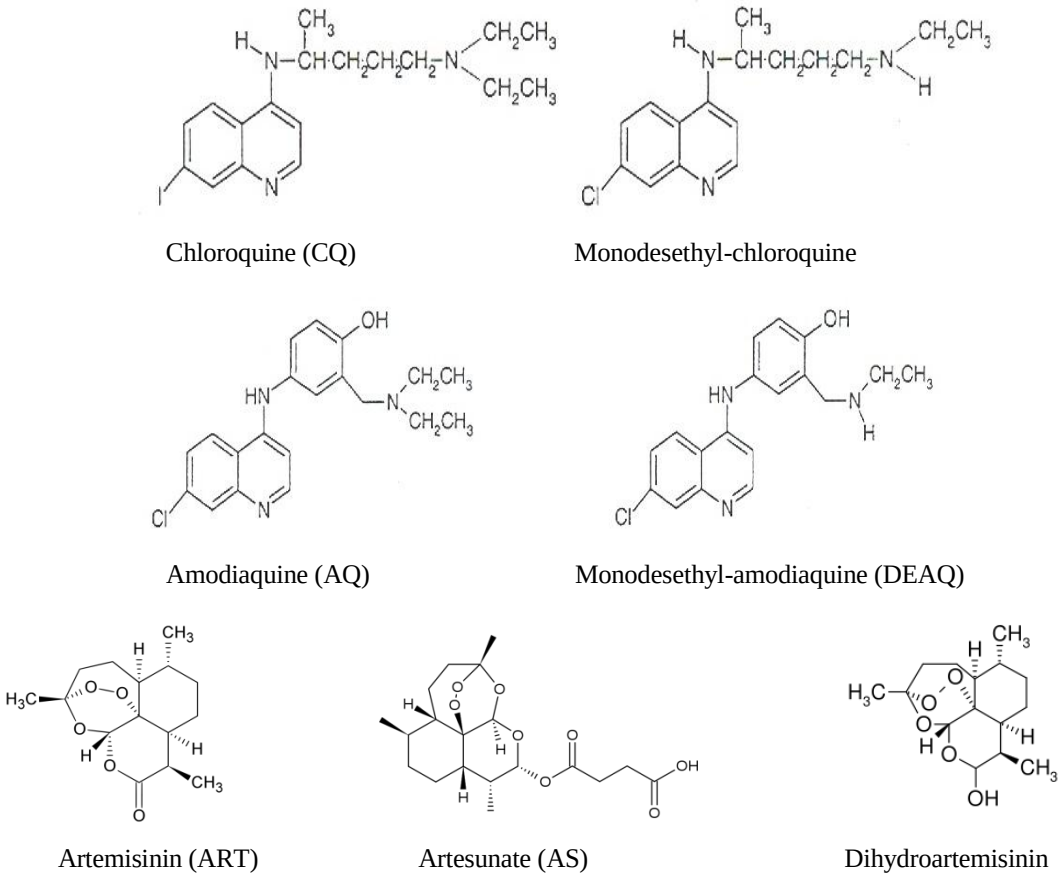


Figure 4. Molecular structures of CQ, AQ, ART, AS and their main metabolites.

1.11 RESISTANCE TO ANTIMALARIAL DRUGS

Antimalarial drug resistance is defined as the ability of a parasite to survive and/or multiply despite the administration and absorption of a drug given in doses at least equal to those usually recommended, but within the limits of tolerance of the subject (WHO, 1986). However, antimalarial treatment is an interaction between human – parasite (immunity), human – drug (pharmacokinetics) and parasite – drug (pharmacodynamics incl drug resistance). Thus, treatment failure is not always caused by drug resistance.

Drug resistance is probably a stepwise process. Therefore, resistance is sometimes divided into resistant and tolerant parasites. Resistant parasites can survive a full dose of treatment. Tolerant parasites are intermediate resistant and killed by a full dose but can survive lower doses. However, these parasites can spread in the human population because they can infect people with residual drug concentrations in the blood after earlier treatments (Hastings and Watkins, 2006).
Recurrent infections after an antimalarial treatment are divided into recrudescent infections which are reappearing base-line parasites and re-infections which are new parasites appearing during follow-up. In clinical trials the true treatment failure rate is represented by the proportion of patients with a recrudescent infection.
Drug resistance is a result of at least one mutation and its selection. Mutations occur randomly in nature. Rarely a mutation might be beneficial e.g. induce decreased susceptibility to a drug and is thereby selected through a survival advantage. Mathematic models show that the most important factor determining the spread of drug resistance is the proportion of infected people being treated i.e. the degree of parasite drug exposure. For instance, in South-East Asia where the majority of infected individuals are symptomatic and treated, the spread of drug resistance is quite exceptional (Farooq and Mahajan, 2004; Noedl et al., 2008; Wongsrichanalai et al., 2002).
The life-span from introduction to development of tolerance/resistance to anti-malarial drugs have varied considerable and might depend on underlying genetic mechanisms and deployment of the drugs.
In the late 1950s two *foci* of CQ resistance were detected. The first *focus* was on the Thai-Cambodian border and during the following two decades resistant strains spread through South-East Asia, Asia, East Africa and West Africa. The second *focus* was in Colombia where from it spread over South America. Analyses of genotype patterns have later identified at least two other *foci* in South America and Papua New Guinea (Mehlotra et al., 2001; Payne, 1987; Sa et al., 2009; Wellems and Plowe, 2001; Wootton et al., 2002).
AQ resistance is widespread in South America where it has been used extensively since the 1950s (Echeverry et al., 2006; Gama et al., 2009). Contrary, AQ has remained relatively effective even against CQ resistant *P. falciparum* malaria in many parts of Africa (Olliaro and Mussano, 2003; Olliaro et al., 1996), despite the information on a certain degree of in vitro cross resistance between the drugs (Childs et al., 1989).
ACT has so far remained very efficacious (Sinclair et al., 2009). However, possible AS resistance has recently been reported from the Thai-Cambodian border (Dondorp et al., 2009; Noedl et al., 2008) and the choice of partner drug is critical to prevent further development of antimalarial drug resistance and for ACT efficacy to endure (Olliaro and Taylor, 2004).

1.11.1 Assessment of *Plasmodium falciparum* drug resistance

Assessment of *P. falciparum* resistance can be determined phenotypically in vivo or in vitro, as well as genotypically. The standard assessment of antimalarial drug resistance is through clinical trials (in vivo), as this reflects the "reality". Standardized protocols now recommend only inclusion of children < 5 years old with fever or a history of fever in the preceding 24 hours, parasite density 2000 – 200 000 parasites/µL and 28 days follow-up (WHO, 2005b).
Treatment outcome was traditionally classified as S, RI, RII and RIII depending on parasitaemia on day 2, 7 and possible recurrence. Presently treatment outcome is classified as adequate

treatment response, early treatment failure and late treatment failure (where the latter can be further divided into late parasitological or clinical failure) depending on parasitaemia and malaria symptoms before or after day 4 and during 28 days follow-up (WHO, 2003). However, clinical trials can be divided into efficacy and effectiveness studies, where in the former the drug intake is supervised and follow-up more controlled. The results can also be interpreted "per protocol" as compared with "intention to treat", where in the former you exclude all loss to follow-up. The results can also vary depending on PCR-adjustment of recurrent infections, consecutive day sampling and length of follow-up (Martensson et al., 2007).
Antimalarial drug resistance can also be assessed through susceptibility to drugs in parasite cultures (in vitro). Threshold minimum concentration for 50% parasite growth inhibition (IC_{50}) values for resistance in vitro have been defined by determining serum drug concentrations in patients infected with sensitive and resistant *P. falciparum.* Conventional threshold IC_{50} values for in vitro resistance are for CQ 100 nM, AQ 30 nM and DEAQ 60 nM (Aubouy et al., 2004; Echeverry et al., 2006), while values for ART and its derivatives are not yet decided. In vitro assays have the possibility to control the exposure to defined drugs, concentrations and durations, as well as combination of these. However, in vitro assays eliminate the influence of host pharmacokinetic properties, such as drug metabolism and elimination, as well as immunity. Therefore in vitro results might not be in accordance with results in vivo (Ringwald et al., 1999).
Finally, recent molecular research has identified specific mutations (markers) associated with decreased drug susceptibility in vitro and/or treatment failure in vivo. In combination with bioinformatic modeling this information gives us a powerful tool for understanding of the molecular basis of parasite resistance to antimalarial drugs. Indices on how these molecular markers might predict antimalarial drug failure rates have been suggested (Djimde et al., 2001b). The genotype resistance index (GRI) and the genotype failure index (GFI) are calculated by dividing the prevalence of the resistant genotype with the prevalence of RI – RIII outcome or early- and late treatment outcome, respectively. However, these indices are likely to be dependent on age, immunity, non-resistance associated factors, prevalence of the mutation and the parasite genetic background and should therefore be used with caution (Hastings, 2007; Plowe, 2003).
In vitro assays and molecular markers should primarily be used as complement to in vivo trials for more efficient epidemiological monitoring and stronger advisory before treatment policy recommendations. A withdrawal of a recommended antimalarial treatment should be considered when the efficacy in clinical trials falls below 90% and before an implementation of a new treatment the efficacy should exceed 95% (WHO, 2006).

1.11.2 Mechanisms of antimalarial drug resistance

Genotyping of the full *P. falciparum* genome have identified about 5300 genes distributed on the 14 chromosomes (Gardner et al., 2002).
The parasite might alter its response to an antimalarial drug through an ability to regulate gene expression. However, this is probable a first and more limited solution. Most frequent mutations associated with drug resistance are single nucleotide polymorphisms (SNPs), insertions/deletions and gene amplifications. SNPs can be located either in the coding area of a gene which might alter the protein structure, or in the regulatory areas surrounding the gene

which might alter the gene expression. Multiple unlinked events might be necessary (epistasis), forming a specific combination of mutations (haplotype). General resistance mechanisms are altered a) pharmacological properties, b) affinity to the drug target and c) transport of the drug (White, 2004). At the time for this thesis some information was available on mutations associated with resistance to mainly CQ, mefloquine and sulphadoxine – pyrimethamine, while information on the molecular background to other antimalarial drugs was more scarce. Genes associated with resistance to the antimalarial drugs explored in this thesis are here presented in more detail.

1.11.3 *Pfcrt*

The *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*) gene produces an export protein (PfCRT) in the parasite digestive vacuole membrane (Figure 5). It belongs to the drug/metabolite transporter superfamily and contains ten transmembrane domains with its N and C termini facing the parasite cytoplasm (Martin and Kirk, 2004; Sanchez et al., 2007; Valderramos and Fidock, 2006). It is debated whether PfCRT is a saturable ATP dependent exporter or a gradient driven channel (Martin et al., 2009; Sanchez et al., 2007; Warhurst et al., 2002). SNPs in several amino acid positions are identified e.g. *pfcrt* C72S, M74I, N75E, K76T, T152A, S163R, A220S, Q271E, N326S/D, T333S, I356T/L and R371I/T. *Pfcrt* K76T (lysine to threonine) is the main molecular marker for CQ resistance (Fidock et al., 2000a; Fidock et al., 2000b; Sidhu et al., 2002). The mutated form has lost a positive charge and is therefore able to export protonated CQ out from its active site in the digestive vacuole. The Ca^{2+}-transporter inhibitor drug Verapamil (VP) may sensitize *P. falciparum* to CQ either by interference in the ATPase activity or by a sterical block (Valderramos and Fidock, 2006). *Pfcrt* K76T seems to always be accompanied by additional mutations depending on the geographical region (Wellems and Plowe, 2001).

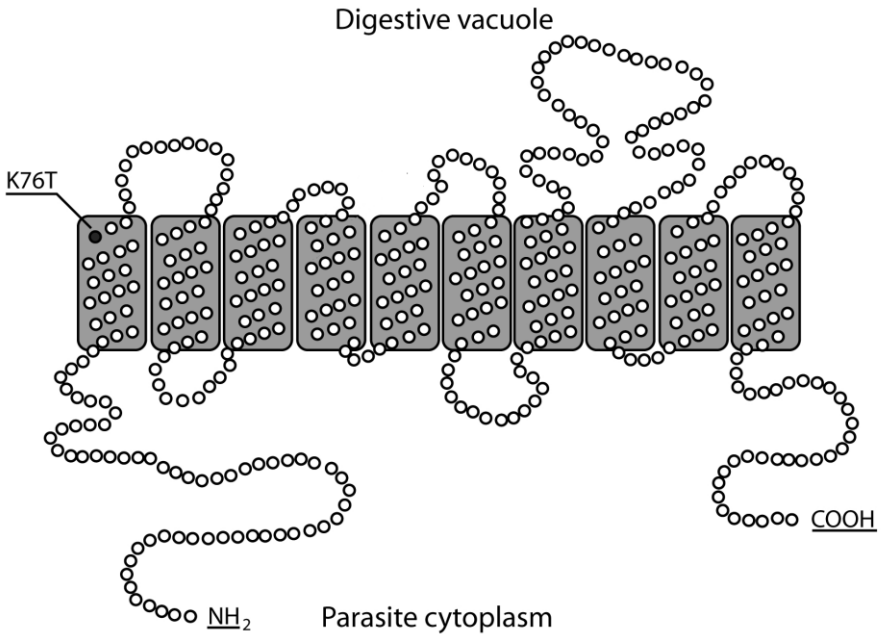


Figure 5. The PfCRT protein with the SNP in position 76 in the first transmembrane domain marked (Valderramos and Fidock, 2006).

1.11.4 *Pfmdr1*

The *Plasmodium falciparum multidrug resistance 1* (*pfmdr1*) gene produces a transport protein (PfMDR1) in the parasite digestive vacuole membrane (Figure 6). It belongs to the ATP-binding cassette (ABC) transporters which are involved in drug resistance in many biological systems e.g cancer (Liu, 2009), bacteria (Poelarends et al., 2003) and parasites (Ouellette and Légaré, 2003). It is a homologue of the mammalian P-glycoprotein 1 (Pgh1) which consists of two homologue regions, each including six transmembrane domains, with its N and C termini and two nucleotide binding domains facing the parasite cytoplasm (Duraisingh and Cowman, 2005; Peel, 2001; Valderramos and Fidock, 2006). Recent data on the subcellular distribution of fluorescein derivatives suggested that PfMDR1 is an importer of various solutes including antimalarial drugs (Rohrbach et al., 2006). SNPs in several amino acid positions are identified e.g. *pfmdr1* N86Y, Y184F, S1034C, N1042D and D1246Y, which are suggested to alter the transport by affecting the substrate specificity (Sanchez et al., 2008). *Pfmdr1* N86Y (asparagine to tyrosine) modulate the CQ resistance in parasites with *pfcrt* 76T in vitro (Reed et al., 2000) and have also been selected for after CQ treatment in vivo (Djimde et al., 2001a). Contrary, *pfmdr1* N86 and an amplification of the gene have been associated with resistance to mefloquine in vitro and in vivo (Price et al., 1999b; Price et al., 2004).

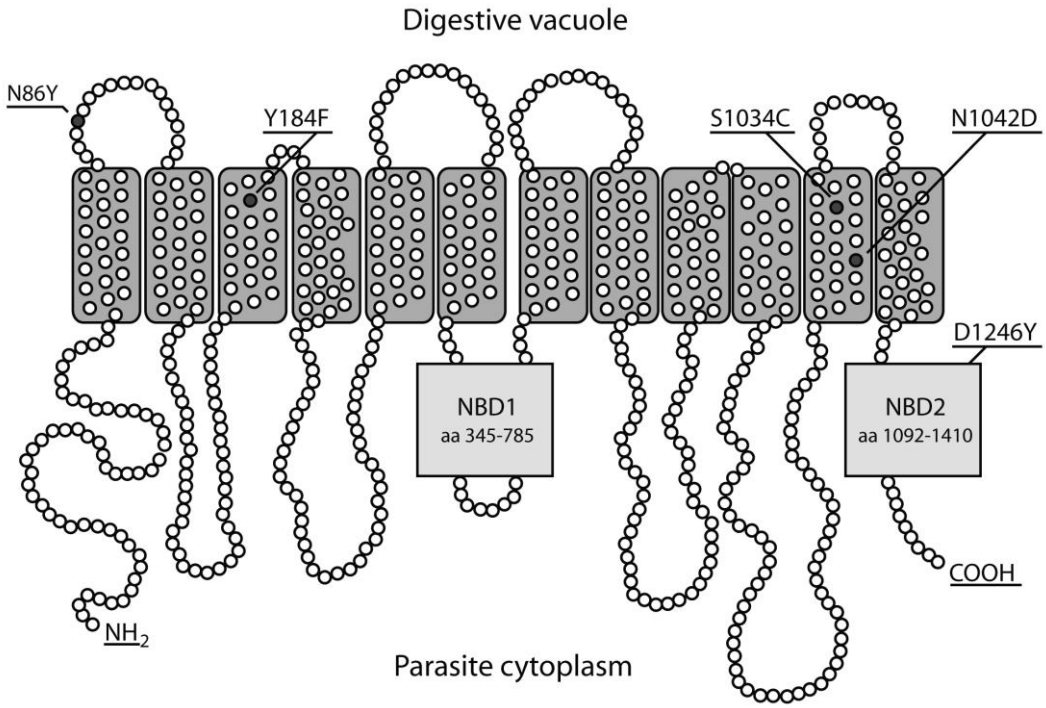


Figure 6. The PfMDR1 protein with SNPs marked. Position 86 facing the digestive vacuole, position 1246 in the nucleotide binding domain (NBD) facing the parasite cytoplasm and position 184, 1034 and 1042 in the transmembrane domains (Valderramos and Fidock, 2006).

1.11.5 *Pfmrp1*

The *Plasmodium falciparum multidrug resistance protein 1* (*pfmrp1*) gene produces an export protein (PfMRP1) in the parasite membrane (Klokouzas et al., 2004). It belongs to the ABC transporters, like PfMDR1. Several SNPs in this gene have been associated with modulated

response to antimalarial drugs in vitro e.g. *pfmrp1* Y191H and A437S (Mu et al., 2003). Another nine putative ABC transporters have been identified in the *P. falciparum* genome, some of which might be associated with drug resistance (Kavishe et al., 2009).

1.11.6 *PfATP6*

The *Plasmodium falciparum sarco/endoplasmatic reticulum* Ca^{+2}*-ATPase (SERCA) 6* (*pfATP6*) gene produce a protein which mediate the Ca^{+2} signalling in the endoplasmatic reticulum (Nagamune et al., 2008) and is suggested to be the target for ART (Eckstein-Ludwig et al., 2003).

1.12 PARASITE FITNESS

It is widely believed that wild-type organisms are normally the most viable in line with Darwin´s survival of the fittest theory and that mutations are acquired to a fitness cost. This would also apply to mutations encoding drug resistance in malaria, where the mutation might be disadvantageous in the absence of the drug. This has been suggested in Malawi where the *pfcrt* 76T and to a lesser extent also the *pfmdr* 86Y and 1246Y frequency decreased following discontinued use of CQ (Kublin et al., 2003; Laufer et al., 2006; Mita et al., 2003). Supportively, intra-seasonal analyses in The Gambia and Sudan showed that the *pfcrt* 76T and *pfmdr1* 86Y frequencies decreased during the dry season when drug pressure was reduced (Abdel-Muhsin et al., 2004; Ord et al., 2007).
In vitro experiments have shown that transfected isogenic parasites with the *pfmdr1* (a.a. 1034, 1042, 1246) CDY haplotype produce less viable merozoites, representing a fitness cost by 25% as compared to parasites with the wild type haplotype *pfmdr1* SND (Hayward et al., 2005). Whether parasite fitness cost also affects gametogenesis and parasite transmission is unclear. In The Gambia parasites carrying the *pfcrt* 76T allele had significantly higher mean gametocyte densities, while the opposite was seen for parasites carrying the *pfmdr1* 86Y allele (Ord et al., 2007).
The impact of fitness cost might depend on the geographical region. In Sub-Saharan Africa, with a continuous relative high frequency of asymptomatic semi-immune malaria infected individuals who are not treated with antimalarial drugs, the proportion of treated patients is small. Thus, most parasites are not under drug exposure and the spread of resistant parasites might be retarded or even impaired if the fitness cost is significant (Mackinnon and Hastings, 1998). To evaluate the impact of mutations on the spread of drug resistance, especially in such high endemic areas, it is thus important to also consider whether they are associated with a parasite fitness cost.

2 AIMS

2.1 GENERAL AIMS

To explore whether mutations, especially in the *pfcrt* and *pfmdr1* genes, are associated with tolerance/resistance to AQ/DEAQ in monotherapy and in ACT, as well as a possible associated parasite fitness cost.

2.2 SPECIFIC AIMS

1. To determine treatment outcome after AQ monotherapy in Kenya (Study I).

2. To evaluate if treatment outcome is related to DEAQ blood concentration (Study I).

3. To determine if mutations in the *pfcrt*, *pfmdr1*and *pfmrp1* genes are associated with AQ and/or DEAQ tolerance/resistance (Study I – V).

4. To estimate the potential cross-resistance between CQ and AQ/DEAQ (Study IV – V).

5. To explore if parasite fitness is affected by mutations associated with AQ/DEAQ resistance (Study V).

6. To estimate the efficacy of AQ as a partner drug in ACT in East Africa (Study I – V).

3 MATERIAL AND METHODS

3.1 IN VIVO STUDIES

With the clinical efficacy trials included in this thesis we tried to determine the treatment outcome after AQ monotherapy or ASAQ combination therapy in East Africa. This information was used to relate with the genotyping data.

3.1.1 Study sites

Study I, II and III were based on three clinical efficacy trials on children < 5 years old with uncomplicated *P. falciparum* malaria in rural districts of East Africa;

1. AQ monotherapy study conducted between November and December 2003 in collaboration with the mobile Scandinavian Doctor Bank in Siaya District in Western Kenya (Holmgren et al., 2006) (Study I, II, III).
2. ASAQ combination therapy study (ASAQ I) conducted between November 2002 and February 2003 at Kivunge Hospital on Unguja Island and Micheweni Hospital on Pemba Island, Zanzibar, Tanzania (Martensson et al., 2005) (Study III).
3. ASAQ combination therapy study (ASAQ II) conducted between January and July 2005 at the same sites on Zanzibar, Tanzania (Study III).

The climate is tropical, malaria holoendemic and the transmission perennial, with peaks during and after the rainy seasons in March-May and October-November. The predominant malaria species and vector is *P. falciparum* and the *A. gambiae* complex, respectively.
At the time for the studies in Kenya and Zanzibar 2002 – 2003 the first-line treatments for uncomplicated malaria were sulphadoxin-pyrimethamine and CQ respectively and second-line treatments were AQ and sulphadoxine-pyrimethamine respectively. The first-line treatments were then changed to Artemether – Lumefantrine (Coartem®) in Kenya (April 2004) and to ASAQ on Zanzibar (Sept 2003). Hence, at the time for the second study on Zanzibar 2005 ASAQ had been the first-line treatment for about a year.

3.1.2 The clinical trials

Children < 5 years old were enrolled if they fulfilled the following inclusion criteria; microscopically confirmed, uncomplicated *P. falciparum* malaria, fever >38ºC or a history of fever in the previous 24 hours, no symptoms or signs of other severe acute or chronic diseases, no history of allergy to AQ or ART and no history of antimalarial treatment the preceding seven days. AQ (10 mg/kg once daily for three days) or ASAQ (AS 4 mg/kg plus AQ 10 mg/kg once daily for three days) were given according to national guide lines under supervision. Follow-up visits were on day 1, 2, 7, 14 and 21 in Kenya and 1, 2, 3, 7, 14, 21, 28, 35 and 42 on Zanzibar. All visits included medical history, clinical examination and capillary finger prick for microscopy, as well as blood spot on filter paper (3MM® Whatman) for molecular analyses. Parasitaemia was determined with light microscopy by an experienced microscopist counting parasites against 200 white blood cells assuming an average of 8000 white blood cells per µL. All clinical and parasitological information was recorded in a standard case report form. Children not returning for their visits were actively followed-up at home. If any symptoms or

signs occurred in between the visits the children were to visit the local health facilities and/or the study sites for an examination and possibly retreatment or rescue treatment. Primary outcome was the first microscopically confirmed recurrent parasitaemia observed after the administration of the drug.

3.2 IN VITRO STUDIES

With the susceptibility testing in study IV and V we tried to specifically determine the influence of mutations in the *pfcrt* and *pfmdr1* genes on parasite response to CQ, AQ and DEAQ and a possible sensitization by VP probably through interference with PfCRT.
With the competition experiment in study V we tried to specifically determine the influence of the SNP *pfmdr1* 1246Y on parasite growth and fitness upon drug exposure and when drug exposure was removed.

3.2.1 Parasite cultures

In study IV we used 15 *P. falciparum* culture adapted isolates collected between 1999 - 2001 in three endemic areas along the Colombian Pacific Coast as well as the reference clones 3D7, HB3, Dd2 and 7G8.
In study V we used five *P. falciparum* clones in which the *pfmdr1* 1034, 1042 and/or 1246 *loci* had been modified through allelic exchange (Reed et al., 2000) which were obtained from The Malaria Research and Reference Reagent Resource Center (MR4®). Three clones derived from the CQ sensitive clone D10 from Papua New Guinea i.e. MRA-563: D10 *pfmdr1* SND (transfection control) ($D10^{SND}$), MRA-564: D10 *pfmdr1* SNY ($D10^{SNY}$) and MRA-565: D10 *pfmdr1* CDY ($D10^{CDY}$). Two clones derived from the CQ resistant clone 7G8 from Brazil i.e. MRA-566: 7G8 *pfmdr1* SND ($7G8^{SND}$) and MRA-567: 7G8 *pfmdr1* CDY (transfection control). The clones were verified with PCR-RFLP and sequencing. However, we chose to use the wild-type 7G8 clone ($7G8^{CDY}$), as the 7G8 transfection control could not be genotypically verified. All clones were defrosted, adapted to continuous culture in supplemented RPMI-1640 and 5% haematocrite and then synchronized with sorbitol, according to well established protocols (Lambros and Vanderberg, 1979; Trager and Jensen, 1976).

3.2.2 Susceptibility testing

In study IV the susceptibility testing was performed at the International Centre for Medical Research and Training (CIDEIM) in Cali, Colombia using a 48-hours ^{3}H-hypoxantine incorporation assay (Cerutti et al., 1999). The in vitro cultures were subcultivated and aliquoted into 96-well microculture plates pre-dosed with ascending concentrations of CQ, AQ and DEAQ. The final concentration of ^{3}H-hypoxanthine was 0.5 μCi/well and that of Albumax I was 0.5%. After incubation at 37°C for 48 hours counts per minute (cpm) were measured in a scintillation counter (Beckman LS7500) and the IC_{50} values were calculated using the PROBIT program in SPSS 7.5 for windows 98 (SPSS. Inc. Chicago 1996).
In study V the susceptibility testing was performed at The Malaria Research Laboratory in Stockholm, Sweden using an HRP2-ELISA based assay (Noedl et al., 2005). In vitro cultured parasites were diluted to an initial parasitaemia of 0.05% and aliquoted into 96-well microculture plates pre-dosed with ascending concentrations of CQ, AQ and DEAQ. After incubation at 37ºC for 72 hours plates were freeze-thawed, transferred and processed in pre-

coated ELISA plates (Cellabs, Australia) for spectrophotometric analysis (Multiskan EX, Thermo Labsystems®, Helsingfors, Finland) of HRP2 produced during parasite growth. The IC_{50} and IC_{90} values were determined using HN-NonLin V1.05 Beta © H. Noedl 2001 (http://malaria.farch.net). The IC_{20} values were calculated using the parasite growth trend line equation (SigmaPlot® 11.0, Systat Software Inc, Californina, USA).
In both studies VP 0.8 μM was also added to a parallel setup of the clones. As for the 7G8 clones, it has been suggested that VP has a less sensitizising effect on clones carrying *pfcrt* (a.a. 72 – 76) SVMNT than CVIET (Mehlotra et al., 2001). Therefore, VP in ascending concentrations of 0.8, 1, 2, 3, 4 and 5 μM was added to a parallel setup of these clones. In this way we tried to find a window with maximum sensitization of PfCRT and before a dominating VP specific antimalarial effect, extracting the effect of the *pfmdr1* SNPs.

3.2.3 Competition experiments

In study V the competition experiments were performed at The Malaria Research Laboratory in Stockholm, Sweden. Parasite densities were thoroughly determined in thin-film slides of synchronized ring-staged cultures of $D10^{SND}$ and $D10^{SNY}$ clones. In two subsequent experiments the cultures were subcultivated to exactly 0.5% parasitaemia and 2 ml of each culture was hybridized into 1:1 mixed cultures.
In a first phase the cultures were run in medium containing previously determined drug concentrations corresponding to the average IC_{20} values (CQ 20 nM, AQ 10 nM or DEAQ 10 nM) in the first experiments and the average IC_{50} values (CQ 30 nM, AQ 20 nM, DEAQ 20 nM) in the second experiment. These concentrations were chosen from an in vivo point of view, where possible remaining parasites after the initial top concentrations might be exposed to lower concentrations of the partner drug. This pharmacokinetic elimination phase is potentially prone to selection of more drug resistant parasites. In the first experiment drugs were removed after six days, while in the second experiment parasitaemia decreased to the extent where we chose to remove drug pressure already after four days. After drug removal all cultures were subcultivated to ≤0.5% and washed thoroughly.
In a second phase each culture was run in drug-free medium, until six days after parasite recovery. Recovery was defined as growing ring-stage trophozoites observed microscopically after drug removal. All cultures were run in duplicates. Parallel control cultures were run in drug-free medium for twelve days and subcultivated to 0.5% on day six. Thin slides and blood on filter paper (3MM® Whatman) for parasite density, morphology and genotyping were collected every second day.

3.3 GENOTYPING

The genotyping has been done at The Malaria Research Laboratory at Karolinska Institutet in Stockholm, Sweden. DNA extracted from blood spots on filter papers have been amplified with polymerase chain reaction (PCR) followed by either restriction fragment length polymorphism (RFLP) and/or DNA pyrosequencing or full sequencing. Gene amplification analysis has been done by TaqMan probe based Real-Time PCR. Finally, we tried to determine a possible association between specific mutations and drug resistance by relating the genotyping data to the results from the susceptibility testing and competition experiments in vitro, as well as the treatment outcome in vivo.

3.3.1 DNA extraction

Blood spots on filter papers were dried and stored in individual, sealable plastic bags in room temperature. In study I and II a Tris-EDTA based DNA extraction method was used (Bereczky et al., 2005). In study III DNA was extracted using the ABI PRISM 6100 Nucleic Acid PrepStation™ (Applied Biosystems, CA, USA). In study IV and V DNA was extracted using the QIAamp® DNA Mini or Micro Kit (Qiagen, USA). Extraction was performed according to the the manufacturers´ protocols with minor modifications for blood on filter papers. Extracted DNA was stored in aliquots at -20ºC.

3.3.2 *Pfmsp2* genotyping

The *Plasmodium falciparum merozoite surface protein 2 (pfmsp2)* gene is highly polymorphic both in sequence and size and was considered as the standard procedure for the distinction between recrudescent and re-infecting parasites within recurrent infections in drug efficacy trials performed in high transmission areas, confirmed in a multi-centre study where *pfmsp2* was shown to be the most informative marker for the multiplicity of this infection (Farnert et al., 2001). A nested PCR amplifying the specific FC27 and IC/3D7 allelic types of this gene was performed as previously described (Snounou et al., 1999). The PCR products were separated in an ethidium-bromide stained 1% agarose gel by electrophoresis and visualized under UV transillumination (GelDoc® 2000, BioRad, Hercules, USA). A recurrent infection exhibiting a similar *pfmsp2* profile as compared with day 0 was considered as a recrudescent infection, an infection exhibiting a new *pfmsp2* profile as compared to day 0 was considered as a re-infection and an infection with a mixed result was considered to include both a recrudescent infection and a re-infection.

Presently, the recommendations are a sequentially analysis of three different genes i.e. *pfmsp2*, *pfmsp1* and *pfGLURP* and infections exhibiting at least one similar profile in all analyses as compared with day 0 should be considered as a recrudescent infection (WHO, 2007).

3.3.3 PCR-RFLP

All PCRs were based on a nested amplification. All PCR reactions included *Taq* polymerase reaction buffer, magnesium chloride, dNTPs, a forward and reverse primer pair and *Taq* DNA polymerase (Promega Corporation, Madison, USA). The nested PCR products were incubated over night with a restriction enzyme specific for each SNP to be analyzed according to the manufacturers' conditions (New England Biolabs). All products were separated in an ethidium-bromide stained 2% agarose gel by electrophoresis and visualized under UV transillumination. Primers and restriction enzymes used are summarized in Table 2.

As the malaria parasite always is haploid in the human host a sample with a mixed SNP result in vivo was considered to include two infections, one with a wildtype allele and one with a mutant allele.

3.3.4 Pyrosequencing

Pyrosequencing is based on direct sequencing of shorter DNA fragments. Biotinylated PCR products are washed and hybridized with a specific sequencing primer before pyrosequencing

by a designed subsequent dNTP entry. Incorporation of a nucleotide causes an enzymatic cascade that generates light. The light produced is proportional to number of nucleotides incorporated and is registered in a camera and presented as a peak in a pyrogram. The method is advantageous in distinguishing in between mixed infections and it is also able to perform allele quantification (Cheesman et al., 2007). Unfortunately, possible dNTP entries might not be able to design for fragments that are diverse for several consecutive amino acid positions e.g. *pfcrt* 72 – 76.

In study III this method was used to distinguish in between mixed results for the *pfmdr1* Y184F SNP using a forward primer (Table 2) and the dNTP entry GTATATATCGT (Pyro Gold Reagents PSQ™ 96MA and PyroMark™ MD System, Biotage AB, Uppsala, Sweden). A pure/mix allele limit was set to 10/90%. In the competition experiments in study V allele quantification of *pfmdr1* 1246Y was determined by pyrosequencing using a reverse sequencing primer (Table 2) and the dNTP entry CTACGTCTA. In the data analysis the results were adjusted against a standard curve, derived from a scale of established mixes of the two alleles (PSQ™ Assay Design).

3.3.5 Sequencing

The full *pfcrt* and *pfmdr1* genes were sequenced in recrudescent isolates in study III and in all isolates and clones in study IV. Primers used are summarized in Table 2.

As for the *pfcrt* gene gDNA was amplified through a nested PCR at a low 60ºC elongation temperature in five blocks or alternatively cDNA was extracted and amplified in three blocks. As for the *pfmdr1* gene gDNA was amplified in three blocks. All products were sent for sequencing analysis (ABI 3700 Capillary DNA Sequencer, Cybergene AB, Huddinge, Sweden or Macrogen Inc. Seoul, South Korea). Sequencing results were analyzed in a bioinformatic software (Sequencher® 4.5, Gene Codes Corporation, USA or ClustalW, © EMBL-EBI).

3.3.6 Real-time PCR

In study II and IV a possible amplification of the *pfmdr1* gene was assessed by TaqMan® probe based real-time PCR (ABI Prism® 7000 sequence detector, Applied Biosystems, USA) as described elsewhere (Price et al., 2004). Amplifications of the *pfmdr1* and *β-tubulin* genes were done in triplicate as a multiplex PCR containing TaqMan® Universal Mix, forward and reverse primers, FAM-labelled *pfmdr1* and VIC-labelled *β-tubulin* probes and DNA template. 3D7 and Dd2 with one and circa three *pfmdr1* copies respectively, were used as calibrators. Fluorescent data were analysed with the comparative ΔC_T (cycle threshold) method (Uhlemann et al., 2005). The results qualified if C_T <35 and ΔC_T spread <1.5 or C_T 35 – 40, SD C_T <0.5 and ΔC_T spread <1.5. Numbers of copies were rounded to the nearest integer.

SNP	Reaction	Forward primer 5´- 3´	Reverse primer 5´- 3´	Restriction enzyme
Pfcrt K76T	First	CCGTTAATAATAAATACAAGAAG	GGTAACTATAGTTTTGTAACATCCG	ApoI
	Nest	TGTGCTCATGTGTTTAAACTT	CAAAACTATAGTTACCAATTTTG	
Pfcrt T152A	First	CTTTTTAGGAACGACACCGA	CCTTGTCATGTTTGAAAAGC	BsrI
	Nest	AGTCATTTTGGCCTTCATA	ACTGCTCCGAGATAATTGTA	
Pfcrt S163R	First	CTTTTTAGGAACGACACCGA	CCTTGTCATGTTTGAAAAGC	HinfI
	Nest	AGTCATTTTGGCCTTCATA	ACTGCTCCGAGATAATTGTA	
Pfcrt gDNA	First	GACATTCCGATATATTATATTTTTAGA	TATATGTGTAATGTTTTATATTGGTAGG	
Seq block 1	Nest	CCGTTAATAATAAATACACGCAG	GGAATAGATTCTCTTATAAATCCATC	
Pfcrt gDNA	First	CCACCTACCAATATAAAACATTAC	CAATTAAGGAACTAATTAAGACAAG	
Seq block 2	Nest	TGTATGTATGTTGATTAATTTGTTTA	AACTAATTAAGACAAGATTAAATATGATA	
Pfcrt gDNA	First	CTCCTTTTTAGATATCACTTATACAAT	AAGGGAAAATTATACATACATACATA	
Seq block 3	Nest	CGGAGCAGTTATTATTGTTGTA	CATTTATTTATTTTTTCTTTCCTAA	
Pfcrt gDNA	First	CCTGTATGCTTTTCAAACATGAC	GCTGTTGCTGGACCTTGT	
Seq block 4	Nest	CATGACAAGGGAAATAGTTT	CAATAGTATATGTCATGGTAGAAAA	
Pfcrt gDNA	First	GTGGTCTTGGTATGGCTAAGTT	GAATCGACGTTGGTTAATTCTC	
Seq block 5	Nest	TCGCATTGTTTTCCTTCT	CGTTGGTTAATTCTCCTTC	
Pfcrt cDNA	First	ATAACAAAATGAAATTCGCAAGT	ACAATTGGAAAAAGGATACCATAG	
Seq block 1	Nest	ATAACAAAATGAAATTCGCAAGT	CAAAATGACTGAACAGGCATC	
Pfcrt cDNA	First	ATAACAAAATGAAATTCGCAAGT	ACAATTGGAAAAAGGATACCATAG	
Seq block 2	Nest	TCAAAAGAACGACACCGAAG	ACAATTGGAAAAAGGATACCATAG	
Pfcrt cDNA	First	TCAAAAGAACGACACCGAAG	GAATCGACGTTGGTTAATTCTC	
Seq block 3	Nest	CCTGTATGCTTTTCAAACATGAC	GAATCGACGTTGGTTAATTCTC	
Pfmdr1 N86Y	First	ATGGGTAAAGAGCAGA AAGAG	CGTACCAATTCCTGAACTCAC	ApoI
	Nest	TGTATTATCAGGAGGAACATTAC	GTAATTACATCCATACAATAACTTG	
Pfmdr1 Y184F	First	AAGAGGTTGAAAAAGAGTTGAAC	CTTATTACATATGACACCACAAAC	Tsp509I
	Nest	ACATATGCCAGTTCCTTTTTAGGTTAAT	AACATAAAGTAACGGAAAAACGCAA	
	Pyro seq	CCAGTTCCTTTTTAGGTT		
Pfmdr1 S1034C	First	GATGATGAAATGTTTAAAGATCC	TCCACCATCATCTCTTACATC	DdeI
	Nest	GTAAATGCAGCTTTATGGGGACTC	TAAGAAGGATCCAAACCAATAGGC	
Pfmdr1 N1042D	First	GATGATGAAATGTTTAAAGATCC	TCCACCATCATCTCTTACATC	AseI
	Nest	GTAAATGCAGCTTTATGGGGACTC	TAAGAAGGATCCAAACCAATAGGC	
Pfmdr1 D1246Y	First	CTACAGCAATCGTTGGAGAAA	GCTCTAGCTATAGCTATTCTC	EcoRV
	Nest	AACCAATCTGGATCTGCAGAAGA	ACATCTTCCAATGTTGCATCTTCT	
	Pyro seq		TTGAAAATAAGTTTCTAAGA	
Pfmrp Y191H	First	GCGTATTTTAGAAGGTCAAAAGATTC	GCATCTGGATTTTGTAAATGATTG	HpyCH4V
	Nest	AGATTCCTGTATATATTTCATTCT	GTAAATGATTGTTATTACTGCA	
Pfmrp A437S	First	GAATCATTTGCTTTTAAATACA	AGTTTGGAAAATTTGCTACA	HpyCH4V
	Nest	GAATGAAAGAAATGAAATATTG	TTCTTCTTTCTTATTTAATCTATCT	

Table 2. Primers and enzymes used for PCR-RFLP, pyrosequencing and sequencing.

3.4 DRUG CONCENTRATION ANALYSIS

In study I analyses of DEAQ concentrations at day 7 were performed from 25 – 50 µL blood on filter papers by liquid chromatography at Dalarna University College, Borlänge, Sweden (Lindegårdh et al., 2002).

3.5 STATISTICS

In study I and III allele frequencies in vivo were compared using Fishers exact χ^2 test (Microstat® software, Ecosoft Inc.). In study V drug susceptibility values and allele proportions in vitro were compared using Student´s *t*-test (SigmaPlot® 11.0, Systat Software Inc, Californina, USA). Statistical significance was defined as $p<0.05$. Outliers beyond $CI_{95\%}$ were removed.

The relative sensitization effect by VP was compared among the clones expressed as a response modification index (RMI) calculated from the equation; RMI = IC_{50} with VP / IC_{50} without VP (Mehlotra et al., 2001).

A one site saturation equation was used in the pharmadynamic analyses on the equilibrium dissociation constant (K_d) and the maximum binding (B_{max}) of VP sensitization (SigmaPlot® 11.0, Systat Software Inc, Californina, USA).

The relative growth (*g*) of the mutant allele in the competition experiment was calculated from the equation; $\log(mut_t/wt_t) = \log(mut_0/wt_0) + t \cdot \log(g)$. *mut* and *wt* are the proportions of the mutant and the wildtype alleles initially and after *t* asexual generations that occurred over the course of the experiments. *g* for the non drug exposed control cultures is equal to the relative fitness (*w*) (Hartl and Clark, 1997; Hayward et al., 2005). The relative growth including the fitness cost (*g´*), was calculated from the equation; $g' = g + (1-w)$.

The relative growth (*g*) of the clone with the mutant allele in the HRP2-ELISA susceptibility analyses was normalized against the background absorbance (abs) and calculated from the equation; g = (10 ^ abs *well* A_{mut} / 10 ^ abs *well* H_{mut}) / (10 ^ abs *well* A_{wt} / 10 ^ abs *well* H_{wt}).

3.6 ETHICS

Study I - IV was approved by The Research Ethics Committee at Karolinska Institutet in Stockholm, Sweden (KI Dnr 03-545, KI Dnr 03-753, KI Dnr 2005/57-31 and KI Dnr 2009/387-31), as well as the local Ethical Research Committées in Nyanza Province, Kenya (GN.153. VOL. 1/50), Zanzibar, Tanzania (ZHRC/GC/2002 and ZMRC/RA/2005) and CIDEIM, Colombia (2006/0603). Informed consents were obtained from the patients or their parents or legal guardians. In study V only reference clones obtained from MR4® were used and no ethical clearance was applied for.

4 RESULTS

4.1 STUDY I

The treatment outcome including numbers of total recurrent infections as well as recrudescent infections and re-infections based on *pfmsp2* genotyping are summarized in Table 3. The PCR-adjusted treatment failure rate was 20% ($CI_{95\%}$ 12-31%). The standard AQ treatment was generally well tolerated with no recorded serious adverse events related to the drug administration.

SNP results are summarized in Figure 7. *Pfcrt* 76T was significantly selected for in total recurrent infections (p=0.02) and specifically in re-infections (p=0.048). There was a tendency of higher frequency of *pfmdr1* 86Y in total recurrent infections (p=0.19), but a significant selection was only observed in recrudescent infections (p=0.048). *Pfcrt* 76T and *pfmdr1* 86Y in combination was also significant selected for in total recurrent infections (p=0.042). However, the predictive value for these SNPs before treatment was poor i.e. for *pfcrt* 76T (p=0.83) and *pfmdr1* 86Y (p=0.19). No difference in frequencies for the other herein analyzed SNPs (*pfcrt* 152A, 163R, 326S and *pfmrp* 191H, 437S) were observed after AQ monotherapy as compared with the base-line frequencies.

DEAQ concentrations on day 7 showed inter individual variability, ranging from <200 to >1000 nM. No statistically significant difference was observed between the drug levels in the patients with adequate treatment response and those with recurrent parasitaemia (p=0.79), or more specifically those with recrudescent parasitaemia (p=0.91).

	Total recurrent	**Recrudescenses**	**Reinfections**	**Mixed**
Day 7 (n)	3	1	2	0
Day 14 (n)	13	5	4	4
Day 21 (n)	12	3	7	2
Total day 7-21 (n)	28	9	13	6
(%)	37%	12%	17%	8%
($CI_{95\%}$)	26-49%	6-22%	10-28%	3-17%

Table 3. Incidence of parasitaemia in 75 children after AQ monotherapy in Kenya.

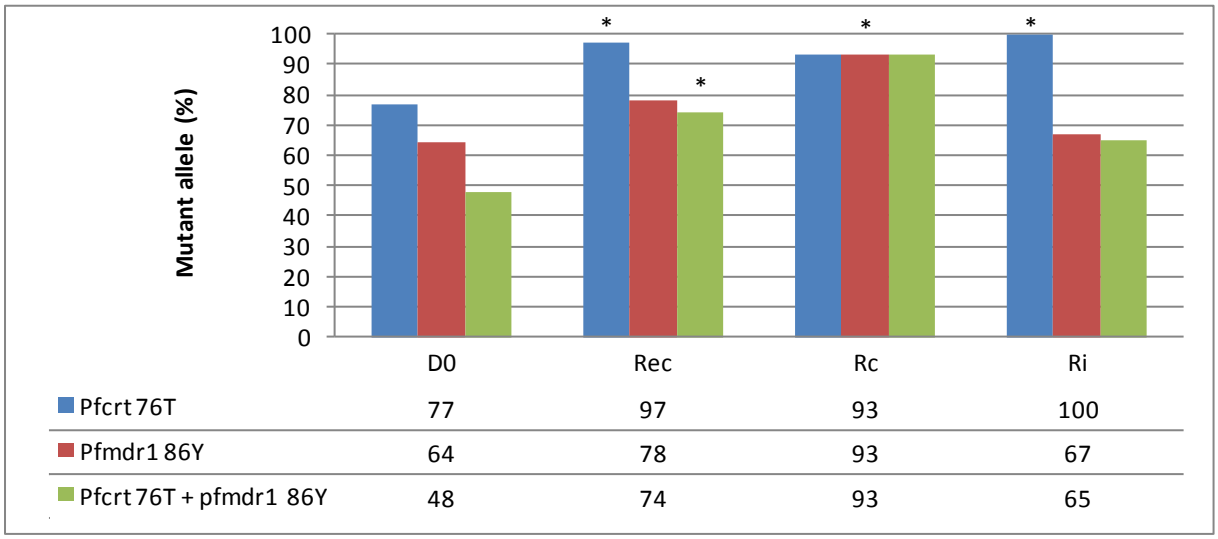


Figure 7. Proportion (%) of the mutant allele among baseline infections (Day 0), total recurrent infections (Rec), recrudescent infections (Rc) and re-infections (Ri) after AQ monotherapy.

4.2 STUDY II

Pfmdr1 copy number results are illustrated in Figure 8. When rounded to the nearest integer, all samples were found to have one *pfmdr1* copy, with the exception of one sample on day 0 (1.4%; $CI_{95\%}$ 0.03 – 7.5%). This sample had at least one parasite strain with multiple *pfmdr1* copies and a mixed *pfmdr1* 86 N/Y genotype. This child had an adequate treatment response.

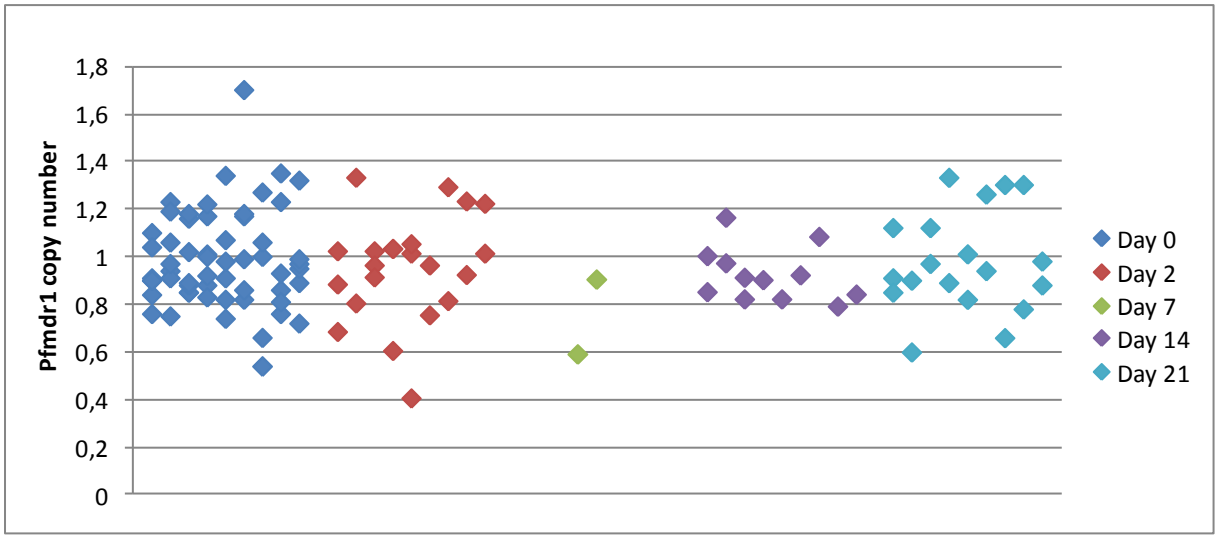
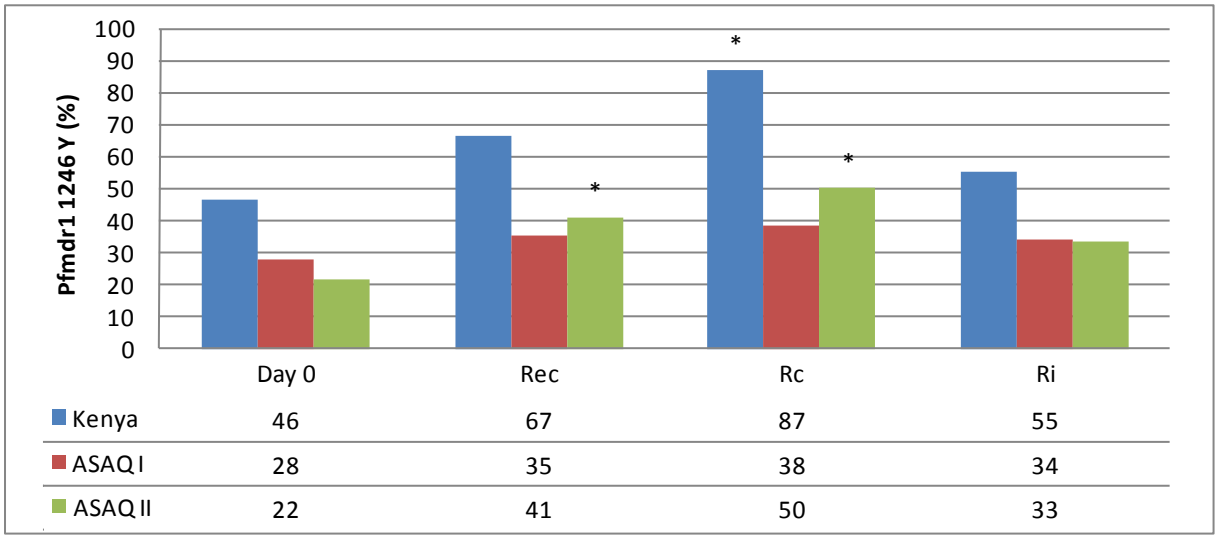


Figure 8. *Pfmdr1* copy number results on day 0 and during follow-up after AQ monotherapy in Kenya.

4.3 STUDY III

As compared to the study from Kenya, the PCR-adjusted treatment failure rates in the ASAQ combination therapy studies from Zanzibar 2002 – 2003 and 2005 were 8% ($CI_{95\%}$ 5 – 12%) and 9 % ($CI_{95\%}$ 5 – 14%) respectively. The base-line frequencies of *pfcrt* 76T were too high (96% in both studies) to allow any statistical analysis of a potential selection among recurrent infections. *Pfmdr1* 86Y was not selected for after combination therapy. On the other hand,

pfmdr1 1246Y and the *pfmdr1* haplotype (a.a 86, 184, 1246) YYY were selected for in recrudescent infections both after monotherapy (p=0.009 and p=0.029 respectively) and combination therapy in 2005 (p=0.017 and p<0.001 respectively) (Figure 9).
No difference in frequencies for the other herein analyzed SNPs (*pfcrt* 326S/D, 333S, 334N, 356T/L, *pfmdr1* 184Y, 1034C and 1042D) were observed after AQ monotherapy as compared with the base-line frequencies. No new mutations could be verified when sequencing the full *pfcrt* and *pfmr1* genes in all recrudescent isolates from the three studies.



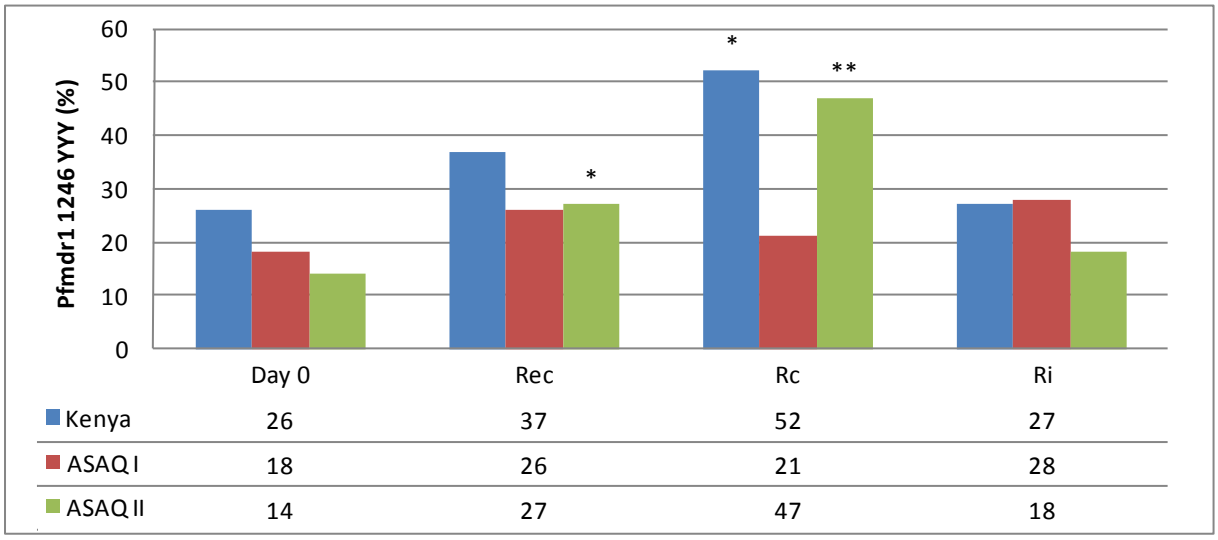


Figure 9. Proportion (%) of *pfmdr1* 1246Y and (a.a. 86, 184, 1246) YYY in base-line infections (Day 0), all recurrent infections (Rec), recrudescent infections (Rc) and re-infections (Ri) after AQ monotherapy (Kenya 2003) and ASAQ combination therapy (Zanzibar 2002 – 2003 (ASAQ I) and 2005 (ASAQ II)).

4.4 STUDY IV

High IC_{50} values for CQ and more varying IC_{50} values for AQ and DEAQ were observed in the 15 Colombian strains (Table 4). 15 SNPs in 12 positions in the *pfcrt* gene were identified. The lowest susceptibility found to AQ was observed in a strain (CA2855) with a haplotype related to the South East Asian clone Dd2 i.e. *pfcrt* (72 – 76) CVIET, 326S, 356T and *pfmdr1* (86, 184,

1034, 1042, 1246) YYSND. However, CA2855 had a single copied *pfmdr1* gene, as compared with Dd2 with a triple copied *pfmdr1* gene. The lowest susceptibility found to DEAQ was observed in a strain (TU741) with the haplotype *pfcrt* CVMNT, 326D, 356L and *pfmdr1* NFSDY, which is related to the South American clone 7G8. In addition, a new mutation *pfcrt* S334N was found in TU741. VP significantly sensitised the response to CQ and DEAQ, while this was not observed for AQ.

Drug	**IC_{50} for Colombian strains (nM)**	**Reference for in vitro resistance (nM)**	**Sensitization effect by VP for Dd2, CA2855 and TU741 (nM)**
CQ	115 - 416	> 100	510 →128
AQ	10 - 65	> 30	21 → 21
DEAQ	38 - 356	> 60	72 → 17

Table 4. In vitro susceptibilities to CQ, AQ and DEAQ (nM) and sensitization by VP (0.8 µM).

4.5 STUDY V

Decreased susceptibilities to AQ ($p<0.001$) and DEAQ ($p<0.001$) were observed in $D10^{SNY}$ as compared with $D10^{SND}$, while this was not significant for CQ (Figure 10). Decreased susceptibility to DEAQ through the full VP concentration range (0 – 5 µM) was observed for $7G8^{CDY}$ as compared with $7G8^{SND}$ ($p<0.001 – 0.03$). VP significantly sensitisized the response to CQ and DEAQ with a maximum from 4 – 5 µM. This was not as significant for AQ (Figure 11). Lower affinity for VP to *pfcrt* was observed for $7G8^{SND}$ as compared to $7G8^{CDY}$, as well as for both clones with DEAQ as compared with CQ.

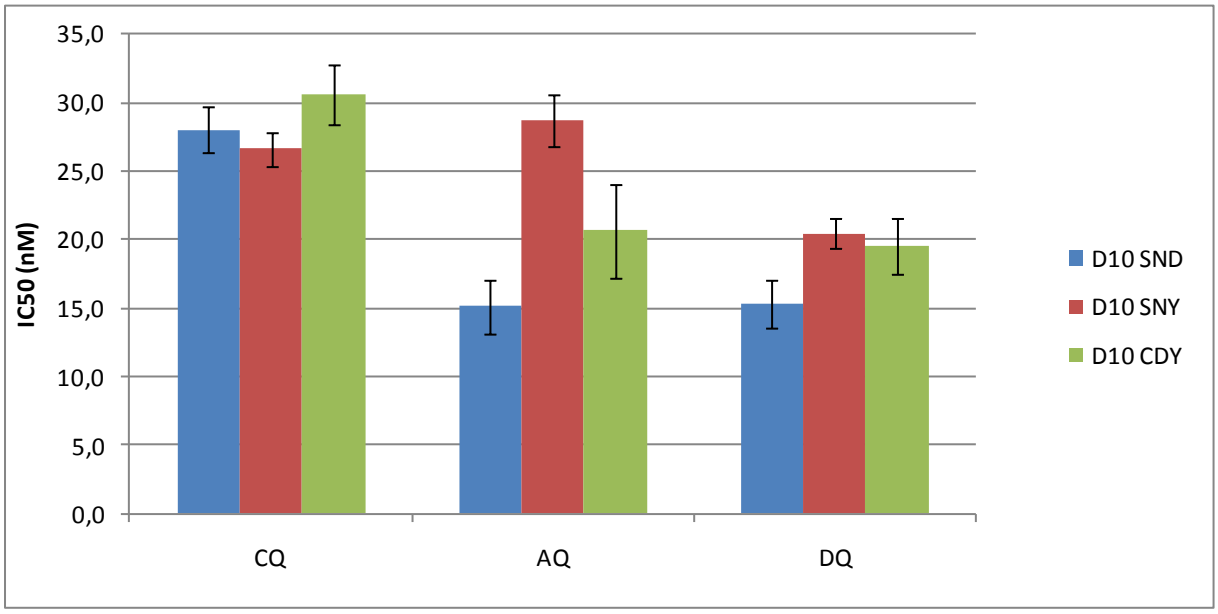


Figure 10. D10 susceptibilities to CQ, AQ and DEAQ (nM; $CI_{95\%}$).

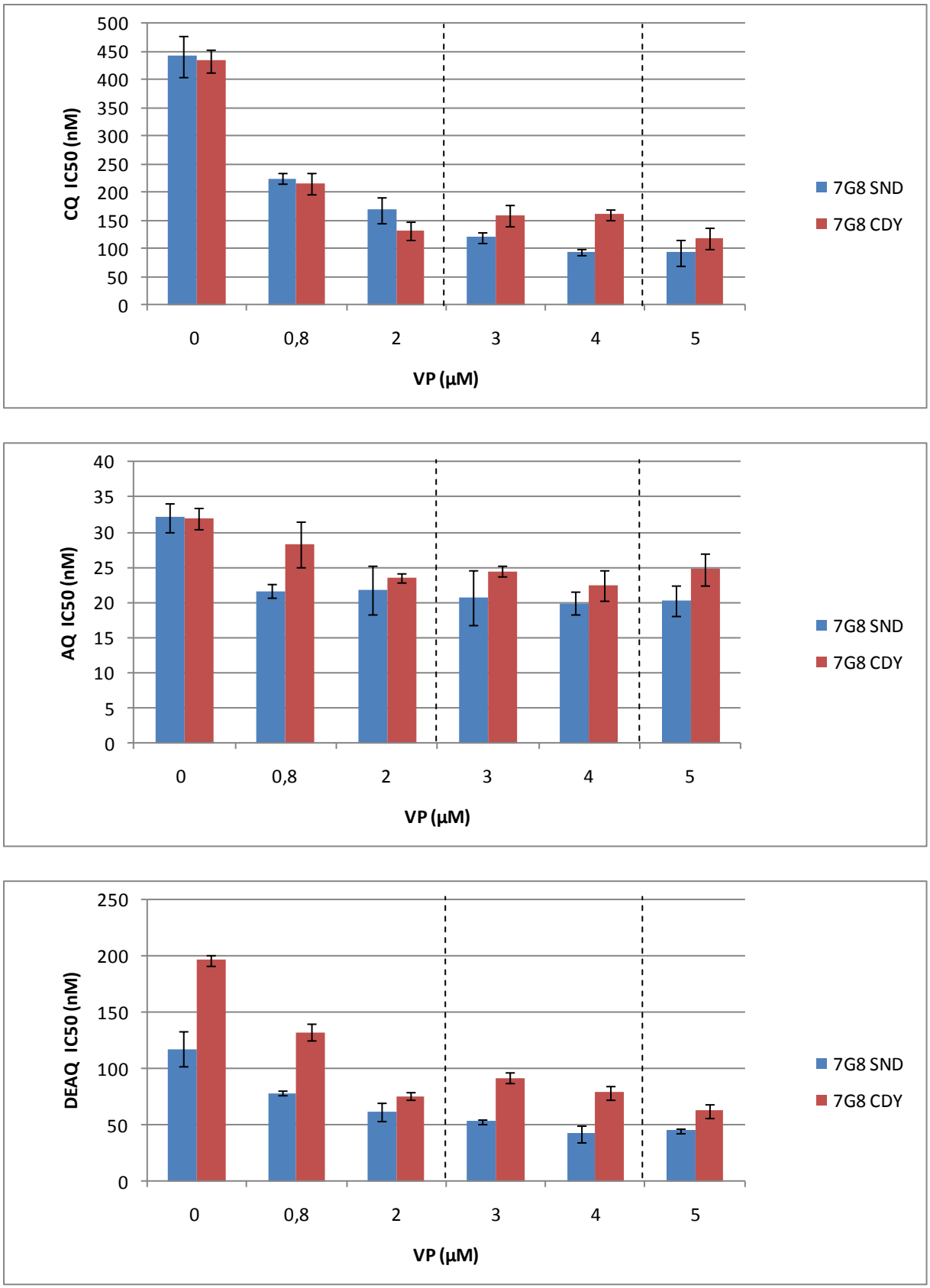
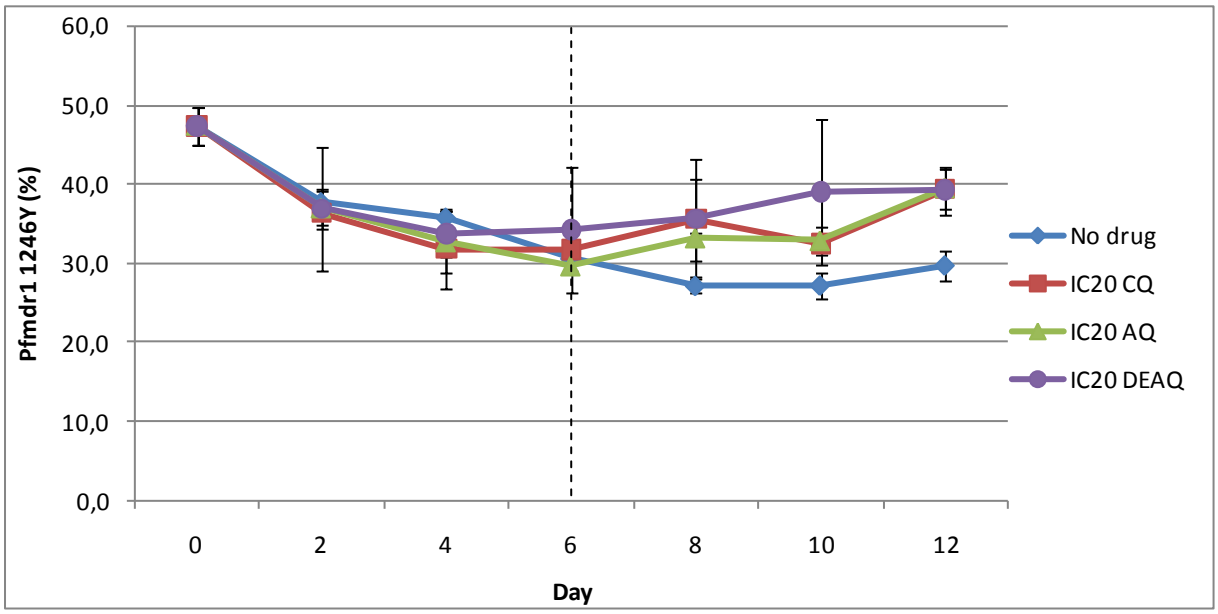


Figure 11. 7G8 susceptibilities to CQ, AQ and DEAQ ± VP (nM; $CI_{95\%}$). The dotted lines indicating the window with maximum sensitization of *pfcrt* and before a dominating VP specific antimalarial effect, extracting the effect of the *pfmdr1* SNPs.

In the competition experiments (Figure 12 and 13) the fitness cost of the mutation *pfmdr1* 1246Y was estimated to 11% (w=0.89) per asexual parasite generation. *Pfmdr1* 1246Y was selected for among parasites which had been exposed to an IC_{20} or IC_{50} drug pressure for six or four days respectively, as compared with none exposed parasites ($p<0.001$ - 0.02). The drug selection benefit over the full culture period was higher in the IC_{50} exposed cultures, as compared with the IC_{20} exposed cultures.



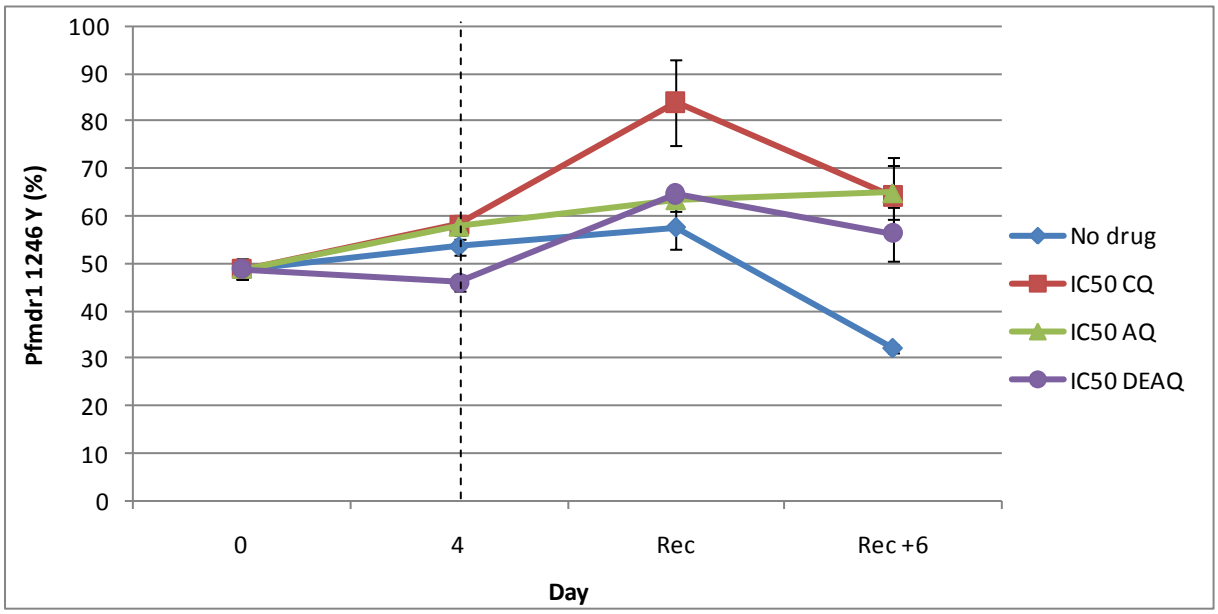
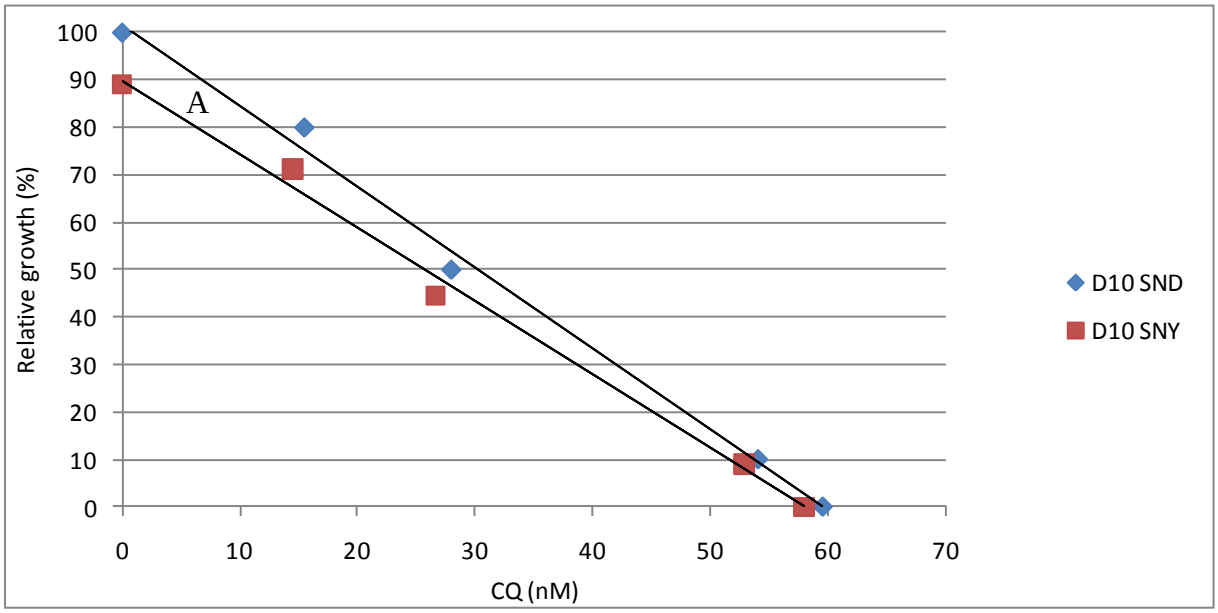
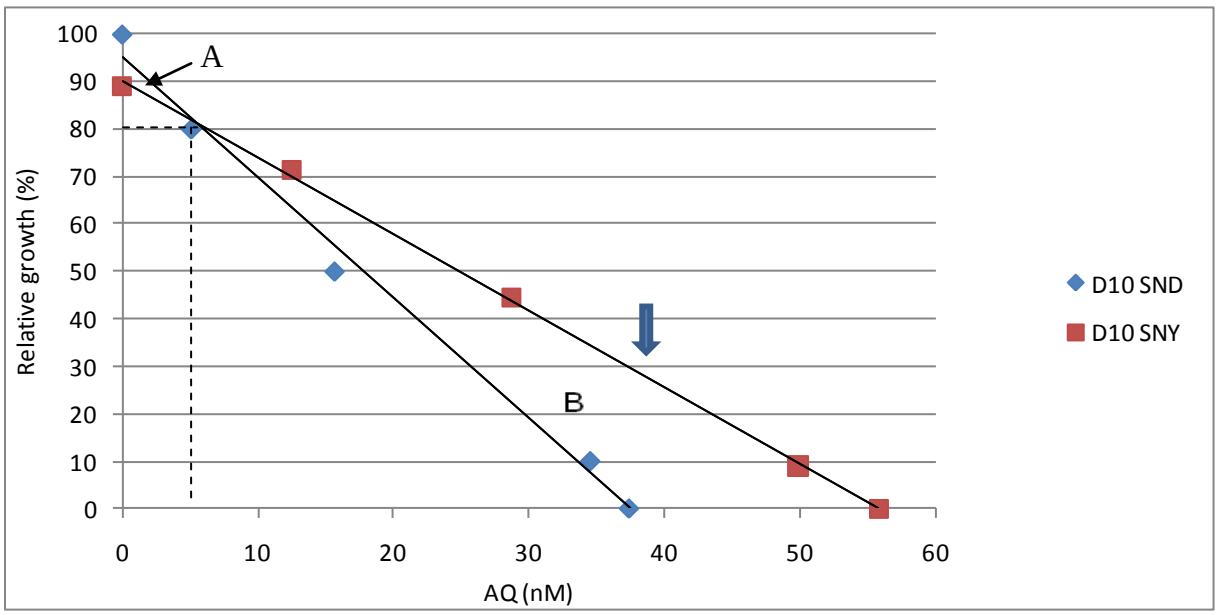


Figure 12 and 13. Proportion of *pfmdr1* 1246Y (%; $CI_{95\%}$) in 1:1 mixed cultures not exposed to drug (control) or exposed to IC_{20} or IC_{50} of CQ, AQ and DEAQ. The vertical line indicating the day when drug exposure was removed. Day of parasite recovery (rec) was day 6, 6, 14 and 18 for the control culture and the cultures exposed to an IC_{50} of CQ, AQ or DQ, respectively.

We developed a new graph model on relative growth of $D10^{SNY}$ vs $D10^{SND}$ where we could estimate the cost-benefit of *pfmdr1* 1246Y over the full drug concentration range (Figure 14). The relative growth trend lines for AQ and DEAQ crossed at approximately IC_{20} and IC_{40} respectively, while for CQ the lines never crossed. The difference between the drug selection benefit (area *B*) and the fitness cost (area *A*) over the full IC scale specify the over-all benefit from *pfmdr1* 1246Y. By normalizing the relative growth and drug concentration against the $D10^{SND}$ clone and using the trend line equations we could calculate these areas to – 7 for CQ, + 16 for AQ and + 6 for DEAQ. The model was confirmed by comparing the figures on relative fitness and relative growth at average IC_{20} and IC_{50} with figures derived from the normalized absorbance values from the HRP2-ELISA susceptibility analyses. Similar cost-benefit graphs were made for $D10^{CDY}$ vs $D10^{SND}$ where we could estimate that the fitness cost of *pfmdr1* 1246Y was eliminated (w=1.04), while the drug selection benefit did not increase as significant.





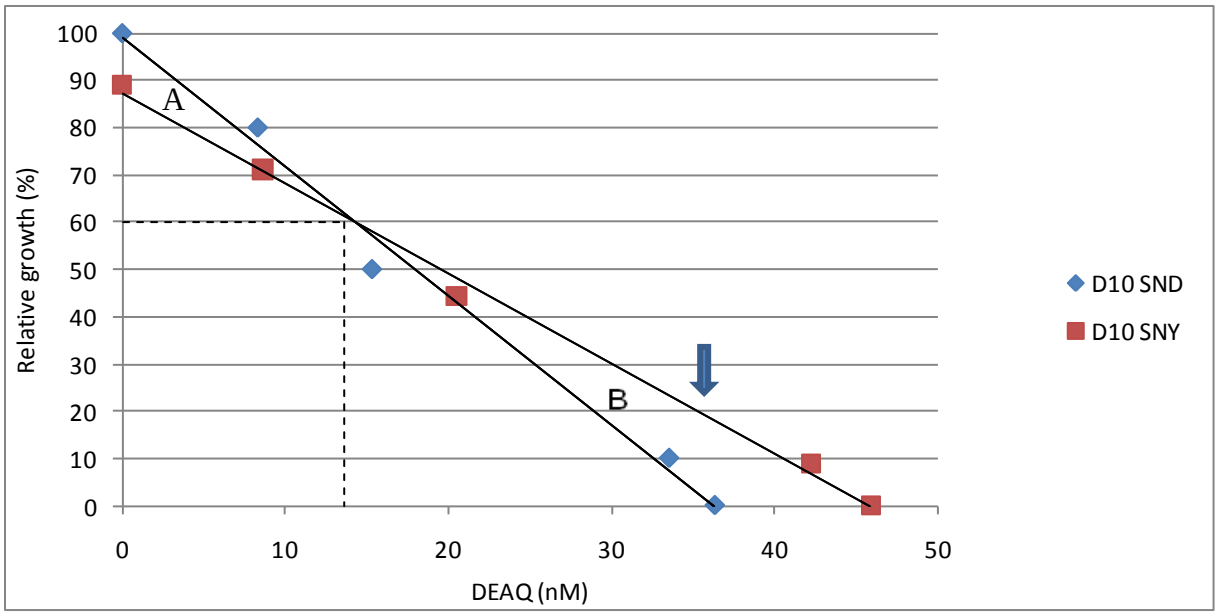


Figure 14. Relative growth for $D10^{SNY}$ vs $D10^{SND}$ over the full concentration range of CQ, AQ and DEAQ. The *y*-axis is estimated parasite growth in proportion to maximum parasite growth of $D10^{SND}$ (%). The *x*-axis is concentration of drug. The plots are based on the IC_{20}, IC_{50} and IC_{90} values derived from the HRP2-ELISA analyses. The relative growth for $D10^{SNY}$ is adjusted for the relative fitness (w=0.89) derived from the competition experiments. The dotted lines indicate the breakpoint values where the drug selective benefit is equal to the fitness cost of *pfmdr1* 1246Y. The arrow indicates the drug concentration where the $D10^{SNY}$ benefits most by *pfmdr1* 1246Y. Area *A* represents the fitness cost and area *B* represents the drug selective benefit of *pfmdr1* 1246Y over the full concentration range.

5 DISCUSSION

5.1 RESISTANCE TO AMODIAQUINE AND DESETHYLAMODIAQUINE

AQ has remained effective in many areas of Africa, despite the fact that it has been used as treatment for uncomplicated *P. falciparum* malaria for decades and at the same time resistance to the structurally related CQ has expanded (Olliaro and Mussano, 2003). This suggests that resistance to AQ is a multi gene *loci* event, associated with a fitness cost and maybe also compensatory mutations.

Our in vitro results suggest that decreased susceptibility to AQ/DEAQ is incurred by *pfmdr1* 1246Y. This is in line with our findings in vivo with a selection of *pfmdr1* 1246Y after AQ monotherapy (Holmgren et al., 2006) and ASAQ combination therapy (Holmgren et al., 2007) in East-Africa. Additionally, *pfmdr1* 1034C and 1042D reduced the fitness cost of *pfmdr1* 1246Y in our cost-benefit graph model in vitro and *pfmdr1* 86Y and 184Y were co-selected with *pfmdr1* 1246Y in our in vivo studies. Further, decreased susceptibility to AQ/DEAQ and high affinity to DEAQ were associated with *pfcrt* (a.a. 72 – 76) CVIET and/or SVMNT in vitro, as well as *pfcrt* 76T being selected for after AQ monotherapy in vivo.

Thus, our in vitro and in vivo results suggest that AQ/DEAQ resistance is associated with *pfmdr1* 1246Y in addition to *pfcrt* (a.a. 72 – 76) CVIET and SVMNT and possibly in a synergistic or compensatory relation with *pfmdr1* 86Y, 184Y, 1034C and 1042D. The average genotype failure index (GFI) for *pfmdr1* 1246Y and AQ treatment failure in our three in vivo studies in East Africa would be 2.8 (Djimde et al., 2001b).

The VP sensitization effect for CQ and DEAQ, but not for AQ, confirms previously published data (Bray et al., 1996; Echeverry et al., 2007; Mehlotra et al., 2001; Sa et al., 2009; Sidhu et al., 2002) and suggests a mechanism of response to AQ less dependent on the *pfcrt* gene. This is in line with our cost-benefit results in vitro where AQ benefit most from *pfmdr1* 1246Y. This suggests a partial cross-resistance between mainly CQ and DEAQ through the *pfcrt* gene, while resistance to AQ might be more dependent on the *pfmdr1* gene.

Resistance to AQ/DEAQ through mutations in the *pfmdr1* gene is probably conferred by affected substrate specificity and reduced import of drug into the active site in the parasite digestive vacuole (Sanchez et al., 2008), while resistance through mutations in the *pfcrt* gene is probably similar to that of CQ.

Our results support the geographic genotype patterns, suggesting two main routes to AQ/DEAQ resistance (Sa et al., 2009). The first route is the South East Asian/African route, mainly originating from CQ resistance. The wild type *pfcrt* haplotype (a.a. 72 – 76) CVMNK is deselected by the imported *pfcrt* CVIET (Ariey et al., 2006), while in parallel the *pfmdr1* haplotype (a.a 86, 184, 1034, 1042, 1246) NFSND is deselected via import or possibly recombination on site to *pfmdr1* 86Y and/or 184Y and with AQ pressure further to the haplotype *pfmdr1* YYSNY. The second route is the South American route more directly associated with AQ/DEAQ resistance. The wild type *pfcrt* haplotype CVMNK is deselected by the on site originated *pfcrt* SVMNT, while in parallel *pfmdr1* NFSND is deselected by recombination on site to *pfmdr1* NFCDY.

So far, the two routes have seemed to exist in parallel without major recombination, except for one report from Papua New Guinea where *pfcrt* SVMNT was seen in combination with *pfmdr1* 86Y and (a.a. 1034, 1042 and 1246) SND and SDD (Mehlotra et al., 2001).

5.2 PARASITE FITNESS COST

The fitness cost of the mutation *pfmdr1* 1246Y was estimated to 11% per asexual parasite generation in vitro. This is in line with earlier competition experiments based on comparison between IC_{50} curves, where 7G8 parasites with *pfmdr1* (a.a. 1034, 1042, 1246) CDY) were outcompeted by parasites with *pfmdr1* SND after 34 days in culture (Hayward et al., 2005). However, our pyrosequencing method gives us a better tool to follow the allele dynamics and to make calculations on the relative growth, not only in cultures but also in field samples. This fitness cost might be too substantial to the parasites in competition with wild type parasites among the relatively high frequency of non treated malaria infections in a high transmission area such as East Africa (Mackinnon and Hastings, 1998). This might explain the selection of *pfmdr1*1246Y after ASAQ combination therapy, whilst the base-line frequency did not increase over three years (Holmgren et al., 2007).

So far it is not clear how *pfmdr1* mutations might cause such a distinct effect on parasite development. Maybe PfMDR1 alterations affect transport of toxic substrates e.g. antimalarial drugs, while distracting the regular transport in to the digestive vacuole, which might deteriorate the parasite to endure in the selection of more fit parasites. Another alternative might be decreased antigenic variation as a result of genetic drift towards linkage disequilibrium and reduced ability of the parasite to persist the immune selection (Babiker et al., 2009).

According to the deselection of mutant parasites after drug pressure was reduced in Africa (Abdel-Muhsin et al., 2004; Kublin et al., 2003; Ord et al., 2007) as compared with South America (Echeverry et al., 2006; Gama et al., 2009), it has been suggested that the former haplotypes are associated with greater fitness cost (Sa et al., 2009). Whether a recombination between the two haplotype routes is possible and what it would mean for parasite resistance to AQ/DEAQ and fitness is unclear.

5.3 THE COST - BENEFIT BALANCE

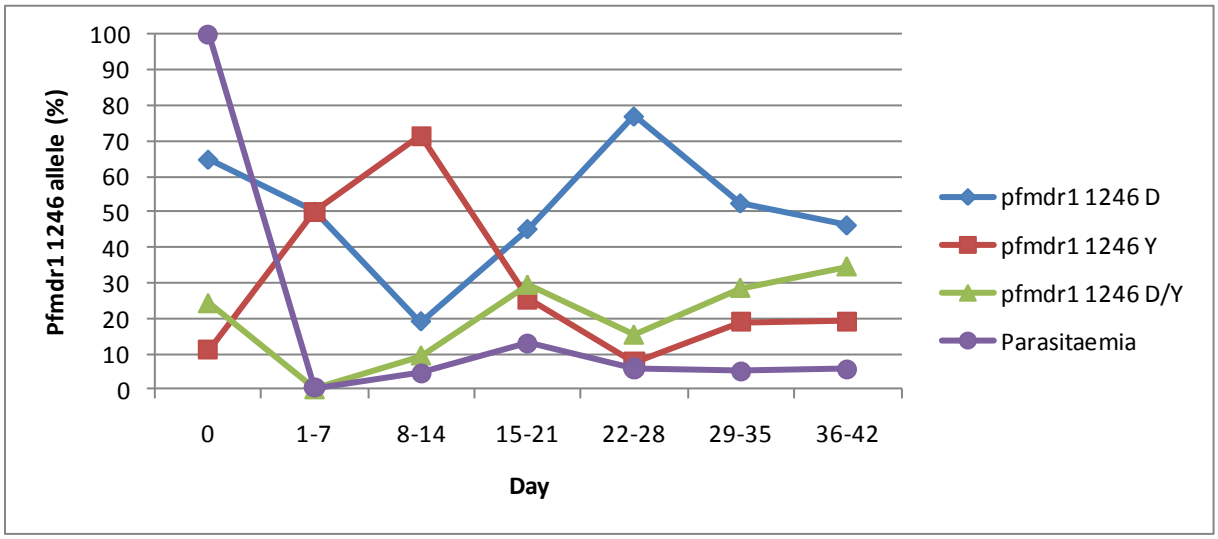
With our new graph model in vitro we could demonstrate that the relative growth for parasites with a particular mutation represents a concentration dependent balance between the fitness cost and the specific drug selection benefit. We calculated that *pfmdr1* 1246Y is most beneficial for parasites exposed to AQ followed by DEAQ, while for parasites exposed to CQ it is only a fitness cost.

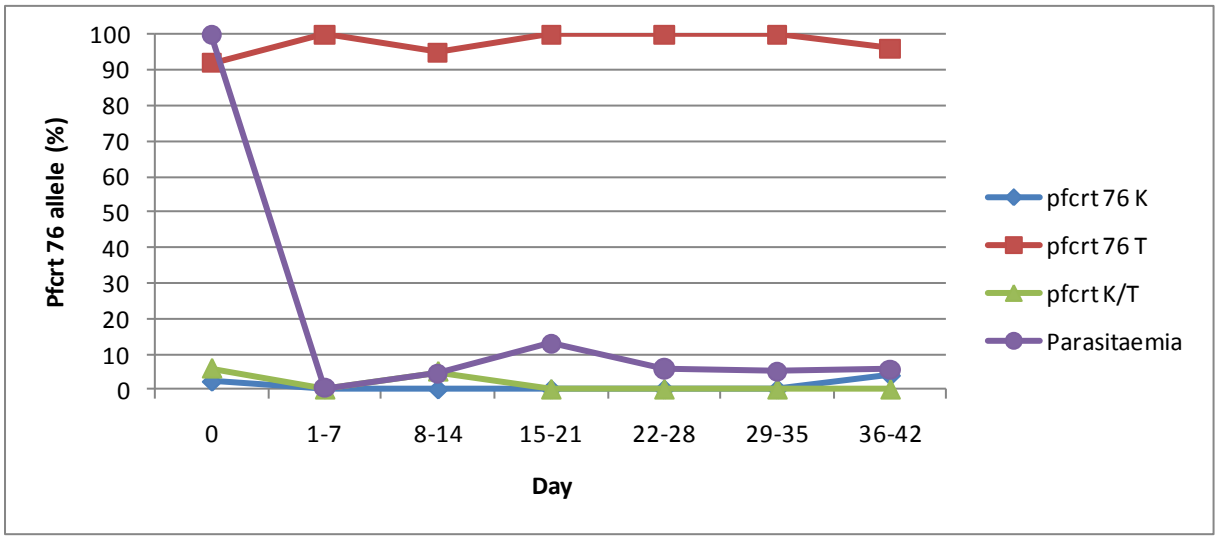
To explore whether this over-all cost-benefit of *pfmdr1* 1246Y in vitro might influence the parasite dynamics after AQ treatment in vivo, where parasites are exposed to various concentrations of the drug, we analysed the *pfmdr1* D1246Y allele dynamics in our three clinical trials in East Africa i.e. the AQ monotherapy study in Kenya 2003 (n=53) (Holmgren et al., 2006) and the ASAQ combination therapy studies on Zanzibar 2002 – 2003 (n=198) (ASAQ I) (Martensson et al., 2005) and 2005 (n=174) (ASAQ II) (Figure 15). Three clear

selection peaks were observed i.e. *pfmdr1* 1246Y on day 8 - 14, *pfmdr1* D1246 on day 22 – 28 and *pfmdr1* 1246 with mixed D/Y in between these peaks on day 15 – 21. This dynamic pattern might be explained by our cost-benefit graph model in vitro. An initial drug selection benefit of *pfmdr1* 1246Y during higher concentration of AQ/DEAQ is followed by a fitness selection of *pfmdr1* D1246 during lower concentrations of the drug. ART in addition to AQ minimized this initial drug selection by a more effective reduction of parasite biomass. Similar analyses were done for *pfcrt* K76T and *pfmdr1* N86Y, where the allele dynamic did not vary as significant (Figure 15), which suggests that the cost-benefit selection pattern is specific for *pfmdr1* D1246Y. Thus, we could demonstrate how the cost-benefit from a mutation in vitro also influences the parasite allele dynamic after antimalarial drug treatment in vivo.

5.4 AMODIAQUINE AS PARTNER DRUG IN ACT

Treatment failure after ASAQ combination therapy is probably conferred by incomplete pharmacodynamic coverage by AS and sufficient degree of resistance to AQ/DEAQ. There is a risk for rapid decay in over all efficacy if the drugs are unable to mutual prevention and drug resistant parasites manage to be selected for. The knowledge on mutations associated with tolerance/resistance to the main ACT drugs has expanded during the last years and is summarized in Table 5. Reduced susceptibility to ART in vitro has been associated with *pfATP6* S769N (Krishna et al., 2006) and with *pfmdr1* N86, D1246 and its amplification (Ashley and White, 2005; Duraisingh and Cowman, 2005; Duraisingh et al., 2000b; Price et al., 2004; Reed et al., 2000). However, these in vitro findings have not been confirmed in clinical trials (Dondorp et al., 2009; Noedl et al., 2008). Even so, the *pfmdr1* correlations are thus reversed for ART as compared with AQ/DEAQ. This is favourable for AQ as partner drug in ACT. Thus, AS in addition to AQ might prevent an increasing treatment failure rate and a spread of drug resistance associated *pfmdr1* SNPs by a more effective reduction of parasite biomass and gametocyte carriage and an opposite *pfmdr1* SNP selection (Ashley and White, 2005; Dokomajilar et al., 2006; Duraisingh and Cowman, 2005; Duraisingh et al., 2000b; Happi et al., 2006; Holmgren et al., 2006; Holmgren et al., 2007; Humphreys et al., 2007; Ochong et al., 2003; Price et al., 2004; Reed et al., 2000). While, in the absence of treatment, *pfmdr1* 1246Y might incur too substantial fitness cost to the parasite to sustain in competition with wild type parasites (Mackinnon and Hastings, 1998). Through a balance between drug selection of more resistant parasites and a natural selection of more fit wild type parasites an area specific steady-state level of AQ/DEAQ resistance may be reached (Hastings, 2006). However, with the widespread deployment of ASAQ in Africa, a possible introduction of South American haplotypes might deteriorate the situation. Even so, we conclude that several factors counteract a selection of AQ resistance, which support sustained efficacy of ASAQ. Thus, we estimate that AQ represents a valuable partner drug option in ACT in East Africa.





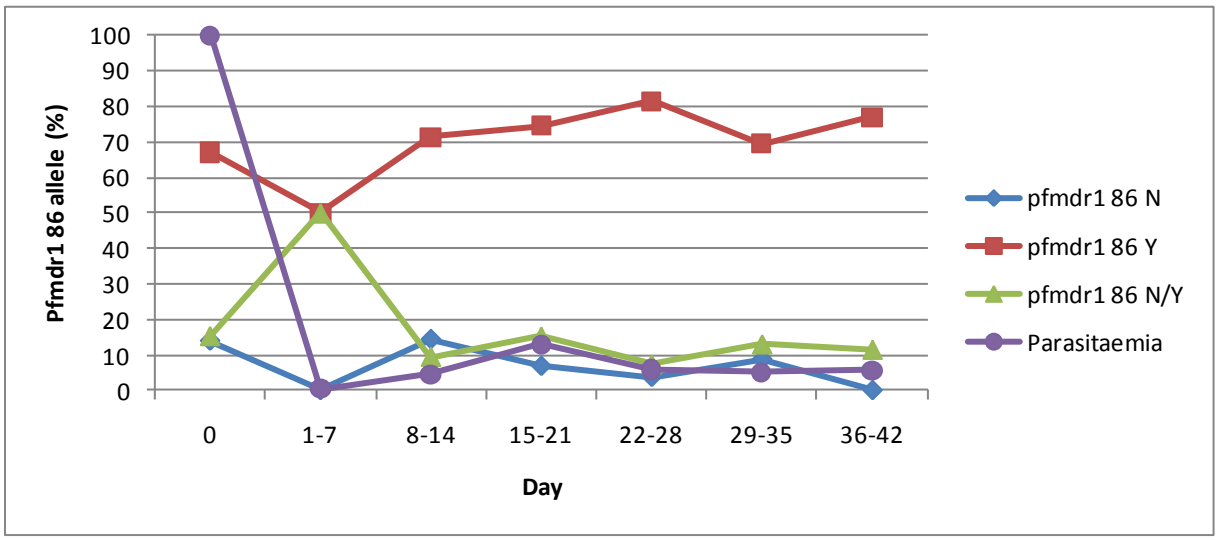


Figure 15. Proportion of the *pfmdr1* D1246Y allele, as well as the *pfcrt* K76T and *pfmdr1* N86Y alleles over the study period in three of our clinical trials on AQ monotherapy or ASAQ combination therapy in East-Africa 2002 – 2005 (n=425). Parasitaemia is the proportion of children with positive parasitaemia as compared with day 0.

Drug	Gene			References
	Pfcrt	***Pfmdr1***	**Other**	
CQ	**76T** **72-76 CVIET** 72-76 SVMNT 152A 163R	86Y 184Y 1034C 1042D 1246Y	*Pfmrp* 191H, 437S	(Djimde et al., 2001a; Fidock et al., 2000b; Johnson et al., 2004; Klokouzas et al., 2004; Lakshmanan et al., 2005; Mu et al., 2003; Picot et al., 2009; Reed et al., 2000; Sidhu et al., 2002)
AQ/DEAQ	76T 72-76 CVIET **72-76 SVMNT** 326S/D 334N 356T/L	86Y 184Y 1034C 1042D **1246Y**		(Danquah et al., 2010; Dokomajilar et al., 2006; Echeverry et al., 2007; Happi et al., 2006; Holmgren et al., 2006; Holmgren et al., 2007; Humphreys et al., 2007; Menard et al., 2006; Nsobya et al., 2010; Ochong et al., 2003; Picot et al., 2009; Sa et al., 2009; Warhurst, 2003)
MQ	76K	86N **Amplification**		(Price et al., 1999b; Price et al., 2004; Sidhu et al., 2002)
Lumefantrine	**76K**	**86N** **1246D** **Amplification**		(Sisowath et al., 2007; Sisowath et al., 2005)
Sulphadoxine-Pyrimethamine			***Pfdhps*** **437G, 540E** ***Pfdhfr*** **51I, 59R, 108N**	(Picot et al., 2009; Wongsrichanalai et al., 2002)
Artemisinins	76K	86N 1246D Amplification	*PfATP6* 769N	(Duraisingh and Cowman, 2005; Duraisingh et al., 2000a; Krishna et al., 2006; Lakshmanan et al., 2005; Price et al., 2004; Reed et al., 2000; Sidhu et al., 2002)

Table 5. Genotypes associated with tolerance/resistance to antimalarial drugs (bold text indicates main markers).

6 CONCLUSIONS

6.1 GENERAL CONCLUSIONS

Several mutations in the *pfcrt* and *pfmdr1* genes were associated with AQ/DEAQ tolerance/resistance and one specific mutation in the *pfmdr1* gene was associated with a significant parasite fitness cost.

6.2 SPECIFIC CONCLUSIONS

1. The treatment failure rate after AQ monotherapy was relatively high (20%) in Kenya.

2. No correlation was found between treatment outcome and variable DEAQ blood concentrations after AQ monotherapy.

3. AQ/DEAQ tolerance/resistance was found to be associated with *pfmdr1* 1246Y in addition to *pfcrt* (a.a. 72 – 76) CVIET/SVMNT and possibly in synergistic or compensatory relation with *pfmdr1* 86Y, 184Y, 1034C and 1042D. Possibly *pfcrt* 326S/D and 356T/L, as well as a newly identified mutation *pfcrt* 334N, are also involved. Treatment failure after AQ monotherapy was not found to be associated with any other of the herein analyzed SNPs in the *pfcrt, pfmdr1, pfmrp1* genes or the rare findings of *pfmdr1* amplifications in Kenya. No new mutations could be verified in the *pfcrt* and *pfmdr1* genes in all recrudescent parasites after AQ or ASAQ therapy.

4. The partial cross-resistance with CQ is probably conferred to mainly DEAQ through the *pfcrt* gene, while resistance to AQ is possibly more dependent on the *pfmdr1* gene.

5. The in vitro fitness cost of the mutation *pfmdr1* 1246Y was estimated to 11% per asexual parasite generation. The relative growth for parasites with a particular mutation is a concentration dependent balance between the fitness cost and the specific drug selection benefit. The in vitro estimated cost-benefit of *pfmdr1* 1246Y correlated with the allele selection dynamics after AQ and ASAQ therapy in vivo.

6. The added effect of ART will potentially prevent against a selection of *pfmdr1* 1246Y by a more effective reduction of parasite biomass and an opposite *pfmdr1* allele selection and in the absence of AQ exposure *pfmdr1* 1246Y will possibly incur too substantial fitness cost to sustain in competition with wild type parasites. We conclude that several factors counteract a selection of AQ resistance, which support sustained efficacy of ASAQ. Thus, we estimate that AQ is a valuable partner drug option in ACT in East Africa.

7 FUTURE PERSPECTIVES

For optimal treatment and to prevent further development and spread of resistance to AQ and DEAQ, as well as to other ACT drugs in the context of *P. falciparum* malaria it is crucial to explore:

1. The cost-benefit of other mutations associated with tolerance/resistance to AQ/DEAQ and other partner drugs with and without ART in vitro.
2. Whether the cost-benefit of mutations associated with drug resistance is also affected by drug exposure time and/or interval in vitro.
3. The cost-benefit selection in vivo by analysing the allele selection dynamics of drug resistance associated genotypes in clinical trials with different ACT.
4. Whether the cost-benefit dynamics of mutations also affect the gametocytogenesis in vitro and in vivo.
5. The epidemiological dynamics after ACT implementation by close screening of the frequencies of mutations associated with drug resistance in the overall parasite population.

For these purposes we intend to use, in addition to traditional methodologies, pyrosequencing for allele quantification in parallel and/or competition cultures in vitro, as well as parasite samples from clinical trials in vivo. New pyrosequencing systems have been designed to analyze *pfmdr1* N86Y, Y184F, D1246Y and the most common *pfcrt* (a.a. 72 – 76) haplotypes i.e. CVMNK, CVIET and SVMNT. For analyzing the results we will include the cost-benefit graph model in vitro and the cost-benefit allele selection dynamics in clinical trials with ACT with extended follow-up. The results will hopefully improve the knowledge basis on mutations associated with antimalarial drug resistance and be advisory in optimizing antimalarial drug policies.

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9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund: Nästan en miljon människor dör och ungefär 250 miljoner insjuknar i malaria varje år. Främst drabbas barn och gravida kvinnor i Afrika. Malaria slår också hårt mot sociala strukturer och ekonomin i ofta redan fattiga länder i tropikerna. Malaria är en blodparasit som smittar via en mygga. Malaria orsakar feber, huvudvärk, muskelvärk och kan ibland försvåras och orsaka blodbrist, andningssvårigheter, njursvikt, leversvikt, kramper, medvetslöshet och död. Man kan skydda sig mot malaria med myggnät och myggspray, men något vaccin finns inte. Malaria kan behandlas med läkemedel ex klorokin, amodiakin, meflokin och ett sulfapreparat. Tyvärr har malariaparasiten de senaste årtiondena utvecklat motståndskraft (resistens) mot de flesta läkemedel, förutom mot det nya läkemedlet artemisinin (kinesisk malört). För att förhindra ytterligare resistensutveckling så rekommenderar nu Världshälsoorganisationen (WHO) att malaria skall behandlas med artemisinin i kombination med ett annat läkemedel. För att de två läkemedlen skall bli effektiva tillsammans och skydda varandra från resistensutveckling, så är det dock väldigt viktigt att välja rätt läkemedelskombination. Läkemedlet amodiakin är en släkting till klorokin, som har använts i Afrika i årtionden och lyckats förbli relativt effektivt. Amodiakin är ett av de läkemedel som rekommenderas i kombination med artemisinin.
Syfte: I denna avhandling har vi studerat amodiakin och dess nedbrytningsprodukt (metabolit), vad gäller effektivitet, genetiska förändringar (mutationer) som orsak till resistensutveckling och vad dessa mutationer kanske kostar parasiten i form av nedsatt kondition (fitness).
Metoder: Studierna baserades på (a) kliniska prövningar i Östafrika och (b) parasitodlingar från Colombia och referensparasitodlingar som fått specifika mutationer infogade i sina gener. Först multikopierades önskade gensekvenser (PCR) och därefter analyserades mutationerna med olika enzymer som klipper generna i olika stora bitar (RFLP) eller genom att varje byggnadsdel (nukleotid) i gensekvensen identifierades (sekvensering). Slutligen jämfördes resultaten från dessa mutationsanalyser med behandlingsresultaten i de kliniska prövningarna och/eller läkemedelskänsligheten och parasittillväxten hos de olika parasitodlingarna för att försöka se ett samband mellan mutationerna och läkemedelsresistens.
Resultat: Behandlingssvikt efter amodiakin var relativt vanligt, medan amodiakin i kombination med artemisinin var effektivt. Resistens mot amodiakin och dess metabolit kunde associeras till framför allt en mutation i en av generna (*pfmdr1*) i kombination med en grupp mutationer i en annan gen (*pfcrt*). Gemensam resistens (korsresistens) föreligger troligen framför allt mellan klorokin och amodiakins metabolit. Den förstnämnda mutationen orsakar en konditionsnedsättning (fitness cost) hos parasiten. När parasiten utsätts för läkemedel innebär mutationen således en fördel i form av resistens. När parasiten inte utsätts för läkemedel är den dock en nackdel i form av nedsatt kondition. Detta kunde observeras i hur bra parasiterna växte i en tävling mellan parasiter med och utan mutationen i parasitodlingar, samt i återfalls-infektionerna i de kliniska prövningarna. Vad mutationen i slutändan innebär för parasiten är alltså en balansgång mellan resistens och nedsatt kondition, som är beroende av den läkemedelskoncentrationen som parasiten utsätts för. Artemisinin och amodiakin skyddar troligen varandra från resistensutveckling. Flera faktorer motverkar således resistensutveckling mot amodiakin, som tycks vara ett bra alternativ i kombination med artemisinin i Afrika.

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