

Department of Medicine Solna, Infectious Diseases Unit
Karolinska University Hospital
Karolinska Institutet
Stockholm
Sweden

**PCR ADJUSTED CURE
RATES IN CLINICAL TRIALS OF
ANTIMALARIAL DRUGS IN AFRICA**

**- INFLUENCE OF EXTENDED FOLLOW-UP AND
CONSECUTIVE DAY BLOOD SAMPLING**

Andreas Mårtensson



**Karolinska
Institutet**

Stockholm 2008

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Universitetservice US-AB

© Andreas Mårtensson, 2008
ISBN 978-91-7409-229-5

ABSTRACT

Malaria treatment guidelines are based primarily on results from clinical trials. Accurate assessment of drug efficacy in such studies therefore represents a prerequisite to ensure effective and lifesaving treatment for children with *Plasmodium falciparum* infections in Africa. The World Health Organization standard procedures for *in vivo* assessment include a patient follow-up duration of at least 28 days. However, during four weeks or more after initiation of treatment in high transmission areas in Africa recurrent infections are inevitable, due to the continuous exposure to infective mosquito bites. Since microscopy cannot distinguish parasites that have escaped drug action (treatment failure/recrudescence) from those arising from new mosquito inoculations (new infections/reinfections), polymerase chain reaction (PCR) based genotyping of malaria infections has been introduced to improve this distinction. PCR adjustment is presently achieved by analysis of two blood samples, one collected at enrolment and the other at time of recurrent parasitaemia. However, despite being a sensitive technique PCR may not identify minority clones in complex malaria infections and parasites sequestering in the deep vascular system during the later part of the erythrocytic life cycle.

This thesis primarily addresses two fundamental aspects of the assessment of PCR adjusted efficacy of antimalarial drugs in Africa, i.e. the influence of extended follow-up beyond 28 days and added consecutive day blood sampling.

The results reveal that both an extension of follow-up and consecutive day blood sampling improve the identification of recrudescences. By pooling data from 1414 children enrolled in three studies included in this thesis the overall sensitivity of the 28 day follow-up schedule to identify recrudescences was calculated to $48/72=67\%$ [95% CI 55-76%] compared with day 42 assessment. Moreover, consecutive day, instead of single day blood sampling improved identification of recrudescences by circa 20%.

The work in this thesis also highlights the potential influence of the number of genetic markers used and the choice of DNA extraction method on the efficacy outcome in the clinical trials

Thus, assessment of PCR adjusted cure rates in high endemic areas depends substantially on methodology and should therefore be interpreted with caution. The results from this thesis should be considered in the design of antimalarial drug trials to improve assessment of antimalarial drug efficacy in Africa.

LIST OF PUBLICATIONS

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

- I. **Mårtensson A**, Strömberg J, Sisowath C, Msellem MI, Gil JP, Montgomery SM, Olliaro P, Ali AS, Björkman A.
Efficacy of artesunate plus amodiaquine versus that of artemether-lumefantrine for the treatment of uncomplicated childhood malaria in Zanzibar, Tanzania. *Clin Infect Dis* 2005; 41(8):1079-86.
- II. Abdulla S, Sagara I, Borrmann S, D'Alessandro U, González R, Hamel M, Ogutu B, **Mårtensson A**, Lyimo J, Maiga H, Sasi P, Nahum A, Bassat Q, Juma E, Otieno L, Björkman A, Beck HP, Andriano K, Cousin M, Lefèvre G, Ubben D, Premji Z.
Efficacy and safety of artemether-lumefantrine dispersible tablet in African infants and children with uncomplicated malaria: a randomised, investigator-blinded, multi-centre comparison with the crushed commercial tablet. *Lancet*. 2008 Oct 14. (Electronically published ahead of print)
- III. Bereczky S, **Mårtensson A**, Gil JP, Färnert A.
Rapid DNA extraction from archive blood spots on filter paper for genotyping of *P. falciparum*. *Am J Trop Med Hyg*. 2005;72(3):249-51.
- IV. **Mårtensson A**, Ngasala B, Ursing J, Veiga MI, Wiklund L, Membi C, Montgomery SM, Premji Z, Färnert A, Björkman A.
Influence of consecutive-day blood sampling on polymerase chain reaction-adjusted parasitological cure rates in an antimalarial-drug trial conducted in Tanzania. *J Infect Dis*. 2007;195(4):597-601.
- V. **Mårtensson A**, Carlsson AM, Ngasala B, Dahlström S, Membi C, Musih MA, Omary S, Montgomery SM, Rombo L, Abdullah S, Premji Z, Gil JP, Björkman A.
Plasmodium falciparum population dynamics during the early phase of antimalarial drug treatment in Tanzanian children with acute uncomplicated malaria. Manuscript.

CONTENTS

1	General Background.....	1
1.1	General introduction to malaria.....	1
1.2	The malaria parasite.....	2
1.3	Life cycle of the parasite	2
1.4	Malaria transmission and epidemiology	5
1.5	Clinical presentation of the disease.....	6
1.6	Malaria diagnosis and treatment in Africa.....	8
1.7	History of antimalarial drugs.....	10
1.7.1	Traditional remedies for fever treatment	10
1.7.2	Driving forces behind antimalarial drug development.....	10
1.7.3	The modern concept for antimalarial drug treatment.....	11
1.8	Objectives of antimalarial drug treatment.....	12
1.9	Antimalarial drug resistance.....	13
1.9.1	Methods for assessment of antimalarial drug resistance.....	14
2	Thesis Specific Background.....	16
2.1	Clinical trials of antimalarial drugs.....	16
2.1.1	Development of the <i>in vivo</i> test	16
2.1.2	<i>In vivo</i> outcome classifications	17
2.1.3	Current recommendations for <i>in vivo</i> trials	18
2.1.4	Analysis of <i>in vivo</i> efficacy.....	19
2.1.5	Standard follow-up schedule for <i>in vivo</i> trials.....	20
2.2	PCR genotyping to distinguish recrudescence from reinfections ...	20
2.2.1	PCR genotyping	20
2.2.2	PCR genotyping of <i>P. falciparum</i>	22
2.2.3	PCR genotyping of <i>P. falciparum</i> in clinical drug trials.....	24
2.2.4	Standardization of PCR genotyping in drug trials.....	27
2.3	Parasite population dynamics in clinical malaria	29
2.4	Antimalarial drugs used in the thesis	30
2.4.1	Artemisinin-derivatives.....	30
2.4.2	Artemisinin-based combination therapies	31
2.4.3	Sulfadoxine-pyrimethamine.....	35
2.5	Policy for introduction and change/review of drug treatment.....	36
2.6	Rationale for the thesis	36
2.7	Methodological research questions.....	36
2.7.1	Influence of extended follow-up beyond 28 days	36
2.7.2	Influence of consecutive day blood sampling	37
2.8	Objectives and aims of the thesis	38
2.8.1	Overall objective	38
2.8.2	Specific aims	38
3	Material and Methods.....	39
3.1	Study sites and population.....	39
3.1.1	Study sites in Zanzibar and mainland Tanzania	39
3.1.2	Study sites in the multicentre trial	40
3.1.3	Study sites in the DNA extraction study	40
3.2	Ethical considerations.....	40

3.3	Clinical trial registration.....	40
3.4	General methodologies in the efficacy trials	41
3.5	Study specific methodologies	42
3.6	Assessment of crude cure and failure rates.....	42
3.7	Assessment of PCR adjusted cure and failure rates	42
	3.7.1 Blood sampling and storage.....	42
	3.7.2 DNA extraction	43
	3.7.3 PCR genotyping	43
	3.7.4 PCR adjusted cure rates	44
4	Results and Discussion.....	45
	4.1 Study I: Efficacy of two ACTs in Zanzibar.....	45
	4.2 Study II: Efficacy and safety of a paediatric ACT formulation	46
	4.3 Study III: Influence of different DNA extraction methods	48
	48
	4.4 Study IV: Influence of consecutive day blood sampling on PCR adjusted cure rates	48
	4.5 Study V: Parasite population dynamics during the early Phase of antimalarial drug treatment.....	50
	4.6 Influence of extended follow-up– pooled data	52
5	Conclusions	53
	5.1.1 Overall conclusion	53
	5.1.2 Specific conclusions.....	53
6	Personal Reflexions and Future Perspectives.....	54
	6.1 Length of follow-up.....	54
	6.2 Consecutive day blood sampling	55
	6.3 Influence of repetitive treatment	56
	6.4 Choice and deployment of ACT based on selection potential of molecular markers of drug resistance.....	57
	6.5 Supporting evidence of drug resistance in vivo	58
7	Acknowledgements	59
8	References	62

LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapies	1
CI	Confidence interval	
<i>crt</i>	Chloroquine resistance transporter gene	
<i>dhfr</i>	Dihydrofolate reductase gene	
<i>dhps</i>	Dihydropteroate synthase gene	
DNDi	Drugs for Neglected Diseases Initiative	
EIR	Entomological inoculation rate	
GLURP	Glutamate rich protein	
<i>glurp</i>	Glutamate rich protein gene	
Hb	Haemoglobin	
ITT	Intention-to-treat	
kDa	Kilo Dalton	
MMV	The Medicines for Malaria Venture Foundation	
<i>mdr1</i>	Multidrug resistance gene 1	
MSP	Merozoite surface protein	
<i>msp</i>	Merozoite surface protein gene	
μ L	Microlitre	
mL	Millilitre	
ng	Nanogram	
OR	Odds ratio	
PCR	Polymerase chain reaction	
RDT	Rapid diagnostic test	
RR	Relative risk	
WBC	White blood count	

1 GENERAL BACKGROUND

1.1 GENERAL INTRODUCTION TO MALARIA

Malaria has undoubtedly played an important role in the history of mankind, not only by being a constant threat to human health and by hampering economical development and growth in areas highly effected by the disease, but also for being more lethal than bullets in war and a major obstacle for colonial empires to expand their territories beyond the costal areas of Africa (Gallup and Sachs 2001; Kitchen, Vaughn et al. 2006)

Despite major attempts to eradicate malaria in Africa between 1955-1978, the disease resurged during the 1980s and 90s concomitantly with rapid spread of resistance to mainstay antimalarial drugs, e.g. chloroquine and sulfadoxine-pyrimethamine (Bjorkman and Bhattarai 2005). However, the advent of a new Millennium has been accompanied by increased awareness and expressed commitment by political leaders in Africa and by the international community to Roll Back Malaria. This has been clearly outlined in the Millennium Development Goals, the Abuja Declaration and more recently in the Global Malaria Action Plan (WHO 2000a; WHO 2003a; WHO 2008a). This new era has coincided with access to powerful tools to control the disease, e.g. modern and efficacious combination treatments based on artemisinin-derivatives as well as improved vector control with long lasting insecticide treated nets and a revival of indoor residual spraying. Integrated, wide scale, high coverage interventions with these tools have recently shown persuasive evidence of marked reduction in overall child mortality and burden of disease in endemic areas of Africa (Bhattarai, Ali et al. 2007), to such an extent that the potential of elimination/eradication of malaria has been suggested (WHO 2008a).

However, despite these promising reports, *Plasmodium falciparum* malaria remains as one of the leading causes of death worldwide from a single infectious pathogen, accounting for approximately 1 million mortalities yearly, mostly among children below 5 years of age in sub-Saharan Africa (WHO 2008b). Moreover, an estimated 247 million malaria infections occurred in 2006 among 3.3 billion people living at risk in 109 endemic countries in Africa, South and Southeast Asia, Oceania, and parts of the Americas, 45 of them being located within the World Health Organization (WHO) Africa region (WHO 2008c)

1.2 THE MALARIA PARASITE

Malaria is a parasitic disease caused by members to the genus *Plasmodium*. More than 120 species have been described in mammals, reptiles and birds. Classically, 4 species have been considered to infect humans, i.e. *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. However, recently *P. knowlesi*, which has the long-tailed and pig-tailed macaque monkeys as natural hosts, has been proposed as a fifth human malaria parasite (Singh, Kim Sung et al. 2004; Cox-Singh, Davis et al. 2008; White 2008). The five human malaria species differ in morphology, life-cycle and clinical presentation/severity.

This thesis involves studies exclusively of *P. falciparum*, which is the most virulent and drug resistance associated human malaria parasite, and as such responsible for a great majority of the global malaria related morbidity and mortality.

1.3 LIFE CYCLE OF THE PARASITE

The lifecycle of human malaria requires the presence of three players, i.e. the parasite (agent), the *Anopheles* mosquito (vector) and man (host). However, considering that the sexual recombination of the parasite takes place within the gut of the mosquito, the arthropod vector by definition also acts as the definite host. Consequently, man is defined as an intermediate host. The French military doctor Alphonse Laveran was the first to discover and describe the malaria parasite in human blood in Algeria 1880. The complete life cycle was described 18 years later (1898) by the British army surgeon Ronald Ross after studies of avian malaria in Calcutta, India. These discoveries rewarded Ross and Laveran the Noble Prize in Physiology and Medicine in 1902 and 1907, respectively.

The life cycle of the malaria parasite is illustrated in Figure 1. Briefly, an infected female *Anopheles* mosquito inoculates sporozoites into the human victim whilst taking a blood meal. The sporozoites readily find their way through the blood stream to the liver, where they invade hepatocytes (liver cells) within 30-60 minutes. The entry into the liver cells is the starting point of the pre-erythrocytic tissue phase, which depending on the malaria species can take one or two different directions. Either, and applicable to all human malaria parasites, there is an initiation of growth followed by asexual replication into mature liver schizonts each containing up to 30 000 merozoites (tissue

schizogony), or, as in the case of *P. vivax* and *P. ovale*, a dormant stage can be entered. The latter stage is called hypnozoite, where the parasite can remain for weeks up to years before it is stimulated to regain physiological activity and to enter tissue schizogony. This phenomenon is the biological explanation behind the clinical relapse occurring in *P. vivax* and *P. ovale* infections (Krotoski 1989). The duration of tissue schizogony is 5-16 days, depending on species. The entire pre-erythrocytic tissue phase is asymptomatic in humans, which is in overt contrast to the next stage of the life cycle, i.e. the erythrocytic phase, which is associated with a wide variety of manifestations, from asymptomatic infections to severe disease and death.

Some of the released merozoites from ruptured liver cells escape from being ingested by Kupffers cells in the liver, whereafter they can attach to and invade their target cells, i.e. erythrocytes (red blood cells). This process, which is a cascade of highly complex events, is rapid and lasts only for approximately 30 seconds. After completion of invasion each parasite grows and multiplies asexually to become a schizont containing 8-24 merozoites (erythrocytic schizogony). When an erythrocyte harbouring a mature schizont ruptures, the merozoites are released into the blood. The liberated merozoites subsequently invade new red blood cells and initiate another cycle of erythrocytic schizogony. The duration of each erythrocytic cycle differs between species, i.e. 24 hours for *P. knowlesi*, 48 hours for *P. vivax*, *ovale* and *falciparum*, and 72 hours for *P. malariae*. This corresponds to the classical pattern of fever paroxysms observed with a periodicity of 24 (quotidian), 48 (tertian) or 72 hours (quartan), respectively. However, this periodic fever pattern is rarely seen in *P. falciparum* infections. Usually all stages of erythrocytic schizogony is present in the peripheral blood with one exception. In *P. falciparum* a special feature has been observed with sequestration of the infected red blood cells in the deep vascular system during the second half of the erythrocytic cycle. Due to this phenomenon, which is closely linked to the pathogenesis of falciparum malaria, normally only ring forms and early trophozoites, but not schizonts, are seen in the peripheral blood of people infected with this species. Other features confined to the erythrocytic schizogony of *P. falciparum* include presence of multiple infections in single red blood cells and the ability to infect erythrocytes of all ages.

Eventually a small fraction of merozoites entering red blood cells will develop into the sexual form of the parasite (gametocytogenesis). If mature gametocytes are ingested by an *Anopheles* mosquito taking a blood meal, the fertilization takes place within the gut

of the vector after fusion of a male and female gametocyte into a zygote. After the sexual recombination is completed an ookinete is formed, which is followed by further development of an oocyst (sporogony), where the parasites (sporozoites) again appear in haploid form. When a mature oocyst ruptures thousands of sporozoites are liberated. They migrate to the salivary glands of the mosquito, awaiting to be transmitted during the next blood meal of the mosquito. The duration of sporogony is about 8-16 days, depending on species and temperature.

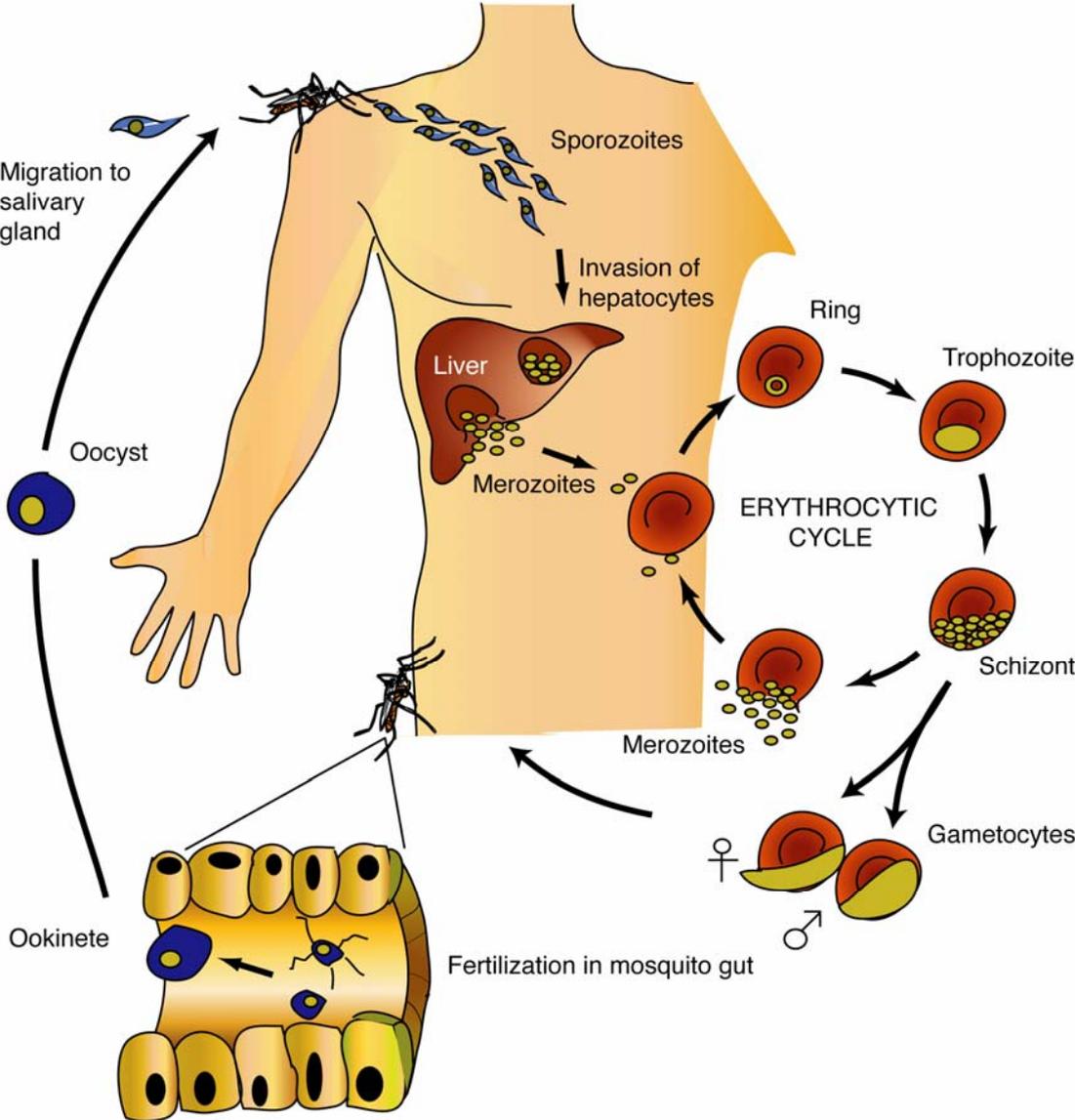


Figure 1. Illustration of the life cycle of the malaria parasite
(Published with kind permission from Dr. Andreas Heddini)

1.4 MALARIA TRANSMISSION AND EPIDEMIOLOGY

Naturally occurring malaria transmission is geographically restricted to areas with presence of the *Anopheles* vector and climatic conditions favourable for the parasite, including mainly tropical and sub-tropical climate. *P. vivax* has the most widely distribution, which prior to the malaria eradication campaigns also included parts of Europe with temperate climate below an altitude of circa 2000 meter. It should be noted that even though the parasite since long is eradicated from Europe, the vector remains present, giving an at least theoretical risk of a future possibility of reintroduction of malaria in Europe.

In Africa the predominant vector belongs to the *A. gambiae* complex. *Anopheline* mosquitoes normally feed between dusk to dawn. However, some species are more likely to feed during early evening others late at night. Additional aspects of feeding habits include whether the local vector prefers to feed on humans (antropophilic) or cattle (zoophilic) and if they favour to feed indoor (endophagic) or outdoor (exophagic). The resting place of the female mosquito after the blood meal can also differ and be either indoor (endophilic) or outdoor (exophilic) (Gibson 1996). Understanding the preferential feeding and resting habits of the local vector is fundamental to tailor cost-effective vector control measures in an area.

Malaria endemicity can be divided in epidemic and endemic transmission. An epidemic is characterized either as an outbreak in an area/population where transmission normally does not occur, or as a period with a sharp increase in number of malaria patients in an area with an underlying/ongoing transmission of parasites, i.e. endemic area. The malaria transmission in endemic areas can be further classified as stable or unstable depending on whether the risk over long periods of time, i.e. many years, is persistently high with peaks related to the seasonal rainfalls or fluctuate considerably. Stable transmission correlates with a continuous exposure to a high number of infective mosquito bites per person per year, i.e. entomological inoculation rate (EIR), which in the most intense transmission settings can exceed 500, corresponding to more than one infective bite per night in average. In these areas, which prevail in most of sub-Saharan Africa and Papua New Guinea, morbidity and mortality of malaria is primarily affecting young children and pregnant women. Children who survive the continuous malaria exposure acquire partial immunity over time, initially with reduced risk of severe disease and death and at older age against

clinical disease. However, sterile immunity is normally not acquired, why a large proportion of the population residing in high endemic areas often carry malaria parasites without having clinical symptoms and thereby remain an important reservoir for transmission. In contrast, unstable malaria transmission occur when EIR is low, normally <5 , but often <1 (WHO 2006). In such areas a great variability in transmission intensity over time is observed. Populations exposed to unstable malaria, which occurs in much of Asia and Latin America where malaria is endemic, do normally not acquire protection and therefore all age groups are at risk for clinical illness as well as for severe disease manifestations and death. Areas of unstable transmission are also epidemic prone.

There are other measurements/classifications of malaria endemicity. One such classical malariometric method is based on the assessment of the proportion of children aged 2-9 years with enlarged (palpable) spleen in a population. This classification was later modified in relation to parasite prevalence found in community based surveys among children aged 2-9 years (Metselaar and Van Thiel 1959). The level of endemicity is categorized in four groups with virtually the same definition used both regarding presence of enlarged spleen and parasitaemia, i.e. hypoendemicity ($\leq 10\%$), mesoendemicity (11-50%), hyperendemicity (50-75%) and holoendemicity ($>75\%$).

Malaria transmission can also occur without the presence of the mosquito vector, i.e. vertical transmission (from mother to foetus/child during pregnancy/delivery) as well as through blood transfusion and inoculation via contaminated needles/syringes. Moreover, controlled inoculations, i.e. therapeutic malaria, were frequently used before the introduction of penicillin for the treatment of patients with neurosyphilis between 1920 and 1950. Undoubtedly, studies of therapeutic malaria have provided important knowledge on parasite biology (Glynn and Bradley 1995).

1.5 CLINICAL PRESENTATION OF THE DISEASE

Malaria literally means bad air in Italian, due to the perception that the febrile condition was associated with foul air in marshlands surrounding Rome. Descriptions of intermittent fevers can be found both in ancient Chinese and Indian medical literature. However, the first to describe the clinical picture more in detail was Hippocrates during the fifth century BC (Warrell and Gilles 2002).

Malaria is an acute febrile illness, with a minimum incubation time of 7 days for *P. falciparum*, which is accompanied by a variety of symptoms or rather combination of symptoms. Beside fever the most common complains include headache, fatigue, anorexia and muscle/joint pains. In African children with clinical malaria abdominal pains, vomiting, diarrhoea, cough and fast breathing are also frequently reported. The symptoms are often accompanied by signs of anaemia and enlarged spleen. The clinical picture is non-specific and therefore difficult to distinguish from other common causes of fever. Particularly, the overlapping clinical presentation of malaria and acute respiratory infections/pneumonia among paediatric patients in Africa represents a dilemma for medical practitioners working in the peripheral health sector without access to confirmatory laboratory diagnostics (O'Dempsey, McArdle et al. 1993; Kallander, Nsungwa-Sabiiti et al. 2004).

Pregnant women represent another important risk group for malaria related morbidity and mortality both affecting the mother and the child (Desai, ter Kuile et al. 2007). Women in high endemic areas are more at risk for asymptomatic infection resulting in maternal anaemia, placental malaria and low birth weight (Steketee, Wirima et al. 1996; Rogerson, Mwapasa et al. 2007), the latter being associated with increased infant mortality. Beside *P. falciparum*, *P. vivax* can also cause anaemia and low birth weight in pregnancy (Nosten, McGready et al. 1999). The most recommended preventive measures to reduce the pregnancy associated burden of malaria in high endemic areas are the use of long lasting insecticide treated nets and intermittent preventive treatment administered at least twice during pregnancy.

The clinical malaria syndrome can be classified as uncomplicated or complicated (severe). Uncomplicated malaria is usually defined as the absence of any symptoms/signs of severe malaria, whereas for severe malaria specific diagnostic criteria are available (WHO 2000b). These criteria include:

- Prostration
- Impaired consciousness
- Multiple convulsions
- Severe anaemia (Haemoglobin (Hb) <50 g/L)
- Hyperparasitaemia (>250 000/ μ L or >5% of red blood cells)
- Hypoglycaemia

- Respiratory distress (acidotic breathing)
- Pulmonary oedema (radiological)
- Circulatory collapse
- Abnormal bleeding
- Jaundice
- Haemoglobinuria
- Hyperlactataemia
- Acidosis

An episode of uncomplicated malaria is, if early and appropriately treated, associated with low risk of fatal outcome, i.e. circa 0.1% for *P. falciparum* (WHO 2006).

However, if initiation of treatment is delayed, the condition can deteriorate rapidly with development of severe manifestations within hours. Severe malaria carries, even if managed in hospital settings with good standard of care, a significant risk of mortality (10-20%), but if untreated the condition is almost always fatal (Waller, Krishna et al. 1995; Warrell 1999). Malaria death in high endemic areas in Africa is usually associated with severe anaemia among the youngest children (≤ 2 years), whereas cerebral malaria (unrousable coma) is a more common cause of death in slightly older African children as well as in all age groups developing severe malaria in low endemic areas. Neurological sequelae are important, but neglected causes of morbidity in African children surviving cerebral malaria with symptoms varying from cognitive impairment to hemiplegia and cortical blindness (Newton, Hien et al. 2000; Mung'Ala-Odera, Snow et al. 2004).

The severity and outcome of a clinical malaria episode is influenced by a number of different factors, such as infecting species, age (acquired immunity), genetic composition (innate immunity), underlying disorders (malnutrition, anaemia etc), co-infections (HIV, measles etc) and for women of childbearing age presence/absence of pregnancy (Marsh 1992; Skinner-Adams, McCarthy et al. 2008).

1.6 MALARIA DIAGNOSIS AND TREATMENT IN AFRICA

Early diagnosis and prompt effective treatment within 24 hours after onset of fever are cornerstones in modern malaria control to prevent progression from uncomplicated to severe malaria. However, considering that a majority of febrile children in Africa are

managed in the peripheral health sector without access to confirmatory diagnostics, malaria diagnosis is usually based on clinical criteria only. In practice this means that a child presenting with fever in sub-Saharan Africa is presumed to have malaria and treated accordingly. Clinical diagnosis based on fever or history of fever in the preceding 24 hours in children is sensitive, but has a low specificity. This results in significant over treatment, which increases the risk of development of antimalarial drug resistance, as well as an insufficient identification and treatment of other, sometimes, life threatening differential diagnoses responsible for the fever episode (Ngasala, Mubi et al. 2008; Msellem, Rotllant et al. Submitted).

Light microscopy of stained blood smears remains the gold standard for confirmatory malaria diagnosis. Microscopy is a sensitive technique with a detection limit of *circa* 50 parasites/ μ L blood under field conditions (Moody 2002). The technique is relatively inexpensive and allows both for species identification as well as for quantification of parasite load/density. However, malaria microscopy requires the presence of well trained technicians with access to functional microscopes and a workload that allows for an adequate assessment of each blood slide subjected for analysis. Moreover, an uninterrupted supply of microscopy equipment and a system for quality assurance are also needed. Therefore, reliable and efficient microscopy services are often difficult to maintain in the peripheral health care sector in Africa (Ngasala, Mubi et al. 2008).

Rapid diagnostic tests (RDTs), which are based on detection of parasite antigens, have recently been introduced as an alternative tool for confirmatory malaria diagnosis (Moody 2002; Murray, Bell et al. 2003). Unlike microscopy, RDTs are simple to perform and do not require skilled technicians. Moreover, and as the name indicates, they are rapidly performed, with a test result available within 20 minutes after blood sampling. However, the present RDTs cannot quantify the parasite load and the cost is relatively high. In addition, RDT remain positive several days after initiation of treatment, which make them unreliable for assessment of treatment outcome. Concerns regarding adherence to test results in clinical practice have also been reported from Africa (Hamer, Ndhlovu et al. 2007; Reyburn, Mbakilwa et al. 2007), but this may often also apply to microscopy based diagnosis. Still, RDTs may represent a future key intervention to improve diagnostic efficiency and to target antimalarial drug treatment to patients with confirmed malaria infection (Mubi, Janson et al. Manuscript; Msellem, Rotllant et al. Submitted).

1.7 HISTORY OF ANTIMALARIAL DRUGS

1.7.1 Traditional remedies for fever treatment

Traditional remedies for treatment of fever have been used since ancient times. Interestingly, the two most potent antimalarial drugs presently available, i.e. quinine and artemisinin-derivatives, both have their roots in traditional medicine. Quinine originates from the bark of the *Chinchona* tree, which has been used by Peruvian Indians for fever treatment for hundreds of years. During the 17th century Jesuit missionaries in South America realized its potential for malaria treatment and consequently supported its use. The drug became known as the “Jesuits Powder”. However, it was not until 1820 when the French scientists Pierre-Joseph Pelletier and Joseph Bienaimé Caventou could isolate the active alkaloids from the bark of the tree. This was followed by the set up of large scale *Chinchona* cultivations in different parts of Asia from where access to the drug could be secured. The improved availability of quinine profoundly influenced history by enabling missionaries, explorers, colonialists and militaries to live and survive in malaria endemic areas (Kitchen, Vaughn et al. 2006).

Similarly, in China, the Sweet wormwood (*Artemisia annua*), has been used for fever treatment for over a thousand years. In 1972, artemisinin (qinghaosu) was isolated from the leaves of *A. annua* by Chinese scientists (Klayman 1985). This was followed by a major undertaking initiated by Mao Zedong between 1979-82, which resulted in development of a number of artemisinin-derivatives, e.g. artesunate, artemether, arteether and dihydroartemisinin (Hien and White 1993; Efferth, Romero et al. 2008).

1.7.2 Driving forces behind antimalarial drug development

The established antimalarial pharmacopoeia is remarkably short considering the global public health magnitude of the disease. This reflects to a large extent that the pharmaceutical industry has been lacking incentives to invest in development of new drugs with questionable commercial potential, due to the blunt reason that people affected by malaria generally are poor. This obstacle for drug development has been increasingly acknowledged and recently resulted in the establishment of the Medicines for Malaria Venture Foundation (MMV) and the Drugs for Neglected Diseases Initiative (DNDi), both being non-profit public-private-partnership with the mission to

support discovery, development and delivery of new effective and affordable antimalarial drugs.

The driving force behind the development of several of the presently used antimalarial drugs has primarily been to ensure protection and treatment of soldiers in war, rather than to save lives of people living at risk in endemic areas. During the First World War shortage of quinine was imminent for countries without direct access to *Chinchona* cultivations. This prompted German researchers to try to develop new synthetic antimalarial drugs in the 1930s, e.g. chloroquine, but which eventually was only further tested in the US and made available as an antimalarial drug after the Second World War. Thereafter, American and British researchers also developed e.g. proguanil in 1944 followed by pyrimethamine in 1952. The latter was subsequently combined with sulfadoxine in a coformulated drug commonly known as Fansidar[®]. Reports of *P. falciparum* chloroquine resistance emerged in late 1950s simultaneously in South America and Southeast Asia. When American troops first encountered chloroquine resistant malaria during the Viet Nam War this spurred the US Army to develop new synthetic compounds, which resulted in the discovery of especially mefloquine and halofantrine.

Chloroquine resistance spread to East-Africa 1978, whereafter it further spread westward throughout Africa. However, despite well established evidence of chloroquine resistance, the failing drug remained as treatment of choice for several years in many parts of Africa, often due to the lack of alternative available and affordable drugs. Later sulfadoxine-pyrimethamine replaced chloroquine in most of sub-Saharan Africa. However, unlike chloroquine, which remained an efficacious antimalarial drug for decades, the therapeutic life span of sulfadoxine-pyrimethamine became short in many parts of the world with a rapid increase in *P. falciparum* resistance sometimes only a few years after its introduction. *P. falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine resulted in disastrous public health consequences in Africa, with a rapid increase in malaria associated morbidity and mortality (Trape 2001; Bjorkman and Bhattarai 2005).

1.7.3 The modern concept for antimalarial drug treatment

To improve treatment efficacy and to delay development of resistance a new concept of antimalarial drug treatment was introduced during the 1990s, i.e. artemisinin-based

combination therapy (ACT) (White, Nosten et al. 1999). The concept of ACT is based on the use of two drugs with different modes of action including an artemisinin-derivative with rapid and effective reduction of parasite bio-mass and gametocyte carriage, combined with a partner drug with longer duration of action. The hypothesis is that these two drugs together will achieve effective clinical and parasitological cure, protect each other from development of resistance and reduce the overall malaria transmission (Price, Nosten et al. 1996; White 1999). ACT is now generally accepted as treatment of choice for acute uncomplicated *P. falciparum* malaria (WHO2006). The proof of concept was first demonstrated in Southeast Asia where the addition of an artemisinin-derivative (artesunate) to mefloquine, both improved treatment outcome and halted/reversed mefloquine resistance (Nosten et al 2000). By June 2008, all except four malaria endemic countries/regions worldwide had complied with the new recommendations and adopted ACT as the first-line treatment (WHO 2008c). However, presently there are only few ACTs recommended by the WHO, i.e. artemether-lumefantrine, artesunate+ amodiaquine, artesunate+mefloquine and artesunate+sulfadoxine-pyrimethamine. The choice of ACT in a country or region depends on a number of considerations, e.g. cost, availability of a coformulation, dosing regimen as well as safety profile and level of underlying resistance to the long acting partner drug in the combination.

Mainland Tanzania and Zanzibar, where a majority of the studies included in this thesis were conducted, have chosen different policies for ACT. In November 2001, Zanzibar Ministry of Health and Social Welfare decided, as one of the first regions in Africa to replace chloroquine and sulfadoxine-pyrimethamine with artesunate+amodiaquine and artemether-lumefantrine as first and second line treatment. The new treatment policy was implemented in 2003, whereas Tanzania started to deploy artemether-lumefantrine as their new first line treatment late 2006.

1.8 OBJECTIVES OF ANTIMALARIAL DRUG TREATMENT

The primary objective of treating patients with severe malaria is to prevent death. In contrast, the primary objective of treating uncomplicated malaria is to cure/eradicate the infection, whereas secondary objectives include reduction of transmission/infectious reservoir and to prevent emergence and spread of antimalarial drug resistance (WHO 2006). The antiparasitic effect of drug treatment can be targeted

against three different stages of the life cycle in humans. The first and most important drug target is to attack the parasite within the red blood cell (blood schizontocidal drugs), which will reduce parasite density and resolve the clinical symptoms and, if an efficacious treatment is administered during long enough time, eventually eradicate the infection. The second drug target is to inhibit the pre-erythrocytic development in the liver (tissue schizontocidal drugs), which is of importance particularly in prophylaxis, and/or to destroy the hypnozoites responsible for the relapses in *P. vivax* and *P. ovale* infections (hypnozoitocidal drugs). Finally, the third drug target is to kill the sexual forms of the parasite (gametocytocidal drugs), which will reduce/block transmission.

An efficacious antimalarial treatment will not only cure the infection responsible for the clinical episode, but does also provide a period of time when reinfections are suppressed/prevented by residual blood concentrations of the drug. This so called post-treatment prophylaxis is of particular importance for children in high endemic areas, providing them an important period of clinical and haematological recovery. The duration of post-treatment prophylaxis depends on several factors, including the dose and elimination half-life of the antimalarial drug(s) used as well as the susceptibility of the reinfecting parasites (White 2008). The post-treatment prophylactic effect is also the rationale behind the strategy of intermittent preventive treatment in e.g. pregnancy. However, with increased duration of residual blood concentration of the drug, the risk of exposed parasites to select for resistance will increase.

1.9 ANTIMALARIAL DRUG RESISTANCE

WHO defines antimalarial drug resistance as the ability of the parasite to survive and/or to multiply despite the administration and absorption of a medicine given in doses equal to, or higher than, those normally recommended but within the tolerance of subject, with the caveat that the form of the drug active against the parasite must be able to gain access to the parasite or the infected red blood cell for the duration of time necessary for its normal action (WHO 1986). However, drug resistance is usually not an absolute phenomenon, rather a process, or a chain of events, where exposed parasites through genetic alterations develop increased levels of tolerance against a particular drug/group of drugs until apparent clinical treatment failure is reached. Development of tolerance is usually at the cost of parasite fitness (Hastings and Ward 2005; Sisowath, Stromberg et al. 2005; Hastings and Watkins 2006; Sisowath, Ferreira

et al. 2007). This hypothesis is strengthened by observations that withdrawal of drug exposure in an area resulted in declining prevalence of mutated parasites towards a return of “wild type” parasites (Kublin, Cortese et al. 2003).

1.9.1 Methods for assessment of antimalarial drug resistance

Antimalarial drug resistance can be assessed with three in principle different methods, i.e. *in vivo* (treatment failure in clinical trials), *in vitro* (parasite susceptibility to antimalarial drugs in cultures) or by identification of molecular markers related to drug resistance. However, all three methods have limitations in their respective assessment of antimalarial drug resistance. For example, *in vivo* parasitological treatment failures may not necessarily reflect “true resistance”. Instead they could be attributed to for example inter-individual pharmacokinetic variations influencing drug absorption, low compliance, incorrect dosing, vomiting, poor drug quality and drug interactions, which all lead to a low bio-availability of the drug (WHO 2006). Therefore, additional measurements of drug levels in clinical trials could be used as an auxiliary tool to improve the association between treatment failures and “true resistance” (White, Stepniewska et al. 2008). Moreover, results obtained from *in vivo* drug trials conducted in high endemic areas are normally influenced by the age of patients enrolled, despite the fact that people living in the same area supposedly are exposed to the same parasite population. This is due to the augmented effect of partial immunity acquired during childhood on parasite clearance after drug exposure. Conversely, *in vitro* assays eliminate the influence of immunity on assessment of drug resistance but as such do not reflect the real life situation, which may explain discordances observed with *in vivo* trials (Ringwald and Basco 1999). However, *in vitro* assays have the advantage of controlled parasite exposure to defined drug concentrations as well as the possibility of performing multiple tests of several drugs in parallel.

Recent achievements in parasite genotyping enable identification of genetic alterations of *P. falciparum* related to antimalarial drug resistance in the fully sequenced parasite genome, which comprises circa 5300 genes distributed on 14 chromosomes (Gardner, Hall et al. 2002). Some of these established genetic markers include harbouring single nucleotide polymorphism (SNPs) in the *P. falciparum* dihydrofolate reductase gene (*dhfr*) and dihydropteroate synthase gene (*dhps*), which are linked to pyrimethamine and sulfonamide resistance, respectively, whereas SNPs in the chloroquine resistance transporter gene (*crt*) and the multidrug resistance gene 1 (*mdr1*) are linked for

example to chloroquine, amodiaquine and lumefantrine tolerance/resistance (Wernsdorfer and Noedl 2003; Sisowath, Stromberg et al. 2005; Holmgren, Gil et al. 2006; Holmgren, Hamrin et al. 2007; Sisowath, Ferreira et al. 2007; Sisowath, Petersen et al. In Press). Moreover, amplifications of *mdr1* are associated with mefloquine and lumefantrine resistance as well as decreased susceptibility of artemisinin-derivatives *in vitro* (Price, Uhlemann et al. 2006), while genetic diversity in the sarco/endoplasmic reticulum Ca²⁺-ATPase orthologue of *P. falciparum* (*pfATP6*) has been proposed as a potential marker for artemisinin resistance (Jambou, Legrand et al. 2005; Golenser, Waknine et al. 2006; Krishna, Woodrow et al. 2006). These and other proposed genetic markers will probably play an important future role in global malaria control, particularly in drug resistance surveillance. However, a weakness with most established molecular markers related to drug resistance is that they do not fully determine the individual treatment outcome, with the possible exception of presence of quintuple mutant parasites (3 *dhps* mutations in codon 108, 51 and 59 plus 2 *dhfr* mutations in codon 437 and 540) in sulfadoxine-pyrimethamine treatment (Kublin, Dzinjalama et al. 2002). This probably also reflects an insufficient understanding of the suggested complex multigenetic origin of antimalarial drug resistance.

In essence the use of *in vitro* drug susceptibility tests and molecular markers should be looked upon as complementary to *in vivo* assessment of antimalarial drug resistance, considering the first two methods provide more of early warning systems of increased parasite tolerance to antimalarial drugs before clinical treatment failures are evident in high endemic areas, whereas *in vivo* testing better reflects the complex real life situation.

2 THESIS SPECIFIC BACKGROUND

2.1 CLINICAL TRIALS OF ANTIMALARIAL DRUGS

Between 1966 and December 2002 a total of 434 antimalarial drug trials, which enrolled 82616 patients, were found during a literature search by Myint *et al.* A majority of these trials involved treatment of *P. falciparum* malaria. The number of trials and average number of patients included in each study during the last 20 years appears to have steadily increased (Myint, Tipmanee et al. 2004).

Clinical trials of antimalarial drugs can be classified in two groups, i.e. efficacy and effectiveness studies. Efficacy trials are characterized by controlled intake of the drug(s) at test, i.e. all drug doses are administered under strict supervision. Conversely, in effectiveness trials drug treatment is taken at home after a standardized description of the drug regimen has been provided to the patient and/or parent(s), i.e. non-supervised drug intake. Results retrieved from studies applying these fundamentally different study designs could be looked upon as representing a “best case scenario” (efficacy trials) or a situation more mimicking real life (effectiveness trials), where the result of the latter intrinsically involves aspects of drug adherence/compliance. During the initial phases of drug development, also including regulatory trials, efficacy data are crucial to obtain, whereas during the post-registration era effectiveness data become increasingly important, since they also include aspects of community uptake of a newly introduced drug.

2.1.1 Development of the *in vivo* test

The first standardized WHO *in vivo* malaria test was developed in 1965 and has since then been revised several times (WHO 2005). The observational time after initiation of treatment has varied over the years between 7 days (“standard test”) and 28 days (“extended test”) depending on to what degree the risk of reinfections (new infections arising from infective mosquito bites) was considered important. To really fulfil the requirement of excluding reinfections the recruited patients could for example be kept in mosquito free environment during the entire follow-up period after treatment. The initially recommended 7 day follow-up was soon abolished in favour of an observational time of at least 14 days, which was believed to provide a reasonable duration for assessment of clinical and haematological response combined with a

limited risk of reinfections to emerge. In 1996 two standardized protocols were developed for assessment of clinical efficacy of antimalarial drugs in areas of high and low to moderate malaria transmission, respectively (WHO 1996). To further improve comparability of results between different transmission settings modifications of the 1996 protocol were agreed upon in 2001 (WHO 2001). This was followed by the launch of a globally standardized protocol in 2003, which is a comprehensive guideline including almost all aspects of “Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated malaria” (WHO 2003b).

However, some differences in 2003 recommendations between drug assessments conducted in high or low to medium transmission areas, respectively, remained in this protocol, e.g. follow-up duration (a minimum of 14 versus 28 days), the age criterion for enrolment (only children <5 years versus the possibility of enrolling also children above 5 years in addition to the main target population, children <5 years), the fever inclusion criterion (documented fever versus documented or history of fever in the preceding 24 hours) and the acceptable range for parasite density at screening (2000-200 000 versus 1000-100 000 parasites/ μ L).

2.1.2 *In vivo* outcome classifications

The classification of treatment outcome *in vivo* has changed considerable over the years. However, in the 2003 document a new strong emphasis was put on re-defining parasite recurrence observed after day 4 during follow-up without being accompanied by symptoms from “Adequate Clinical Response” to “Parasitological failure”. This clearly underscores that parasitological cure was considered the cardinal indicator for a successful antimalarial drug treatment worldwide.

The presently recommend four outcome categories are defined below. For definitions of severe malaria, please see section 1.5 above. General danger signs include persistent vomiting; inability to sit, stand, drink or breastfeed and/or lethargy or otherwise impaired consciousness.

Early treatment failure:

- Danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia.
- Parasite density on day 2 higher than on day 0, irrespective of axillary temperature.
- Presence of parasitaemia on day 3 with axillary temperature ≥ 37.5 °C.

- Parasite density on day 3 \geq 25% of day 0.

Late clinical failure:

- Danger signs or severe malaria in the presence of parasitaemia between day 4 and day 28, without previously meeting any criteria of early treatment failure.
- Axillary temperature \geq 37.5 °C (or history of fever) in the presence of parasitaemia between day 4 and day 28, without previously meeting any criteria of early treatment failure.

Late parasitological failure:

- Presence of parasitaemia between day 4 and day 28 with temperature $<$ 37.5 °C, without previously meeting any criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response:

- Absence of parasitaemia on day 28, irrespective of axillary temperature, without previously meeting any criteria of early treatment failure, late clinical failure or late parasitological failure.

2.1.3 Current recommendations for *in vivo* trials

The currently available recommendations derive from 2005 (WHO 2005). These recommendations interestingly also include comments from the drafting committee of “Guidelines for the treatment of malaria”, which were issued in 2006 (WHO 2006). Noteworthy is the new suggestion to instantly treat all patients with recurrent asymptomatic parasitaemia during follow-up based on observations from Tanzania and Uganda where circa 50-60% of patients developing late parasitological failure progress to clinical malaria within 28 days (Mutabingwa, Nzila et al. 2001; Njama-Meya, Kanya et al. 2004). Still, the most important message is the recommendation of a follow-up duration in antimalarial drug trials of at least 28 days, regardless of transmission setting. However, during four weeks or more after initiation of treatment in high transmission areas in Africa recurrent infections are inevitable, due to the continuous exposure to new infections through infective mosquito bites (Figure 1). Since microscopy cannot distinguish parasites that have escaped drug action (treatment failure/recrudescence) from those arising from new mosquito inoculations (new infections/reinfections), polymerase chain reaction (PCR) based genotyping of the

malaria parasite has been introduced as a tool in clinical drug trials to improve this distinction. Such PCR adjusted cure rate is now the recommended primary end point in antimalarial drug trials conducted in Africa (WHO 2006).

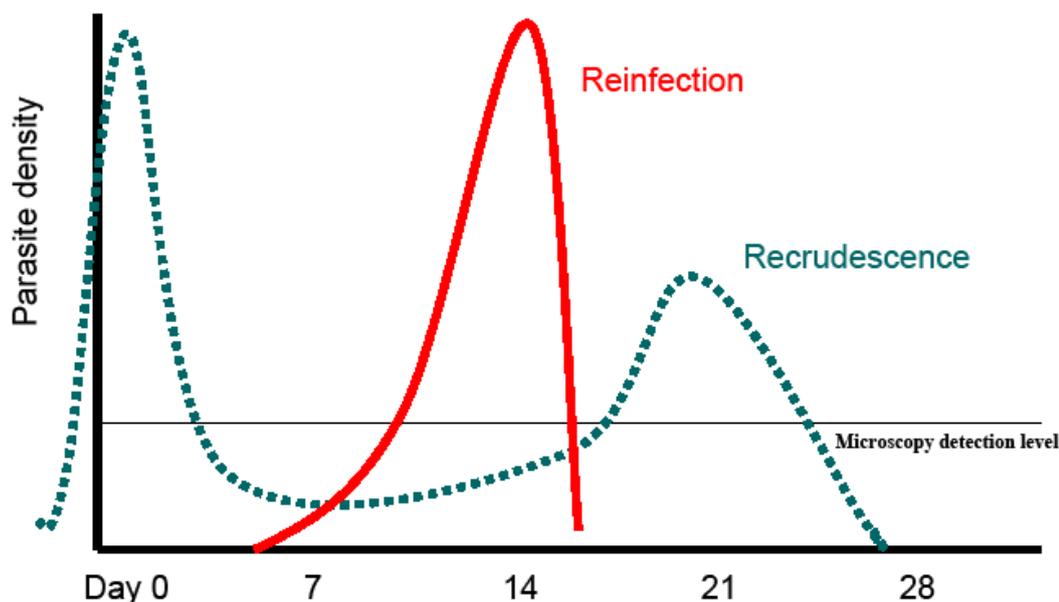


Figure2. Recurrent parasitaemia during follow-up after treatment can either represent a new infection/reinfection arising from a new infective mosquito bite or a treatment failure/recrudescence. The latter infection was in this case suppressed below microscopy detection limit by treatment given day 0-2, but not eradicated from the body. The infection reappeared above microscopy level beyond day 14 during follow-up. (The figure is adopted from a WHO power point document)

2.1.4 Analysis of *in vivo* efficacy

The efficacy outcome can be analysed with three in principle different methods; per-protocol, intention-to-treat and/or survival/life table analysis. The per-protocol approach only includes analysis of data from patients with evaluable treatment outcomes, all remaining patients are excluded from the analysis. Conversely, in the intention-to-treat approach all patients randomly allocated to treatment are included in the analysis, regardless of whether an evaluable treatment outcome is available. The purpose of this conservative approach with regards to successful treatment outcome is to retain the integrity of the randomization procedure. Survival/life table analysis allows patients lost to follow-up or withdrawn from the study to contribute to the

analysis until day of exit from study, when they are censored. Clearly, the choice of analytic method used may profoundly influence the outcome. However, the analytic method presently considered to provide the most precise estimate of treatment failure is survival analysis, which is also the recommended method by WHO (WHO 2003b; (Stepniewska and White 2006; Ashley, Pinoges et al. 2008)

2.1.5 Standard follow-up schedule for *in vivo* trials

The standard follow-up schedule with an extension to 42 days, including the timing of different key activities during an antimalarial drug trial, is outlined in Table 1.

Day of visit	0	1	2	3	7	14	21	28	35	42	Any other day
Drug treatment	+	+	+								
Medical history/ adverse events	+	+	+	+	+	+	+	+	+	+	+
Physical examination	+	+	+	+	+	+	+	+	+	+	+
Body temperature	+	+	+	+	+	+	+	+	+	+	+
Blood slide for parasite count	+		+	+	+	+	+	+	+	+	+
Haemoglobin/ haematocrit	+							+		+	(+)
Blood sample for PCR genotyping	+				+	+	+	+	+	+	> Day 7

Table 1. Extended follow-up schedule in antimalarial drug trials of ACT in Africa with the respective key activities indicated for each visit (For artesunate+amodiaquine and artesunate+sulfadoxine-pyrimethamine a follow-up duration of 28 days is recommended, whereas for artemether-lumefantrine 42 days is considered more appropriate).

2.2 PCR GENOTYPING TO DISTINGUISH RECRUDESCENCE FROM REINFECTIONS IN ANTIMALARIAL DRUG TRIALS

2.2.1 PCR genotyping

The discovery of the PCR technique is a landmark in biomedical research, which rendered the inventor Kary B. Mullis the Nobel Prize in Chemistry 1993.

The principle of PCR is based on amplification of well characterized sequences in specific genetic loci (Mullis, Faloona et al. 1986; Saiki, Gelfand et al. 1988). This is achieved by repeating a cyclic reaction several times resulting in an exponential multiplication of the targeted genetic fragment, up to millions of copies within a few hours. Each cycle, which takes only few minutes to complete, involves three major steps where rapid change in temperature plays a crucial role to enable the entire biochemical reaction to be completed:

- **Denaturation:** By rapid heating up to 94-95°C the double stranded DNA is separated to become single stranded DNA.

- **Annealing:** Denaturation is followed by a sudden temperature decrease to circa 55-58°C, which allows specifically designed primers (forward and reverse) incorporated in the reaction to bind to an exact complementary DNA sequence both upstream and downstream of the single stranded DNA fragment of interest. The enzyme (*Taq*-polymerase) can attach to the small piece of double stranded DNA, which has been formed by the primer and its complementary DNA sequence and starts copying the template.

- **Extension:** After annealing the temperature is again increased to circa 72°C which is the optimal temperature for the *Taq*-polymerase to extend the sequence by adding deoxynucleotides (dNTP) to the complementary template. However, primers that have attached to DNA without an exact match will fall off and consequently not give rise to a duplication of the fragment. After extension, the temperature is again increased to 94°C to denature the newly formed double stranded DNA, whereafter the next cycle continues.

After completion of the PCR reaction the presence and size determination of the PCR product must be confirmed. This can be done with different techniques, one of the most simple and user-friendly being gel electrophoresis. In brief an agarose gel, which has been stained with ethidium bromide to facilitate visualization of DNA, is put in an electrophoresis chamber and covered with buffer. Thereafter, the PCR product and a molecular weight standard (base pair ladder) are loaded on the gel in parallel wells. After electricity is applied the DNA product migrates towards the positive electrode, with a speed inversely proportional to its size, i.e. small fragments move faster/longer

in the gel. After adequate migration in the gel the molecular weight of the PCR product can be assessed/visualized under ultraviolet transillumination in comparison with the base pair ladder.

2.2.2 PCR genotyping of *P. falciparum*

The introduction of PCR genotyping in malaria research has paved the way for major improvements in the understanding of parasite biology. For example a number of highly diverse genetic markers of *P. falciparum* have been identified. These markers have been extensively studied and characterized as potential vaccine candidates. The most commonly used markers for genotyping of *P. falciparum* are surface antigens, e.g. the *merozoite surface protein 1 (msp1)* and *2 msp2*, and the *glutamate-rich protein (glurp)* (Snounou and Beck 1998).

The merozoite surface protein 1 (MSP1) is a 200 kDa polypeptide and MSP2 a 28 kDa glycoprotein, which both are expressed on the merozoite surface. Their respective genes are located on chromosome 9 and 2. The *msp1* gene consists of 17 blocks, of which 7 show different degrees of diversity, whereas *msp2* consists of 5 blocks (Tanabe, Mackay et al. 1987; Smythe, Coppel et al. 1991). Block 2 of *msp1* and block 3 of *msp2*, which both are flanked by conserved regions, are associated with the highest polymorphism in the respective genes and therefore normally targeted for genotyping. The *msp1* can be divided in three allelic types/families, i.e. K1, MAD 20 and R033, and *msp2* in two allelic families, i.e. FC27 and 3D7/IC. The glutamate rich protein (GLURP) is a 220 kDa polypeptide, which is expressed during all stages in the life cycle in humans and released during the rupture of schizonts (Borre, Dziegiel et al. 1991; Hogg, Thompson et al. 1993). The gene is located on chromosome 10 and consists of two repeat regions (RI and RII) and appears only as one allelic type/family. The RII region is most diverse and therefore normally targeted for genotyping.

These three genetic markers are unlinked, i.e. located on different chromosomes, single copy genes with extensive polymorphism, both with regards to sequence and size, which is mostly generated by intragenic repeats that are variable in copy number and length of the repeat unit (Farnert, Arez et al. 2001). These features make them attractive candidates for studies where identification and enumeration of genetically distinct *P. falciparum* parasite sub-populations are of interest. As such they have proven to be

useful tools in a number of molecular epidemiological studies conducted in different epidemiological settings.

Several other genetic markers have also been used for the purpose of *P. falciparum* genotyping, e.g. the *circumsporozoite protein (csp)* and microsatellites (Ohrt, Mirabelli-Primdahl et al. 1997; Nyachieo, C et al. 2005; Mwangi, Omar et al. 2006; Kanya, Yeka et al. 2007). Of interest from the clinical trial perspective, a combination of different microsatellites used alone or in addition to conventional markers appears to be suitable alternatives for distinguishing recurrent infections as recrudescences or reinfections.

2.2.2.1 Nested PCR genotyping

One of the most commonly used techniques for genotyping of *P. falciparum* malaria is by means of nested PCR. This approach involves an initial PCR amplification of the outer region of the targeted genetic sequence, followed by a second amplification with family specific primers. The use of nested PCR increases sensitivity of the reaction and is therefore essential for detection of genotypes present in minute concentrations.

The nested PCR products from *P. falciparum* genotyping of *msp1*, *msp2* and *glurp*, which vary in size between circa 150-1200 base pair, are usually distinguished from each other based on size polymorphism after separation on agarose gel by electrophoresis (see description above). A weakness with this technique is the relatively low discriminatory power/resolution. This is particularly cumbersome with large PCR fragments, where small size differences may not be possible to detect with gel electrophoresis. A way to improve the discriminatory power is to exchange agarose gels with polyacrylamide gels. Furthermore, the use of digitalized software programs can improve assessment of fragment size.

2.2.2.2 Other genotyping techniques

Restriction fragment length polymorphism (RFLP) use a restriction enzyme (*Hinf I*) to digest the nested PCR products, whereafter the fragments are separated on high resolution gels. The RFLP technique has been developed for the *msp2* marker, which has proven to be useful for improved discrimination between fragments with small differences in size (Felger, Irion et al. 1999).

Fragment sizing by capillary electrophoresis performed in an automated DNA sequencer is another method that has been used for *m*sp2 genotyping (Jafari, Le Bras et al. 2004; Falk, Maire et al. 2006). This is a highly discriminatory method, which allows detection of size differences down to single base pairs.

Several other interesting techniques have been used for genotyping *P. falciparum*. e.g. the heteroduplex tracking assay of *m*sp1 and *m*sp2, which is a quantitative method with a high sensitivity using radioisotopes (Kwiek, Alker et al. 2007) and microsatellite typing followed by fragment sizing by capillary electrophoresis (Greenhouse, Myrick et al. 2006).

2.2.3 PCR genotyping of *P. falciparum* in clinical drug trials

PCR adjustment of efficacy rates is presently achieved based on analysis from two blood samples, one collected on day of enrolment and the other on initial day of microscopy confirmed recurrent parasitaemia during follow-up. However, until recently PCR adjustment has been hampered by lack of consensus on how to use and interpret the retrieved results. For instance different groups have incorporated different number and combination of genetic markers in their analysis and even definitions of recrudescence and reinfection from given PCR results have been inconsistent (Cattamanchi, Kyabayinze et al. 2003; Collins, Greenhouse et al. 2006). This is probably a reflection of an insufficient scientific evaluation and standardization of the method, which to a large extent preclude comparability of result from different clinical trials. This is well exemplified by a review of 116 antimalarial drug trials conducted in endemic areas between 1997 and 2007, where *m*sp1 was used in 78%, *m*sp2 in 97% and *glurp* in 53% of the studies. Furthermore, *m*sp2 was used alone in 19%, whereas a combination of two (*m*sp1 and *m*sp2) or three markers (*m*sp1, *m*sp2 and *glurp*) were used in 25 and 53%, respectively (MMV/WHO 2008). (Please note that from the referenced document it is not clear which combination the remaining 3% (100-19-25-53=3%) of the studies used.)

PCR technique is in theory simple and straight forward but in real life situations, i.e. when used to distinguish recrudescence from reinfections in clinical trials of antimalarial drugs, the technique is well known to be associated with potential pitfalls/limitations:

- PCR products with identical size may differ in DNA sequence, which is an inherent limitation of a technique based solely on size polymorphism.
- The discriminatory power (see above) of size determination is a limitation, particularly with regards to distinguishing small size differences with gel electrophoresis among large PCR products.
- A particular problem is the non-ability to amplify minority clones in complex infections, a common feature in children residing in high-transmission areas in Africa, due to template competition.
- A positive PCR reaction may represent presence of gametocytes rather than asexual parasitaemia. This means that remaining gametocytes from a previous and successfully treated infection may falsely be identified as a recrudescence.
- A positive PCR may not necessarily reflect presence of living parasites. Instead it could be due to remaining DNA debris from previously killed parasites.
- Another key issue for PCR adjustment is the constraints imposed by the biology of the *P. falciparum* parasite, with sequestration in the deep vascular system during the later part of the erythrocytic life cycle, when they may not be detectable in capillary blood samples (see also below section 2.3)
- The use of one or a combination of genetic markers may substantially change the interpretation of PCR adjusted cure, with lower rates retrieved with a single marker and higher rates retrieved with multiple markers (Mugittu, Priotto et al. 2007).
- Uncertain/non-conclusive PCR outcomes will always appear in major clinical trials. This could be due either to missing filter paper, either at enrolment or at recurrent parasitaemia, or absence of amplified DNA (negative PCR). An uncertain PCR result could either represent a recrudescence or a reinfection. Depending on the fraction and handling of uncertain PCR outcome in the analysis this may substantially influence the assessment of the drug(s) at test.

Moreover, the accuracy of PCR-adjusted results in antimalarial drug trials do not only rely on the PCR technique *per se*, but on a chain of different activities preceding the DNA amplification and separation of PCR products, all being associated with potential risk of influencing the final PCR outcome and thus the interpretation of the efficacy of the drugs at test.

- Blood sampling and storage has previously been described to influence PCR outcome, with whole blood samples frozen immediately after collection being superior with regards to sensitivity of PCR as compared with blood samples collected on untreated filter paper and stored in room temperature (Farnert, Arez et al. 1999). Still, the possibility to extract and amplify parasite DNA from minute blood volumes (few drops of blood) collected from a capillary finger prick and stored on filter paper has both practical and logistical advantages, which makes filter paper attractive for storage of blood in antimalarial drug trials conducted in remote areas. However, recently commercial filter paper collection cards, e.g. FTA (Whatman), have been introduced in clinical drug trials for blood collection. The cost of these cards is relatively high but they appear to improve PCR sensitivity compared with untreated filter papers (MMV/WHO 2008).
- Duration of storage and climatic condition during storage of filter paper have also been shown to influence the PCR outcome, with long duration of storage in tropical climate being associated with a 10-100 fold loss in PCR sensitivity (Farnert, Arez et al. 1999). Major clinical trials of antimalarial drugs usually take long to conduct in the field, why genotyping often is done several months after the first blood sample has been collected, which may hamper the accuracy of PCR adjusted efficacy.
- The DNA extraction method used may influence the final PCR outcome, since different extraction methods may differ in the ability to provide a pure high quality DNA template to be incorporated into the PCR reaction. Moreover, no DNA extraction method can fully compensate for insufficient/low quality of biological material.

2.2.4 Standardization of PCR genotyping in antimalarial drug trials

The need of standardization of PCR genotyping in antimalarial drug trials has been long identified. Therefore, the MMV and WHO cosponsored an expert meeting held in Amsterdam 29-31 May, 2007, with the aim of achieving consensus regarding the use of parasite genotyping in antimalarial drug trials. The report from this meeting, which has recently been published, represents a crucial step forward to improve comparability of PCR adjusted cure rates between different studies (MMV/WHO 2008). The remaining part of this paragraph is a résumé of the key recommendations from the meeting with regards to *P. falciparum* genotyping.

- Blood samples for genotyping should be collected regularly on days 0, 7, 14, 21, 28 and any other day of fever.
- The use of commercial blood filter paper collection cards is recommended, which is mandatory for all regulatory trials, and consequently untreated filter papers should be avoided.
- The methods used for sampling, storage and transit should be described in the trial report.
- To allow genotype analysis to distinguish recrudescence from reinfection one blood sample should be collected immediately before initiation of treatment on day of enrolment (Day 0) and another on the first day of parasite recurrence (Day X) from day 7 and onwards during follow up after a successful initial parasite clearance. These two blood samples should be analysed with PCR genotyping including three polymorphic markers, i.e. *msp1*, *msp2* and *glurp*.
- The outcome from sequential analysis, starting either with *msp2* or *glurp* and end with *msp1*, should be the primary end point in antimalarial drug trials. As soon as a recurrent infection is defined as new infection the genotyping should

be stopped, whereas the other (recrudescences and non-conclusive PCRs) are carried forward to analysis with the next marker.

- The genotyping technique used for each marker should be the most discriminatory technique available. Capillary electrophoresis is recommended, but if not available nested PCR genotyping should be performed with family specific primers for *msh1* and *msh2*. For the analysis of *msh2* RFLP is an option to be used in order to increase discriminatory power.

- If agarose gel electrophoresis is used for size determination analysis should either include the use of software on digitalized images, or independent ocular analysis by two readers.

- A “recrudescence” is defined as at least one common/matching allele/genotype detectable in the blood sample collected on Day 0 and Day X in all three genetic markers. Conversely, in a “new infection” all alleles/genotypes detected on Day 0 and Day X are different for one or more of the genetic markers analysed. Illustration of different genotype pattern is illustrated in Figure 2.

- If PCR cure rates are below 90% it is recommended that additional information are provided to assess the likelihood of misclassifications of new infections as recrudescences. Such information should include: mean multiplicity of infection determined from 50 randomly selected Day 0 samples, allelic frequency of the dominant genotype and presence of gametocytaemia on day of failure.

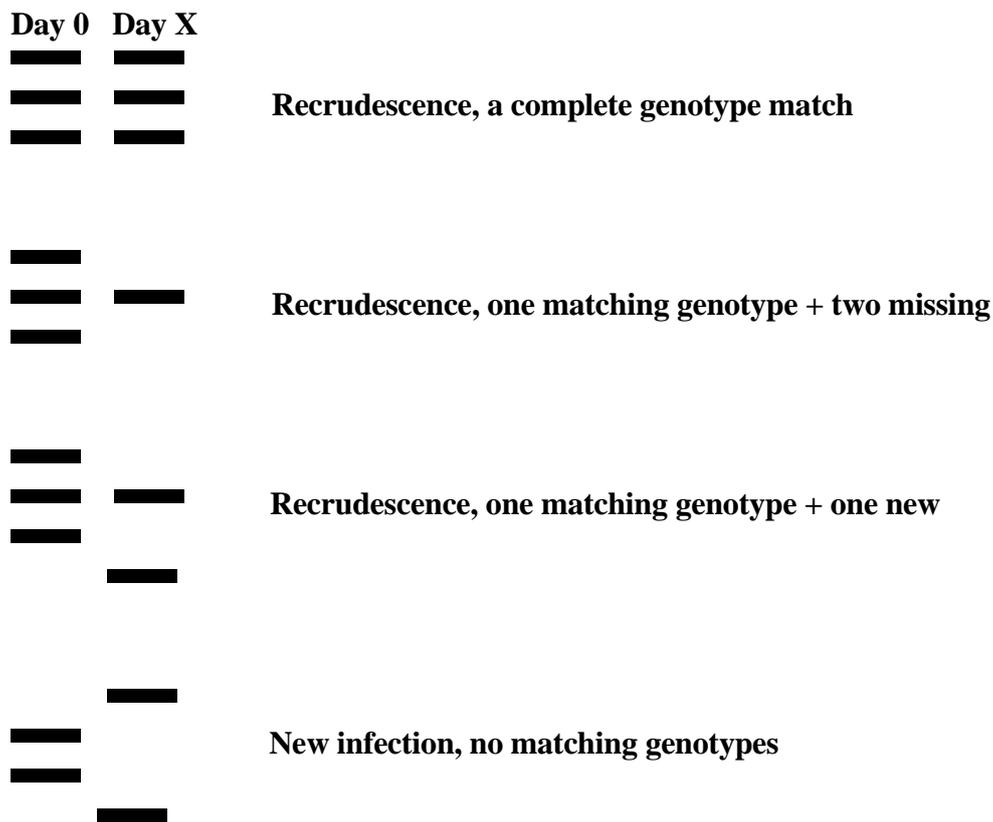


Figure 3. Illustration of different genotype pattern on day of enrollment (Day 0) and initial day of parasite recurrence (Day X) during follow up in one genetic marker. The outcome classification of recrudescence and new infection/reinfection is according to recently described consensus definition (The illustration is adopted from MMV/WHO 2008)

2.3 PARASITE POPULATION DYNAMICS IN CLINICAL MALARIA

Molecular epidemiological studies have demonstrated that children residing in high endemic areas in Africa often harbour complex asymptomatic *P. falciparum* infections with a mixture of genetically distinct *P. falciparum* parasite sub-populations (Beck, Felger et al. 1997; Berezky, Liljander et al. 2007). These asymptomatic infections are characterized by extensive dynamics in detection of the individual parasite genotypes with PCR technique over time, often following a 48 hour pattern (Farnert, Snounou et al. 1997), but sometimes appearing and reappearing within hours (Jafari-Guemouri, Boudin et al. 2006). However, to date only few studies have addressed the importance and magnitude of a potential similar phenomenon in children with acute uncomplicated malaria in high transmission areas.

In adult travellers returning from endemic areas with clinical malaria Färnert *et al* reported a consistent genotype pattern during consecutive day blood sampling (Farnert

and Bjorkman 2005), whereas Jafari *et al* detected different genotypes at different time points using a quantitative method (Jafari, Le Bras et al. 2004). In Gabonese children treated with intravenous quinine the same genotype pattern was observed during the infection, but in some patients alleles disappeared and reappeared over time (Missinou, Kun et al. 2004). However, the complexity of infection in this small cohort was relatively low with 7/17 (41%) children harbouring multiclonal infections as assessed with the *mSP2* marker only. More recently, a cluster of *P. falciparum* patients was detected in United Kingdom among travellers returning from Africa. In 1 out of 6 patients a different genotype pattern was observed in two consecutive blood samples collected just prior to initiation of quinine treatment (Day 0) and the day after (Day 1), both with regards to *mSP1* and *mSP2* analysis (Rodger, Cooke et al. 2008). In a further Ugandan study where *P. falciparum* parasites from 98 children with malaria were cultured over nine days the authors observed an appearance of new genotypes after day 0 in 20 cultures as assessed with *mSP2* genotyping. A majority of the new genotypes were detected on day 1 (Nsobya, Kiggundu et al. 2008).

These results indicate an at least low to moderate level of parasite population dynamics in detection of *P. falciparum* sub-populations in clinical malaria, even though it generally appears that the dynamics in asymptomatic infections are considerably more extensive (Farnert 2008). However, to what extent these observations from clinical malaria may have implications for assessment of PCR adjusted efficacy of antimalarial drugs in African children remains debatable (MMV/WHO 2008). Particularly, from the clinical trial perspective, studies assessing PCR adjusted cure rates from consecutive day samples collected both at enrolment and at parasite recurrence during follow-up, are critically needed to allow evidence based decisions. This question was initially identified a research priority by the WHO in 2003 and has since then been reiterated in 2005 and 2008 (WHO 2003b; WHO 2005; MMV/WHO 2008).

2.4 ANTIMALARIAL DRUGS USED IN THE THESIS

2.4.1 Artemisinin-derivatives

The artemisinin-derivatives are the most potent schizontocidal drugs presently known, with a parasite reduction ratio of 10^4 each 48 hour erythrocytic cycle (White 1997). A reason for their rapid action is that they are active against all parasite stages in the red

blood cell (Skinner, Manning et al. 1996). They are also gametocytocidal, but have no effective against the pre-erythrocytic tissue phase. The mechanism of action is not completely understood, but all derivatives appears to inhibit an essential sarco/endoplasmic reticulum Ca^{2+} -ATPase orthologue of *P. falciparum* (*pfATP6*) (Eckstein-Ludwig, Webb et al. 2003). Artemisinin-derivatives are remarkably safe and well tolerated (Price, van Vugt et al. 1999; Eckstein-Ludwig, Webb et al. 2003; Ashley and White 2005). However, concerns have been raised regarding neurotoxicity as well as foetal abnormalities and death in early pregnancy observed in animal studies (WHO 2003b), but this has never been reproduced in humans with the exception for one retrospective case-control study suggesting an association of audiometric changes after artemether-lumefantrine treatment (Toovey and Jamieson 2004). This finding has not been confirmed in later conducted prospective studies (McCall, Beynon et al. 2006; Gurkov, Eshetu et al. 2008). Monotherapy with artemisinins is associated with autoinduction of its own metabolism, necessitating an at least 7 day treatment course to minimize recrudescence rate (Hassan Alin, Ashton et al. 1996).

Artemisinins are recommended for treatment both of severe malaria with intravenous artesunate, intramuscular artemether, or as a pre-referral rectal administration of artemisinin-based suppositories, as well as for uncomplicated malaria where derivatives such as artesunate and artemether play key roles as rapid acting components in ACT (Nosten, van Vugt et al. 1998; Checkley and Whitty 2007; Gomes, Ribeiro et al. 2008).

2.4.2 Artemisinin-based combination therapies

Thousands of children with uncomplicated malaria have been enrolled in clinical trials of ACTs in Africa. By adding treatment with an artemisinin-derivative to a longer acting partner drug during three days, the artemisinin acts over two asexual erythrocytic cycles (2 x 48 hours) and thereby ensures rapid and substantial reduction of the parasite load, leaving a residual parasite burden of maximum of 10^5 parasites to be killed off by the partner drug during subsequent cycles (White 1997). Such addition of three days artesunate treatment to standard antimalarial drugs reduced treatment failures and gametocyte carriage significantly in a meta-analysis (Adjuik, Babiker et al. 2004). This has been followed by several reports comparing different ACTs, which generally have proven them to be safe and efficacious (Mutabingwa, Anthony et al. 2005; van den Broek, Kitz et al. 2006; Faye, Ndiaye et al. 2007; Nosten and White 2007; Adjei, Kurtzhals et al. 2008; Falade, Ogundele et al. 2008). Presently, two main ACT

candidates for wide scale use in Africa are available: artesunate+amodiaquine and artemether-lumefantrine. Unfortunately, little attention has been given to development of specific paediatric formulation developed to facilitate administration of ACT to small children is presently registered. Instead, conventional tablets are normally crushed and dissolved in water before being given to children. This exposes the often bitter taste of the long acting partner drugs, particularly amodiaquine and lumefantrine, which hampers drug administration in children. However, recently DNDi and MMV have been involved in development of soluble/dispersible formulations of artesunate+amodiaquine and artemether-lumefantrine together with Sanofi-aventis and Novartis Pharma, respectively.

2.4.2.1 Artesunate+amodiaquine

Artesunate+amodiaquine is currently available both in a loose combination or as co-blister packaged tablets. Recently a coformulation has been registered (Coarsucam[®]), which in October 2008 became the first fixed-dose combination with a soluble formulation, specifically designed for children, to be granted a pre-qualified status by the WHO.

Artesunate is a water soluble semi-synthetic artemisinin derivative. It was developed during the 1970s in China. It is rapidly absorbed. Peak plasma concentration occurs after circa 90 minutes after oral administration and almost all is converted to the active metabolite dihydroartemisinin, which has a rapid elimination with a terminal half-life of approximately 45 minutes (Navaratnam, Mansor et al. 2000).

Amodiaquine is a 4-aminoquinoline, which resembles chloroquine both structurally and by mechanism of action. It is readily absorbed and metabolized into desethyl-amodiaquine, which appears to have a terminal half-life of circa 3-12 days in African children with uncomplicated malaria (Hietala, Bhattarai et al. 2007). Peak plasma concentration is reached after circa 4 hours. The drug accumulates in the parasite food vacuole and acts by interfering with parasite haem detoxification. The drug has schizontocidal effect, but has neither effect against tissue schizogony nor on hypnozoites. Despite, some degree of cross-resistance with chloroquine, amodiaquine has the advantages of being more palatable and thereby easier to administer to children (Olliaro, Nevill et al. 1996).

Amodiaquine has a complex history as an antimalarial drug. It was synthesized in the 1940s and has thereafter been widely used for treatment of uncomplicated malaria. However, in the mid-1980s reports emerged of fatal adverse events, i.e. agranulocytosis and toxic hepatitis, in travellers using the drug as prophylaxis (Neftel, Woodtly et al. 1986). Estimations suggest that the risk of developing agranulocytosis following amodiaquine prophylaxis was in a magnitude of 1/2000 (Hatton, Peto et al. 1986). These reports prompted WHO to remove amodiaquine from the essential drug list in 1988. Following a systematic review which concluded that amodiaquine is superior to chloroquine for treatment of uncomplicated malaria together with a similar safety profile as chloroquine and sulfadoxine-pyrimethamine, WHO reinstated amodiaquine for treatment of *P. falciparum* malaria in 1996 (Olliaro, Nevill et al. 1996).

Artesunate+amodiaquine has been the choice of many African malaria control programmes as new first line treatment (Bosman and Mendis 2007). However, a concern with this ACT is that amodiaquine has been used as monotherapy prior to the implementation of the combination in many parts of Africa. This is a factor that could compromise its therapeutic life span due to underlying low grade resistance. Therefore, it is important with close surveillance of efficacy and pharmacovigilance of the combination. Another limitation with this ACT is that up to recently no coformulation has been available, which may have resulted in some degree of monotherapy with either of the drugs in areas where the loose or co-blistered combinations have been deployed.

A standard treatment with artesunate+amodiaquine is administered once daily for three days. The target dose of artesunate is 4mg/kg/day and for amodiaquine 10mg/kg/day.

2.4.2.2 Artemether-lumefantrine

Artemether-lumefantrine is a fixed drug combination being pre-qualified by WHO. The drug was jointly developed by the Academy Medical Sciences, Beijing, China and Novartis Pharma (Wernsdorfer 2004). Each tablet contains 20 mg artemether and 120 mg lumefantrine.

Artemether is, similarly with artesunate, a semisynthetic derivative of artemisinin developed during the 1970s in China, but unlike artesunate, it is highly lipophilic. Artemether is rapidly absorbed and metabolized to the active metabolite

dihydroartemisinin following oral intake. Peak plasma concentrations occur after 2-3 hours. Some 95% of the drug is plasma bound. Elimination is rapid, with a terminal elimination half-life of circa 1 hour.

Lumefantrine, formally known as benflumetol, belongs to the aryl-aminoalcohol group of antimalarial drugs, which also includes mefloquine, halofantrine and quinine. It was developed in China, but first mentioned in the scientific literature as recently as 1990 (Wernsdorfer 2004). It is schizontocidal, but has no effect on gametocytogenesis or liver schizogony. Drug absorption is highly variable, particularly during acute illness but improves during recovery. Absorption can be significantly augmented by intake of a normal meal/fatty food (Ezzet, van Vugt et al. 2000; Ashley, Stepniewska et al. 2007). Plasma peak concentration is reached circa 10 hours after administration. The terminal elimination half-life is 4-5 days (Ezzet, Mull et al. 1998). Lumefantrine has despite its structural similarities with halofantrine not been associated with cardiotoxicity (van Vugt, Ezzet et al. 1999).

In initial clinical trials of artemether-lumefantrine conducted in areas with multidrug resistant *P. falciparum* malarial, a four dose regimen given over two days was associated with insufficient cure rates, why the treatment was extended to 6 doses resulting in much improved treatment outcome (van Vugt, Brockman et al. 1998; van Vugt, Looareesuwan et al. 2000; Lefevre, Looareesuwan et al. 2001; Hutagalung, Paiphun et al. 2005). The standard treatment course of artemether-lumefantrine is administered in 6 doses over three days, i.e. at 0, 8, 24, 36, 48 and 60 hours after initiation of treatment, with children <15 kg receiving 1 tablet/dose, 15-24 kg 2 tablets/dose and 25-34 kg 3 tablets/dose.

Artemether-lumefantrine has been introduced as first line treatment for uncomplicated malaria in several countries of sub-Saharan Africa (Bosman and Mendis 2007). Artemether-lumefantrine had the advantage over other ACTs by being a fixed-drug combination, where the long acting partner drug previously has not been used as monotherapy. The combination has proven to be highly efficacious by day 28 in several clinical trials (Falade, Makanga et al. 2005; Koram, Abuaku et al. 2005; Dorsey, Staedke et al. 2007; Zongo, Dorsey et al. 2007). Interestingly, a day 7 lumefantrine cut-off plasma concentration of 280 ng/ml has been shown to correlate with treatment outcome in Thailand (Ezzet, van Vugt et al. 2000). Effectiveness trials conducted in

Africa with 28 days follow-up has given similar high cure rates as efficacy trials, but notably with significantly lower levels of plasma lumefantrine concentrations on day 7 (Piola, Fogg et al. 2005).

A paediatric formulation of artemether-lumefantrine has been developed by Novartis Pharma. It is a dispersible tablet with cherry flavour. Drug development has reached regulatory Phase III studies.

2.4.3 Sulfadoxine-pyrimethamine

Sulfadoxine-pyrimethamine is a co-formulated drug, each tablet containing 500 mg sulfadoxine and 25 mg pyrimethamine. It has the great advantage of being administered as a single dose treatment. It is a blood schizontocidal drug. Despite the fact that the drug consists of two different compounds, sulfadoxine-pyrimethamine is not per definition a combination therapy, since the compounds do not have unrelated mechanisms of action. Instead they act synergistically as anti-folates, sulfadoxine by inhibiting the enzyme dihydropteroate synthase and pyrimethamine by inhibiting dihydrofolate reductase. As previously described, genetic alterations in these particular genes are related to parasite resistance to the drugs.

Both compounds are relatively well absorbed after oral intake. Around 90% of the drugs are protein bound. In a recent pharmacokinetic study median peak plasma concentration occurred after circa 24 and 9 hours for sulfadoxine and pyrimethamine, whereas the median terminal half-life was 6.7 and 3.2 days, respectively (Barnes, Little et al. 2006). The drug is relatively well tolerated when used for malaria treatment. However, infrequent, but serious adverse events including severe skin reactions as well as haematological and hepatic toxicity do occur. Their incidences when used for malaria treatment are not known in detail, but generally appear to be less frequent than during prophylaxis (Taylor and White 2004). Sulfadoxine-pyrimethamine is administered according to bodyweight in standard doses. For children weighing 5-10 kg, 11-20 kg and 21-30 kg a single dose of ½ tablet (250 mg/12.5 mg), 1 tablet (500 mg/25 mg) and 1 ½ tablet (750 mg/37.5 mg) is given, respectively.

The therapeutic life span of sulfadoxine-pyrimethamine in *P. falciparum* infections has unfortunately been short in many places. However, two important indications remain, i.e. for intermittent preventive treatment in e.g. pregnancy and as the long acting partner

drug in combination with artesunate in areas with low grade underlying sulfadoxine-pyrimethamine resistance.

2.5 POLICY FOR INTRODUCTION AND CHANGE/REVIEW OF ANTIMALARIAL DRUG TREATMENT

Currently WHO recommends that a new antimalarial treatment should have an average cure rate of $\geq 95\%$ as assessed in clinical trials before its implementation, whereas a review/change of treatment policy should be initiated when the efficacy of a recommended treatment falls below 90% (WHO, 2006). This is a major change towards more stringent and ambitious criteria for policy decisions on antimalarial drugs from previous recommendations, where a cut-off point of $\geq 25\%$ of total failures or $\geq 15\%$ of clinical failures were recommended for policy change (WHO 2003b).

2.6 RATIONALE FOR THE THESIS

Policymakers in Africa rely primarily on results from *in vivo* trials of antimalarial drugs for treatment recommendations. Accurate results from efficacy trials therefore represent a prerequisite to ensure effective and lifesaving treatment for malaria sick children.

The introduction of ACT, a novel and extensively more expensive treatment strategy than both chloroquine and sulfadoxine-pyrimethamine, puts a heavy extra burden on malaria control programmes and their drug resistance surveillance systems in Africa. This further emphasizes the need for optimized monitoring of efficacy of antimalarial drugs used for treatment of acute uncomplicated *P. falciparum* malaria in Africa. This thesis primarily addresses two fundamental methodological aspects of the assessment of PCR adjusted efficacy, i.e. the influence of extended follow-up beyond 28 days and consecutive day blood sampling.

2.7 METHODOLOGICAL RESEARCH QUESTIONS

2.7.1 Influence of extended follow-up beyond 28 days

The length of follow-up after treatment is critical for assessment of efficacy of antimalarial drugs. The follow-up duration should preferably be tailored to correspond

to the time needed to completely clear the drug at test and its active metabolites from the blood, i.e. clearance time. Clearance of a drug is usually estimated as 6 times the elimination half-life (WHO 2003). Based on estimations of clearance of the long acting partner drug in ACT, it has been suggested that an appropriate follow-up is 28 days after artesunate+amodiaquine and sulfadoxine-pyrimethamine treatment, 42 days after artemether-lumefantrine and 63 days after artesunate+mefloquine treatment (WHO 2003; (Ringwald 2004). However, an extension of follow-up both increases cost of the trial as well as reduces the willingness to participate and to complete the stipulated follow-up. Moreover, it results in a significant number of patients experiencing recurrent parasitaemia during follow-up, necessitating PCR genotyping to distinguish treatment failure from new infections in high endemic areas. These factors need to be carefully assessed and weighted against the possibility of establishing a more accurate estimation of drug efficacy.

A recent meta-analysis from several randomised clinical trials conducted in settings with low to medium malaria transmission showed that assessment day 28 missed 15% of total failures identified by day 42 (Stepniewska, Taylor et al. 2004). However, the impact of extended follow-up beyond 28 days on PCR adjusted efficacy rates of ACT in high transmission areas in Africa, where partial immunity influences treatment outcome, has previously not been thoroughly evaluated.

2.7.2 Influence of consecutive day blood sampling

PCR analysis from a single blood sample may not provide a complete picture of all parasite sub-populations present within a malaria infection (Farnert, Snounou et al. 1997). Hence, apparently new genotypes in a follow-up sample may have been missed in a previous blood sample from the same individual. In the present WHO standard protocol for assessment of antimalarial drug efficacy paired single day blood samples, collected from day of enrolment and initial day of recurrent parasitaemia (1+1 day), are analysed with PCR to distinguish recrudescence from reinfections. However, the influence of PCR genotyping from blood samples collected on consecutive days both at enrolment and parasite recurrence during follow-up in antimalarial drug trials needs to be elucidated (WHO 2003b, MMV/WHO 2008).

2.8 OBJECTIVES AND AIMS OF THE THESIS

2.8.1 Overall objective

- To evaluate the WHO standard protocol used for assessment of PCR adjusted efficacy of antimalarial drugs in Africa with regards to methodological aspects, i.e. consecutive day blood sampling and length of follow-up.

2.8.2 Specific aims

- To study to influence of extended follow-up from 28 to 42 days in clinical trials of ACTs in Africa on assessment of PCR adjusted cure rates.
- To study the need for blood sampling on consecutive days, both at study inclusion and at parasite recurrence during follow-up to improve PCR adjustment of cure rates.
- To study *P. falciparum* parasite population diversity and dynamics during the early phase of drug treatment in children with uncomplicated malaria in a high transmission setting.
- To study the PCR adjusted efficacy of two major ACT candidates for the treatment of acute uncomplicated childhood *P. falciparum* malaria in Africa.
- To study the efficacy and safety of a new dispersible formulation of artemether-lumefantrine in children with acute uncomplicated malaria.
- To assess the influence of different DNA extractions methods on PCR outcome from blood samples collected on filter paper.

3 MATERIALS AND METHODS

3.1 STUDY SITES AND POPULATION

Three of the in total four antimalarial drug trials included in this thesis were conducted solely in East Africa, i.e. Zanzibar or mainland Tanzania, whereas the remaining study was a multicentre trial comprising 8 sites in 5 sub-Saharan African countries.

Study participants were, with the exception of 15 people from Mali providing blood samples to Study III (see below), recruited among children presenting at the respective study sites with symptoms and signs compatible with uncomplicated clinical malaria and screened for eligibility. If they fulfilled the stipulated inclusion, but none of the exclusion criteria they and their parents/guardians were asked to participate in the study.

3.1.1 Study sites in Zanzibar and mainland Tanzania (Study I, IV and V)

Study I was conducted in Zanzibar, which is situated just off the coast of mainland Tanzania. Two study sites were used in this trial, i.e. Kivunge Hospital, Unguja Island and Micheweni Hospital, Pemba Island. Both hospitals are located in densely populated rural areas. The study was conducted between November 2002 and February 2003, i.e. prior to implementation of ACT in Zanzibar. At the time of study the government supplied chloroquine and sulfadoxine-pyrimethamine to the study sites. Other antimalarial drugs, but no ACTs, were available in the private sector.

Study IV and V were conducted in Fukayosi Primary Health Care Centre, which is located in a relatively scarcely populated rural area in Bagamoyo District, Tanzania. Study IV and V were conducted in April–July 2004 and June 2006, respectively, when sulfadoxine-pyrimethamine and amodiaquine were still deployed as first- and second line treatments. No artemisinin-containing therapies were available through the government health care services in the district during the conduct of the trials

In the three mentioned study sites *P. falciparum* is the predominant malaria species and *A. gambiae* complex the main vector. Malaria transmission is perennial, with peaks related to the seasonal rainfalls in March-May and October-December. Malaria transmission prior to the conduct of the respective trials has been reported to be high.

However, precise data on parasite prevalence and EIR during the conduct of the respective trials are not available, which makes it difficult to characterize the malaria transmission intensity.

3.1.2 Study sites in the multicentre trial (Study II)

This study was conducted between August 2006 and March 2007 in 8 health care facilities in 5 malaria endemic countries namely Benin (1), Kenya (3), Mali (1), Mozambique (1), mainland Tanzania (1) and Tanzania/Zanzibar (1). The trial centres were, with the exception of one Kenyan site, located in rural and periurban areas, representing a variety of different malaria epidemiological settings. The Zanzibar part of this study was conducted in Kivunge Hospital.

3.1.3 Study sites in the DNA extraction study (Study III)

Study III included a selection of 15 blood samples collected during Study I. The remaining 15 blood samples were selected among a group of people participating in a cross-sectional survey conducted in a mesoendemic area of Mali in 2001.

3.2 ETHICAL CONSIDERATIONS

All clinical trials included in this thesis have been conducted in accordance with Good Clinical Practice and the Declaration of Helsinki (ICH-GCP 1996; WMA 2008). Ethical approval has been obtained in parallel for each sub-study, both from the country of origin as well as from Sweden. With regards to Study II approval has also obtained from the ethical review boards of the University of Heidelberg School of Medicine (Germany), Centers for Disease Control and Prevention, Atlanta (USA), Walter Reed Army Institute of Research, Silver Spring (USA), Hospital Clínic Provincial de Barcelona (Spain), and the Universitair Ziekenhuis Antwerp (Belgium), representing the primary affiliations of researchers involved in this trial. Parents/guardians provided informed consent for their children to participate prior to enrolment in the respective studies.

3.3 CLINICAL TRIAL REGISTRATION

Following new recommendations issued by the members of the International Committee of Medical Journal Editors (ICMJE) in September 2004 (De Angelis,

Drazen et al. 2004), all clinical trials included in this thesis conducted after 2004 have been registered on www.ClinicalTrials.gov, which was applicable to Study II and V.

3.4 GENERAL METHODOLOGIES IN THE EFFICACY TRIALS

Four antimalarial drug trials were included in this thesis, which all enrolled children with acute uncomplicated *P. falciparum* malaria. One study had a short follow-up of 72 hours (Study V), whereas the remaining three (Study I, II and IV) were comparative clinical trials adopting an extended follow-up duration of 42 days. In these studies the follow-up visits after initiation of treatment were conducted on days 1, 2, 3, 7, 14, 21, 28, 35 and 42, except in Study II, which only had one follow-up visit after day 28, i.e. day 42. Beside the scheduled visit, parents/guardians were encouraged to return to the study site if their children developed any complains after initial clearance of parasites, e.g. fever. Children not returning for scheduled visit were actively followed-up at home.

On every scheduled and extra visit a medical history was taken, a physical examination performed, including measurement of body temperature and a thick blood smear collected for assessment of presence and density of *P. falciparum*. Blood smears were examined with light or sunlight microscopy in the field by experienced microscopists counting parasites against 200 white blood cells (WBC). Parasite density was quantified (parasites/ μ L) by assuming an average of 8000 WBC per μ L blood, except in Study II when absolute WBC counts were available. Quality control was performed in all studies according to WHO standards by independent readers blinded to the initial outcome (WHO 2003b).

All study drugs were administered under supervision in standard doses according to body weight, except in Study IV where for logistic reasons only the first of the two daily doses of artemether-lumefantrine were administered under supervision. All concomitant medications were documented.

Adverse events were assessed by means of clinical and laboratory investigations and reported with regards to severity and causality to the study drug. Children developing severe malaria during follow-up were withdrawn and given rescue treatment with quinine.

3.5 STUDY SPECIFIC METHODOLOGIES

Study specific objectives and methodologies are described in the respective methods section of the papers and partly in section 4 below.

3.6 ASSESSMENT OF CRUDE CURE AND FAILURE RATES

For assessment of crude (non-PCR adjusted) cure rates in Study I, II and IV the standard WHO treatment outcome classification for antimalarial drug trials, see section 2.1.2 above, was used, but adapted to the extended follow-up of 42 days (WHO 2003b; WHO 2005).

Crude cure refers to adequate clinical and parasitological response, whereas crude failure corresponds to the sum of early treatment failure, late clinical failure and late parasitological failure.

3.7 ASSESSMENT OF PCR ADJUSTED CURE AND FAILURE RATES

All DNA extraction and parasite genotyping was performed at the Malaria Research Laboratory, Department of Medicine, Karolinska Institutet, except for Study II where this was done centrally at the Swiss Tropical Institute, Basel, Switzerland. All blood samples subjected to PCR analysis from the same patient were processed in parallel during all aspects of the laboratory work, from DNA extraction to separation of PCR products on agarose gels.

3.7.1 Blood sampling and storage

Blood samples were collected on 3MM[®] (Whatman) filter papers for parasite genotyping, except in Study II where commercial filter paper collection cards (FTA[®]; Whatman) were used and in the 15 Mali samples from Study III, which were collected on 903[®] (Schleicher & Schuell) filter paper. After blood collection in the field the filter papers were thoroughly dried, put in individual zipper plastic bags and stored in room temperature. At the end of each trial all filter papers were transported to Stockholm, where they were again stored in room temperature until time of DNA extraction.

3.7.2 DNA extraction

DNA extraction was performed with different methods. In Study I methanol extraction was used (Gil, Nogueira et al. 2003), whereas in Study IV and V DNA was extracted using the ABI PRISM[®] 6100 Nucleic Acid PrepStation (Applied Biosystems, Fresno, CA, USA) as previously described (Dahlstrom, Veiga et al. 2008).

Study III was conducted following repetitive failures to amplify DNA from archive blood spots. Consequently, a new DNA extraction method was developed based on Tris-EDTA (TE) buffer. Briefly, Tris-EDTA buffer, composed of 10 mM Tris, pH 8.0 (Tris-base plus Tris-HCl) and 0.1 mM EDTA in distilled water, was prepared and kept at room temperature. A filter paper punch from a dried blood spot was placed in an Eppendorf tube, soaked in 65 µL of TE buffer, and incubated at 50°C for 15 minutes. Thereafter, the filter paper punch was pressed gently several times against the bottom of the test tube using a pipette tip, followed by heating at 97°C for 15 minutes to elute DNA. Condense on the lid and walls of the tube were removed by centrifugation for 2–3 seconds. Extracted DNA was kept at 4°C for use within few hours or else stored frozen at –20°C.

3.7.3 PCR genotyping

All parasite genotyping was performed based on standard nested PCR protocols for *msp1* and *msp2* with regards to oligonucleotide primer sequences, mastermix concentrations and cycling conditions (Snounou, Zhu et al. 1999). An initial denaturation period of 5 min at 95°C was followed by annealing for 2 min at 58°C, extension for 2 min at 72°C and denaturation for 1 min at 94°C. Twenty-five cycles were performed for the first reaction. For the nest reaction the cycling conditions were the same for *msp2*, but for *msp1* the annealing temperature was 61°C. All nested reactions included 30 cycles. The last extension was carried out for 5 min both after initial and nested PCR reaction, whereafter the temperature was reduced to 20°C. One negative control and three and two positive controls representing the respective allelic types of *msp1* (K1, MAD20 and R033) and *msp2* (FC27 and 3D7/IC) were incorporated in every PCR run.

The *msp1* and *msp2* PCR products were loaded on 3% and 2% agarose gels, respectively, stained with ethidium-bromide, separated by electrophoresis and visualized under ultraviolet trans-illumination (GelDoc[®], Biorad, Hercules, USA). All

samples from one patient were run in adjacent lanes on the gel. Analyses of number of genotypes and size polymorphism were done by ocular assessment of digitalized photos in Study I and IV, whereas in Study V this was computerized using Quantity One[®] software (Biorad, Hercules, USA).

3.7.4 PCR adjusted cure rates

The strategy for distinguishing PCR adjusted cure rates varied between the trials. In Study I this was done solely based on analysis of the *msp2* marker. In Study IV stepwise genotyping of *msp2* followed by *msp1* was used. Finally, in Study II stepwise genotyping by an initial RFLP analysis of *msp2* (Felger and Beck 2002) was followed by nested PCR analysis of *msp1* and *glurp* (Snounou, Zhu et al. 1999).

Patients with microscopy confirmed parasitological failure during follow-up was subjected to PCR analysis. Recrudescence was defined as at least one matching allelic band at baseline and parasite recurrence in all markers analyzed in the study. Patients with missing filter paper sample or negative PCR were considered as uncertain PCR-adjusted outcome. Patients with parasitological failure defined as new infections by PCR were considered treatment successes. PCR adjusted cure refers to patients with adequate clinical and parasitological response and parasitological failures defined as new infections by PCR.

4 RESULTS AND DISCUSSION

4.1 STUDY I: EFFICACY OF ARTESUNATE+AMODIAQUINE AND ARTEMETHER-LUMEFANTRINE IN ZANZIBAR

This was the first clinical trial comparing PCR adjusted efficacy of artesunate+amodiaquine and artemether-lumefantrine during an extended follow-up of 42 days combined with a systematic analyses of genetic markers related to quinoline resistance. The study was conducted to provide baseline information on PCR adjusted efficacy of the respective drugs before their introduction as new first- and second line treatments for uncomplicated *P. falciparum* malaria in Zanzibar. A total of 408 children were enrolled, of whom 208 were assigned artesunate+amodiaquine and 200 artemether-lumefantrine. Children aged 6–8 months and/or weighing 6–8 kg were assigned to receive ASAQ for 3 days, whereas children aged 9–59 months and weighing ≥ 9 kg were randomly assigned either ASAQ or AL for 3 days in standard doses. The adjustment of age and weight inclusion criteria, which were due to the fact that artemether-lumefantrine was not registered for treatment in children ≤ 9 months and/or ≤ 9 kg by the time of the trial, was accounted for by multiple regression analysis of the efficacy outcome.

Four key results/messages were retrieved from this study:

- Both drugs were highly efficacious by day 28 with PCR adjusted cure rates of 94% for artesunate+amodiaquine and 97% for artemether-lumefantrine. However, the corresponding PCR adjusted cure rates by day 42 decreased to 91% and 94%, respectively. With a more conservative approach, i.e. defining all uncertain PCR outcomes (N=10) as recrudescence, the PCR adjusted cure rates by day 42 decreased further to 88% and 92% for artesunate+amodiaquine and artemether-lumefantrine, respectively. In light of the stringent WHO criteria for introduction of a new treatment policy the efficacy data retrieved by day 28 justify the introductions of both ACTs as the new treatment policy in Zanzibar. However, if the day 42 assessments were to be used, which is the recommended follow-up duration at least for artemether-lumefantrine, the efficacy of both drugs falls below the acceptable cut-off point of 95% efficacy, although this should be put in perspective of that only one genetic marker was used to assess PCR adjusted cure rates in this trial. (see also section 4.5)

- Among the total PCR adjusted recrudescences 5/18 (28%) and 7/12 (58%) were detected beyond day 28, i.e. between day 29-42, after artesunate+amodiaquine and artemether-lumefantrine treatment, respectively. These results support the present recommendation of an extended follow-up to day 42 in trials of artemether-lumefantrine and further suggest that a follow-up duration of 28 days in trials of artesunate+amodiaquine may not be sufficient for assessment of efficacy.
- A statistically significant difference in crude cure rates by day 42 was observed between the treatments by day 42. This was to a large extent explained by a stronger prevention against reinfections provided by artemether-lumefantrine, with reinfection rates by day 42 of 36% after artesunate+amodiaquine compared with 17% after artemether-lumefantrine [OR 0.37; 95% CI 0.22–0.60; $P < 0.001$]. The clinical importance of such prolonged post-treatment prophylaxis needs to be further elucidated in the African context.
- A significant selection of *P. falciparum mdr1* 86N was observed in isolates with reinfection after artemether-lumefantrine treatment, compared with baseline distribution (2.2-fold increase; $P < 0.001$). No selection was observed in *crt* codon 76, probably due to a high baseline prevalence of 76T (97% of the *P. falciparum* isolates). This indicates that the long acting partner drug in ACT is vulnerable to selection of molecular markers related to drug tolerance/resistance, which highlight the importance of close drug resistance surveillance of ACT in Africa.

4.2 STUDY II: EFFICACY AND SAFETY OF A PAEDIATRIC ACT FORMULATION

This was a randomized, investigator-blinded, multicentre, parallel-group trial conducted in 8 study sites in 5 sub-Saharan African countries. The study aimed to demonstrate non-inferiority (using a margin of -5%) of a new dispersible formulation versus conventional crushed tablets of artemether-lumefantrine for the treatment of children with acute uncomplicated *P. falciparum* malaria based on PCR-adjusted cure rates. A total of 899 children (110 in Benin, 193 in Kenya, 225 in Mali, 102 in Mozambique, 240 in Tanzania main land, and 29 in Zanzibar) aged <3 months to 12

years and weighing 5 kg to 35 kg, were enrolled, of whom 447 were assigned the dispersible tablet and 452 the crushed tablet.

Four key results/messages were retrieved from this study:

- The PCR corrected cure rates by day 28 (primary end-point) was 97.8% after treatment with the dispersible tablet and 98.5% after crushed tablet. Applying a conservative analysis of PCR adjusted cure rates by day 42, considering patients with non-conclusive PCR results as recrudescence, the efficacy decreased to 91% and 93%, respectively. Similarly with findings from Study I, these results indicate a high, and non-inferior, efficacy by day 28. However, when follow-up was extended to 42 days the PCR adjusted efficacy rates, as assessed with a conservative approach, fell below the acceptable limit of 95% efficacy for introduction as new treatment policy.
- Crude cure rates were similar between the groups, with ACPR by day 28 of 92.1% with the dispersible tablet and 90.5% with crushed tablet, whereas the corresponding day 42 results were 77.7% and 74.5%, respectively.
- Tolerability was adequate in both treatment groups, with no difference in pattern and overall frequency of adverse events. No signs of neuro/ototoxicity or cardiotoxicity were observed. These results support the safety profile of the new formulation previously documented in African children with conventional administration of the crushed tablets.
- The lumefantrine pharmacokinetic profile was similar between the treatments.

In summary the new dispersible tablet showed high efficacy and non-inferiority by day 28 as well as a similar safety and pharmacokinetic profile as the conventional crushed tablet. The new formulation has the potential of facilitating drug administration/compliance in small children considerably. However, considering that this is the first paediatric formulation available of artemether-lumefantrine, the effectiveness of the new formulation should preferentially be assessed before its general recommendation for wide scale use.

4.3 STUDY III: INFLUENCE OF DIFFERENT EXTRACTION METHODS ON PCR OUTCOME FROM BLOOD SAMPLES STORED ON FILTER PAPER

This study aimed to assess the influence of different DNA extractions methods on PCR outcome from blood samples collected on filter paper by evaluating a new Tris-EDTA (TE) buffer-based method against the commonly used methanol and Chelex methods (Plowe, Djimde et al. 1995; Gil, Nogueira et al. 2003). The assessment was done by systematically performing parallel analyses of all samples in duplicate from 15 blood spots stored on 3MM[®] (Whatman) and 903[®] (Schleicher & Schuell) filter paper, respectively. Moreover, to improve comparability a standardized filter paper size, corresponding to similar quantities of blood, was incorporated in the respective DNA extraction analysis. This was achieved by using a sterile biopsy punch with a diameter of 4 mm to cut the filter paper.

Two key results/messages were retrieved from this study:

- Sensitivity and reproducibility varied considerably between extraction method, filter paper type and parasite density.
- The TE buffer based method generally performed best. The PCR sensitivity for blood samples collected on 3MM was 100%, 73% and 93% for the TE, methanol, and Chelex method, respectively, whereas the corresponding sensitivity was 93%, 73% and 0% for the 903 filter paper. Thus, the choice of DNA extraction method may considerably influence PCR outcome from blood samples collected and stored on filter paper.

4.4 STUDY IV: INFLUENCE OF CONSECUTIVE DAY BLOOD SAMPLING ON PCR ADJUSTED CURE RATES

This open-label exploratory clinical trial aimed to assess the influence of consecutive day blood sampling both at study inclusion and parasite recurrence on PCR adjusted parasitological cure rates of sulfadoxine-pyrimethamine and artemether-lumefantrine for the treatment of uncomplicated *P falciparum* malaria during an extended follow-up of 42 days. The primary endpoint was PCR adjusted parasitological cure rates of the respective treatments using WHO's standard protocol for PCR genotyping with single

day blood sampling (1+1 day) compared with an elaborated protocol using blood sampling on 4 consecutive days at study inclusion and 2 consecutive days at time of recurrent parasitaemia (4+2 days). Some 106 children were enrolled, 56 were randomly allocated sulfadoxine-pyrimethamine and 50 artemether-lumefantrine. Three children were lost to follow-up, all in the sulfadoxine-pyrimethamine group. A majority of enrolled children experienced recurrent infections during follow-up, 40/53 (75%) in the sulfadoxine-pyrimethamine and 38/50 (76%) in the artemether-lumefantrine group.

Four key results/messages were retrieved from this study:

- PCR genotyping with *msp2* from blood samples collected on days 1-3 identified 32 additional parasite genotypes in 26/106 (25%) children compared with those seen at day 0, of which 21, 8 and 3 were detected on days 1, 2 and 3, respectively. This indicates that a single blood sample may not provide a complete picture of all parasite populations present in a child with clinical malaria infection in a high endemic area.

- Initial *msp2* genotyping identified 27 and 33 recrudescences using single and consecutive day sampling, respectively. Following additional *msp1* genotyping 17 and 21, respectively, of these episodes were still classified as recrudescences. This indicates a similar sensitivity of the standard single day PCR protocol, $27/33=82\%$ [95% CI 68-96]. and $17/21=81\%$ [95% CI 63-99], in both genotyping steps. These results suggest two important issues. Firstly the number of genetic markers incorporated into the analysis will significantly change assessment of PCR adjusted cure rates. By adding a second genetic marker a substantial number of recrudescences were reclassified as reinfections, suggesting that the use of a single genetic marker may underestimate PCR adjusted cure rates. However, the increased specificity in detection of recrudescences achieved with multiple genetic markers are at the cost of sensitivity, by reduced chance of detecting minority clones in multiple genetic markers, which conversely may overestimate cure rates. Secondly, consecutive day sampling improved detection of recrudescences, indicating that the use of paired single day blood sampling may overestimate drug efficacy.

- Another important advantage with the elaborated protocol in this study was the improved ability to retrieve PCR adjusted outcomes in children with non-conclusive PCR results from paired blood sampling. This additional benefit of the enhanced protocol may represent an important factor for improving the robustness of PCR adjusted cure rates in clinical trials.
- A majority of both the additional genotypes in this study were detected on the first day after enrolment and the additional recrudescences were identified by combining PCR-results from 2 consecutive days both at enrolment and recurrent parasitaemia. Based on this evidence future studies assessing the efficiency of consecutive day blood sampling should primarily target a blood sampling model of 2+2 days.

4.5 STUDY V: PARASITE POPULATION DYNAMICS DURING THE EARLY PHASE OF ANTIMALARIAL DRUG TREATMENT

This open-label, exploratory clinical trial aimed to explore *P. falciparum* population dynamics during the early phase of antimalarial drug treatment in African children with acute uncomplicated malaria. A total of 50 children aged 1-10 years were hospitalized and received supervised treatment with artemether-lumefantrine. Blood samples were collected 11 times, at time of diagnosis (-2 hours) and 0, 2, 4, 8, 16, 24, 36, 48, 60 and 72 hours after initiation of treatment. The screening blood sample (-2 hours) was collected from a capillary finger prick and the remaining were drawn from an intravenous cannula inserted in an anti-brachial vein. Parasite population dynamics were assessed using nested PCR genotyping of *msp1* and *msp2*. Individual genotypes were defined by binning 20 base-pairs intervals together. Initially, we identified the median genotype for each family of the respective genetic markers. The absolute size of the identified median band +/- 10 base-pairs formed the initial bin. Thereafter, each 20 base pair interval below and above the median band were defined as representing a distinct genotype.

Three key results/messages were retrieved from this study:

- A total of 32% and 24% patients had different genotype patterns detected in the two pre-treatment samples (-2 and 0 hours) for *msp1* and *msp2*, respectively. This comprised 19 genotypes in *msp1* and 18 in *msp2*, of which 8 genotypes in

each marker were detected at -2 hours but were absent 0 hours, whereas the remaining appeared at 0 hours without having been identified -2 hours. Similarly, 28/50 (56%) had at least two fold differences in parasite density measured by microscopy before initiation of treatment, of whom 21 showed an increase of more than 100% and 7 a decrease of at least 50%

- PCR-analyses from 9 sequential blood samples collected after initiation of treatment identified 20 and 21 additional genotypes in 15/50 (30%) and 14/50 (28%) children with *mSP1* and 2, respectively, non-detectable in the pre-treatment samples (-2 and 0 hours combined).
- Some 75% and 67% of the new genotypes were identified within 24 hours, whereas 85% and 90% within 48 hours for *mSP1* and *mSP2*, respectively.

The results from this study suggest uncomplicated *P. falciparum* malaria to be a complex condition, where individual parasite genotypes increase and decrease in a non-synchronized way in the peripheral blood and thus within short time periods may be present in different proportions of the overall parasitaemia. This dynamic/chaotic situation appears to be especially pronounced before initiation of treatment. However, it should be noted that the two pretreatment samples arose from different sampling routes, which may have influenced the outcome.

A majority of the new genotypes detected during follow-up were identified within 24 hours, which is in line with results from Study IV, indicating 24 hours after treatment onset to be an appropriate time for collection of an additional blood sample in future studies assessing the influence of consecutive blood sampling on PCR adjusted cure.

It remains open whether the complex genotype pattern observed in this study reflects true parasite population dynamics or limitations of the PCR-technique to detect all minority genotypes present due to e.g. template competition in complex infections or even PCR artefacts. This underlines the importance of interpreting PCR outcomes with caution and suggests that the present use of PCR-adjustment from paired single day blood samples in antimalarial drug trials may overestimate drug efficacy in high-endemic areas in Africa.

4.6 INFLUENCE OF EXTENDED FOLLOW-UP – POOLED DATA FROM STUDY I, II AND IV

The influence of extended follow-up was assessed by pooling efficacy data from the total cohort of 1414 patients enrolled in Study I, II and IV. Number of patients with recrudescence identified with PCR genotyping by day 28 and day 42 for each study and treatment arm as well as proportion of recrudescences identified by day 28 among total failures observed by day 42 are presented in Table 2. The overall sensitivity of the 28 day follow-up schedule to identify recrudescences was calculated to $48/72=67\%$ [95% CI 55-76%]. In two out of three studies a high proportion of recrudescences occurred beyond day 28, whereas in the third study the additional benefit from the extended follow-up was limited. However, this study was small, 106 patients in total, and only one child treated with artemether-lumefantrine had PCR adjusted treatment failure. Moreover, the comparator in this trial, sulfadoxine-pyrimethamine, was by the time of the trial already a failing drug in Tanzania, which may explain that recrudescences generally occurred early during follow-up also in this treatment arm.

	Study I		Study II		Study IV		Total number
	ASAQ	AL	AL DT	AL CT	SP	AL	
Day 28	13	5	9	6	14	1	48
Day 42	18	12	14	11	16	1	72
Proportion	0.72	0.42	0.64	0.55	0.88	1.0	0.67

Table 2. Number of patients with recrudescence identified with PCR genotyping by day 28 and day 42 and proportion of recrudescence identified by day 28 among the total observed by day 42.

In Study I PCR adjustment was based on *msp2* analysis only, in Study II by stepwise genotyping of *msp2*, *msp1* and *glurp* using the modified intention to treat analysis, and in Study IV by stepwise genotyping of *msp2* followed by *msp1* using the standard protocol. (ASAQ= Artesunate+amodiaquine, AL=artemether-lumefantrine, DT=dispersible tablet, CT=crushed tablet, SP=sulfadoxine-pyrimethamine)

Thus, the analysis of pooled data suggests that the duration of follow-up represent a key determinant for assessment of antimalarial drug efficacy in Africa.

5 CONCLUSIONS

5.1.1 Overall conclusion

- Methodological aspects, i.e. consecutive day blood sampling and length of follow-up, substantially influence assessment of PCR adjusted efficacy and the findings suggest that the present WHO standard protocol may overestimate PCR adjusted cure rates of antimalarial drugs in Africa. Thus, PCR adjusted cure rates should be interpreted with caution.

5.1.2 Specific conclusions

- Extended follow-up from 28 to 42 days provides a more robust efficacy assessment of both ACTs at test in this thesis, by identification of a significant number of late recrudescences appearing between days 29-42.
- PCR analysis of consecutive day blood samples both at enrolment and parasite recurrence reveals more complex infections and changes the interpretation of cure rates by identification of more recrudescences in a high endemic area.
- PCR analysis from multiple blood samples collected during the three days of drug treatment identifies even more complex genotype pattern than daily consecutive blood samples.
- Artesunate+amodiaquine and artemether-lumefantrine were both highly efficacious as assessed by day 28 for the treatment of children with acute uncomplicated *P. falciparum* malaria in Zanzibar, which justified their introduction as new first- and second line treatments.
- The new dispersible formulation of artemether-lumefantrine was highly efficacious and non-inferior to the conventional formulation with crushed tablets by day 28 for the treatment of acute uncomplicated childhood malaria in Africa.
- The choice of DNA extraction method may considerably influence PCR outcome from blood samples stored on filter paper.

6 PERSONAL REFLEXIONS AND FUTURE PERSPECTIVES

PCR adjusted cure rate is generally accepted as primary end-point in antimalarial drug trials conducted in Africa (WHO 2006; MMV/WHO 2008). However, the results from this thesis demonstrate that PCR adjusted cure rates may not be as exact, straightforward and easily interpretable as one may think. Instead, it appears that PCR adjusted cure rates are vulnerable to a number of methodological factors studied in this thesis, each being associated with a considerable risk of influencing the final PCR outcome. Still, it should be acknowledged that the use of PCR adjustment is essential and presently represent the best method available to distinguish treatment failures from new infections. However, improved awareness of the potential pitfalls of the technique strengthens the suggestion that PCR adjusted cure rates should be interpreted with caution.

The use of different number and combinations of genetic markers in the different sub-studies of this thesis should be acknowledged as a weakness, but this mirrors the general confusion and lack of standardization which has prevailed after the introduction of PCR genotyping in antimalarial drug trials. However, considering the consistent results retrieved in the thesis with regards to the influence of extended follow-up and consecutive day blood sampling, regardless of the number of genetic markers incorporated in the analysis, the evidence presented appears overall convincing. In light of all this it is praiseworthy that there is now a general consensus within the malaria research community of the importance of improved standardization of the use of parasite genotyping in antimalarial drug trials to allow future comparability of results (MMV/WHO 2008).

6.1 LENGTH OF FOLLOW-UP

It is customary to report end-points as crude and PCR-adjusted day 28 efficacy rates (MMV/WHO 2008). Conversely, a follow-up duration of 42 days in trials of artemether-lumefantrine is recommended based on estimations of clearance of the long acting partner drug (WHO 2003b; WHO 2006). However, the latter has not been accompanied by clear recommendations to primarily assess the efficacy of this

combination at the final day of the proposed 42-day follow-up. This is a dilemma and an inconsistency which needs to be addressed. Clearly, and as the results from this thesis point out, the PCR adjusted cure rate by day 28 generally seems to produce efficacy results of artemether-lumefantrine exceeding 95%. However, when follow-up is extended to 42 days the PCR adjusted cure rates, even in Study II with the use of three genetic markers, tend to approach or even fall below the recommended cut-off efficacy value for a new treatment regimen to be introduced as policy.

Obviously, it would be a sensitive issue if a modern and highly promoted ACT candidate does not necessarily fulfil the new stringent and ambitious efficacy criteria by day 42 for introduction as new treatment policy. However, considering the imprecise nature of PCR adjusted cure rates, particularly in high endemic areas, this probably says more about the insufficiency of the method than the efficacy of the drug. Regardless, it is utterly important to standardize the primary end-point in any antimalarial drug trial to correspond with clearance of the long acting partner drug at test to achieve as accurate assessment of efficacy as possible. Consequently and if logistically feasible any comparative clinical trial should always aim at a follow-up long enough to reach clearance of the drug with longest terminal half-life involved in the trial. This will not only improve efficacy assessment and assure a fair comparison of the drugs, but also allow a more robust assessment of post-treatment prophylaxis of the drugs. Moreover, and considering the observations in this thesis, it is important to further explore if an extended follow-up even beyond day 42 in trials of artemether-lumefantrine is needed. This is based on the assumption that children with partial immunity may suppress recrudescences during even longer periods of time than the actual drug clearance. We have taken on this research question in a recently completed trial in the field with assessment of PCR adjusted cure rates of artemether-lumefantrine up to day 56 (see also below).

6.2 CONSECUTIVE DAY BLOOD SAMPLING

Growing evidence, including results from this thesis, supports the picture of at least low to moderate dynamics in PCR detection of different parasite genotypes over time in children with acute uncomplicated malaria in high transmission areas. Regardless if the reason for this is parasite population dynamics or limitations of the PCR technique, the inability to detect all parasite populations present in an individual in a single blood

sample may overestimate PCR adjusted efficacy in clinical trials. Our data suggest that consecutive day sampling (2+2), i.e. Day 0 + 24 hours and Day X + 24 hours, may be proposed as the way forward, but in the recent MMV/WHO document aiming at standardization of the use of PCR genotyping in clinical trials it was considered that more evidence is needed before the 2+2 day sampling could be recommended (MMV/WHO 2008). The concern was especially the practicability of the proposed sampling strategy, since the use of consecutive day sampling will require a change in the standard protocol with additional blood sampling, e.g. on day 1. However, considering that efficacy trials of ACT require the patients to be seen for drug administration on day 1 together with the recently realised importance of strictly measuring parasite clearance time for assessment of *in vivo* artemisinin-tolerance, it appears that an additional blood sampling on day 1 may anyway materialize in the near future. The results from the thesis indicate that a high proportion, circa 80%, of patients remain positive by PCR on day 1 even after controlled intake of ACT, which makes the blood samples from day 1 suitable for PCR analysis.

The most appropriate place for an initial introduction of consecutive day blood sampling would suggestively be in trials where high accuracy of PCR adjusted cure is most desirable. That would apply primarily to regulatory trials as well as pre-implementation studies of new policy candidates. However, for general surveillance of drug efficacy it appears to be less important to add consecutive day blood sampling before its broader feasibility has been thoroughly assessed. It should also be noted that both clinical trials assessing consecutive day blood sampling and parasite population dynamics during the early phase of antimalarial drug treatment (Study IV and V) in this thesis were conducted in a high endemic area during the yearly transmission peak. Therefore, the retrieved results from these studies may not necessarily be applicable to lower transmission areas in other parts of Africa.

6.3 INFLUENCE OF REPETITIVE TREATMENT

In clinical practice a child re-attending the health facility with clinical malaria infection within 14 days after the previous treatment is considered as a treatment failure, which should be rescued with the second line drug. In contrast a recurrent infection after 14 days or more is considered a new infection and is therefore retreated with the first line drug. However, retreatment with the same drug within short periods of time results in

repetitive exposure of the long acting partner drug in ACT to small children with potential risk of drug accumulation. The influence of such drug accumulation on efficacy, safety and selection of parasite genotypes with increasing drug tolerance or even drug resistance is an important field of research to explore. Since repetitive drug treatment is a real life phenomenon it appears important that studies addressing this topic do not only include efficacy, but also effectiveness assessment. Such trials need to include measurement of lumefantrine concentrations at least on day 7 after initial and repetitive treatment. This is now feasible also under field conditions with a newly developed method based on capillary blood sampling of only 100 μ L blood collected on pre-treated filter papers (Blessborn, Romsing et al. 2007). Therefore, and as an initial offspring from this thesis, we have combined the interest of extending follow-up beyond 42 days (see section 6.2), with assessment of repetitive treatment with artemether-lumefantrine in a two arm study (efficacy versus effectiveness), which has recently been completed in Tanzania.

6.4 CHOICE AND DEPLOYMENT OF ACT BASED ON SELECTION POTENTIAL OF MOLECULAR MARKERS OF DRUG RESISTANCE

The choice of ACT in a country/region is a dilemma, where several factors must be allowed to influence decision making. Emerging data on selection potential of identifiable parasite genotypes related to drug tolerance/resistance is probably an important factor that needs strict consideration in this regard. The finding from Study 1 of *P. falciparum mdr1* 86N selection in reinfections after artemether-lumefantrine treatment, initially published by Sisowath et al, represents the first observation in Africa of this kind, which subsequently has been confirmed by other groups (Sisowath, Stromberg et al. 2005; Dikomajilar, Nsohya et al. 2006). Interestingly, new molecular data from Study IV has identified an additional selection of *crt* 76K after artemether-lumefantrine treatment (Sisowath, Petersen et al. In Press). This was probably not detected in Study 1 due to the high baseline prevalence of *crt* 76T in Zanzibar. Considering that amodiaquine/desetyl-amodiaquine selects in the opposite direction, towards *mdr1* 86Y and *crt* 76T (Holmgren, Gil et al. 2006; Holmgren, Hamrin et al. 2007) it appears from this perspective that Zanzibar made an attractive choice with two ACTs as first- and second line treatment that counteract/balance each others genetic selection potential. These findings and their clinical importance represent an important

future field of research with potentially high impact on future decision making and deployment of ACT in Africa.

6.5 SUPPORTING EVIDENCE OF DRUG RESISTANCE IN VIVO

In vivo trials will probably remain gold standard for assessment and surveillance of antimalarial drug resistance, where PCR adjustment has a fundamental role to play in establishment of parasitological cure rates. However, the potential weaknesses of the *in vivo* methodology in general and PCR adjusted cure rates in particular, which have been outlined in this thesis, calls for supporting evidence to strengthen the association between treatment failure and drug resistance. This may be achieved in the future by a triangulation, including both systematic measurements of drug concentrations of the long acting partner drug on day 7 together with detection of established molecular markers related to drug resistance at enrolment and recurrent parasitaemia in addition to the PCR adjustment. The first tool (measurement of drug concentrations) is already available and has been advocated to be used in clinical trials (White, Stepniewska et al. 2008). With the new capillary method for lumefantrine blood sampling it could be applied even in remote areas for trials of artemether-lumefantrine as described above (Blessborn, Romsing et al. 2007). However, the second parameter (molecular markers) will first require an improved understanding of the correlation between recrudescences and genetic drug resistance markers before it can be fully utilized. An initial determination of drug level could exclude treatment failures likely to be due to low bioavailability. For the remaining patients with recurrent parasitaemia during follow-up and adequate drug levels on day 7 the correlation between PCR adjusted recrudescences (with presence of confirmed and/or potential genetic markers related to drug resistance) and reinfections (with presence of genetic markers related to drug sensitivity) could be further assessed. This appears to be an interesting potential future approach to strengthen the association between treatment failure and drug resistance and thus a more accurate assessment of true recrudescence versus reinfection.

7 ACKNOWLEDGEMENTS

Three persons have played a fundamental role in the completion of this thesis. Firstly, Sofia my dear wife and medical colleague with whom I have had the great privilege of working with on three different continents and who introduced me to malaria treatment. The patience, unconditional love, support and positive input you have given me during the PhD project is remarkable. This thesis is as much yours as it is mine! Secondly, Dr Sándor, the king of *msp2*, my friend and colleague. You introduced me to the world of PCR genotyping. Your guidance, help and warm sense of humor made my life bearable despite all obstacles during the first two years of PhD training. Thirdly, Anders my main supervisor, friend and mentor. You have opened the door to the world of science and carefully guided me on my way. The atmosphere you create in the lab is unique and based on your humble approach to people, science and life. It's because of you I like to stick around! I do hope we will continue to explore the fascinating world of science together for many more years to come! Big thanks!

I have had the privilege of having three official and one unofficial co-supervisor during this project, which proves that the cliché 'the more the merrier' is true. Thanks to Professor Lars Rombo for being an unlimited source of inspiration. Your help and good advice truly reflects the mind of a real scientist. Professor Scott Montgomery for showing me my way through the SPSS jungle. Without your help, guidance and problem solving I would have been lost! Docent Anna Färnert for always being ready to scrutinize my applications and manuscripts. Your clear scientific mind, writing skills and general encouragement have really facilitated this thesis. Thanks! Dr Pedro Gil for taking on the assignment of being my unofficial supervisor without even knowing it. Thanks for always showing great interest in my work, for fascinating scientific discussions, help and advice throughout my training!

Room mates in M9: Sándor (again), Achuyt and Johan! Thanks for countless laughs!

My gratitude to all friends and colleagues in the Department of Medicine Solna, Malaria Research Laboratory, IHCAR and collaborators in Africa and Asia for their support: Professors Zul Premji, Amos Massele, Christina Broliden, Göran Tomson, Bo Lindblad, Vinod Diwan, Hans Rosling, Asim Beg, Akira Kaneko, Salim Abdulla and

Berit, Billy, Guida, Mubi, Anja, Isabel, Christin, Sabina, Gabrielle, Najia, Pedro F, Annika J, Abdullah Ali, Membi, Mwinyi, Johan S, Marika, Anne, Eva-Maria, Maja, Lotta, Deler, Kjerstin B and many many more.....

Special thanks to all the staff at the Emergency Medicine Unit, Kullbergska Hospital for great support, especially to doctors Jonny, Calle, Håkan, Gunvor och Andrey!

Many thanks to colleagues in other hospitals where I have worked over the years: Lund University Hospital, Norrköping Hospital, Bethania Hospital (Pakistan), Mnene Mission Hospital (Zimbabwe), Torsby Hospital and Kiruna Hospital.

To Mahidol University, Bangkok, Thailand, for excellent teaching in Tropical Medicine and for accepting Sofia and I to the same DTM&H course.....

To family and friends who never gave up on me! Thanks for your support!

To my children David, Sara and Maria for asking the best and most provocative questions during this PhD project.

During this PhD project two persons dear to me died: Firstly, my mother in law Maria Habib, who died in a car accident on the way back from Saint Josephs Catholic Church, Lahore, Pakistan. I will never forget the Gospel tunes that embraced me when I entered the Catholic Church in Bagamoyo, Tanzania, to pay homage to your departed soul. These divine songs will always make me remember your love to Sofia, myself and our children. May the Good Shepard keep you in his eternal love! Secondly, Magnus my friend and schoolmate, who fought his cancer with stoic pride for several hard years. You lived and died like a true man with dignity and integrity. Respect!

Last, but not the least I would like to express my sincere thanks to all the children and their parents as well as the staff members at Fukayosi Health Care Centre, Kivunge and Micheweni Cottage Hospitals. Without your dedicated participation this thesis would not have been possible!

This PhD project and its sub-studies received financial support from:

- Swedish Development Cooperation Agency-Department for Research Cooperation (SIDA-SAREC; SWE 2003-278, 2005-017 and 2005-004596)
- R&D-Unit, Sörmland County Council (Project grant 82090)
- United Nations Development Program–World Bank–World Health Organization Special Program for Research and Training in Tropical Diseases (A20674)
- The European 5th Framework Project (QLK2-CT20021-1503-RESMALCHIP),
- Novartis Pharma, Basel, Switzerland
- Medicine for Malaria Venture, Geneva, Switzerland,
- Golje's Foundation
- Irma and Arvid Larsson-Röst's Foundation
- Anders Otto Sward's Foundation.

8 REFERENCES

- Adjei, G. O., J. A. Kurtzhals, et al. (2008). "Amodiaquine-artesunate vs artemether-lumefantrine for uncomplicated malaria in Ghanaian children: a randomized efficacy and safety trial with one year follow-up." Malar J **7**: 127.
- Adjuik, M., A. Babiker, et al. (2004). "Artesunate combinations for treatment of malaria: meta-analysis." Lancet **363**(9402): 9-17.
- Ashley, E. A., L. Pinoges, et al. (2008). "Different methodological approaches to the assessment of in vivo efficacy of three artemisinin-based combination antimalarial treatments for the treatment of uncomplicated falciparum malaria in African children." Malar J **7**: 154.
- Ashley, E. A., K. Stepniewska, et al. (2007). "How much fat is necessary to optimize lumefantrine oral bioavailability?" Trop Med Int Health **12**(2): 195-200.
- Ashley, E. A. and N. J. White (2005). "Artemisinin-based combinations." Curr Opin Infect Dis **18**(6): 531-6.
- Barnes, K. I., F. Little, et al. (2006). "Sulfadoxine-pyrimethamine pharmacokinetics in malaria: pediatric dosing implications." Clin Pharmacol Ther **80**(6): 582-96.
- Beck, H. P., I. Felger, et al. (1997). "Analysis of multiple Plasmodium falciparum infections in Tanzanian children during the phase III trial of the malaria vaccine SPf66." J Infect Dis **175**(4): 921-6.
- Berezcky, S., A. Liljander, et al. (2007). "Multiclonal asymptomatic Plasmodium falciparum infections predict a reduced risk of malaria disease in a Tanzanian population." Microbes Infect **9**(1): 103-10.
- Bhattarai, A., A. S. Ali, et al. (2007). "Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar." PLoS Med **4**(11): e309.
- Bjorkman, A. and A. Bhattarai (2005). "Public health impact of drug resistant Plasmodium falciparum malaria." Acta Trop **94**(3): 163-9.
- Blessborn, D., S. Romsing, et al. (2007). "Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of lumefantrine in capillary blood on sampling paper." J Pharm Biomed Anal **45**(2): 282-7.
- Borre, M. B., M. Dziegiel, et al. (1991). "Primary structure and localization of a conserved immunogenic Plasmodium falciparum glutamate rich protein

- (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle." Mol Biochem Parasitol **49**(1): 119-31.
- Bosman, A. and K. N. Mendis (2007). "A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies." Am J Trop Med Hyg **77**(6 Suppl): 193-7.
- Cattamanchi, A., D. Kyabayinze, et al. (2003). "Distinguishing recrudescence from reinfection in a longitudinal antimalarial drug efficacy study: comparison of results based on genotyping of msp-1, msp-2, and glurp." Am J Trop Med Hyg **68**(2): 133-9.
- Checkley, A. M. and C. J. Whitty (2007). "Artesunate, artemether or quinine in severe *Plasmodium falciparum* malaria?" Expert Rev Anti Infect Ther **5**(2): 199-204.
- Collins, W. J., B. Greenhouse, et al. (2006). "The use of genotyping in antimalarial clinical trials: a systematic review of published studies from 1995-2005." Malar J **5**: 122.
- Cox-Singh, J., T. M. Davis, et al. (2008). "*Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening." Clin Infect Dis **46**(2): 165-71.
- Dahlstrom, S., M. I. Veiga, et al. (2008). "Diversity of the sarco/endoplasmic reticulum Ca(2+)-ATPase orthologue of *Plasmodium falciparum* (PfATP6)." Infect Genet Evol **8**(3): 340-5.
- De Angelis, C., J. M. Drazen, et al. (2004). "Clinical trial registration: a statement from the International Committee of Medical Journal Editors." N Engl J Med **351**(12): 1250-1.
- Desai, M., F. O. ter Kuile, et al. (2007). "Epidemiology and burden of malaria in pregnancy." Lancet Infect Dis **7**(2): 93-104.
- Dokomajilar, C., S. L. Nsoya, et al. (2006). "Selection of *Plasmodium falciparum* pfm_{dr1} alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic." Antimicrob Agents Chemother **50**(5): 1893-5.
- Dorsey, G., S. Staedke, et al. (2007). "Combination therapy for uncomplicated *falciparum* malaria in Ugandan children: a randomized trial." Jama **297**(20): 2210-9.
- Eckstein-Ludwig, U., R. J. Webb, et al. (2003). "Artemisinins target the SERCA of *Plasmodium falciparum*." Nature **424**(6951): 957-61.

- Efferth, T., M. R. Romero, et al. (2008). "The antiviral activities of artemisinin and artesunate." Clin Infect Dis **47**(6): 804-11.
- Ezzet, F., R. Mull, et al. (1998). "Population pharmacokinetics and therapeutic response of CGP 56697 (artemether + benflumetol) in malaria patients." Br J Clin Pharmacol **46**(6): 553-61.
- Ezzet, F., M. van Vugt, et al. (2000). "Pharmacokinetics and pharmacodynamics of lumefantrine (benflumetol) in acute falciparum malaria." Antimicrob Agents Chemother **44**(3): 697-704.
- Falade, C., M. Makanga, et al. (2005). "Efficacy and safety of artemether-lumefantrine (Coartem) tablets (six-dose regimen) in African infants and children with acute, uncomplicated falciparum malaria." Trans R Soc Trop Med Hyg **99**(6): 459-67.
- Falade, C. O., A. O. Ogundele, et al. (2008). "High efficacy of two artemisinin-based combinations (artemether-lumefantrine and artesunate plus amodiaquine) for acute uncomplicated malaria in Ibadan, Nigeria." Trop Med Int Health **13**(5): 635-43.
- Falk, N., N. Maire, et al. (2006). "Comparison of PCR-RFLP and Genescan-based genotyping for analyzing infection dynamics of Plasmodium falciparum." Am J Trop Med Hyg **74**(6): 944-50.
- Farnert, A. (2008). "Plasmodium falciparum population dynamics: only snapshots in time?" Trends Parasitol **24**(8): 340-4.
- Farnert, A., A. P. Arez, et al. (2001). "Genotyping of Plasmodium falciparum infections by PCR: a comparative multicentre study." Trans R Soc Trop Med Hyg **95**(2): 225-32.
- Farnert, A., A. P. Arez, et al. (1999). "Sampling and storage of blood and the detection of malaria parasites by polymerase chain reaction." Trans R Soc Trop Med Hyg **93**(1): 50-3.
- Farnert, A. and A. Bjorkman (2005). "Limited advantage of multiple consecutive samples for genotyping Plasmodium falciparum populations during the first days of treatment." Am J Trop Med Hyg **73**(1): 204-6.
- Farnert, A., G. Snounou, et al. (1997). "Daily dynamics of Plasmodium falciparum subpopulations in asymptomatic children in a holoendemic area." Am J Trop Med Hyg **56**(5): 538-47.
- Faye, B., J. L. Ndiaye, et al. (2007). "Efficacy and tolerability of four antimalarial combinations in the treatment of uncomplicated Plasmodium falciparum malaria in Senegal." Malar J **6**: 80.

- Felger, I. and H. P. Beck (2002). Genotyping of *Plasmodium falciparum*: PCR-RFLP analysis. Malaria methods and protocols. Methods in molecular medicine. e. Doolan D, Humana Press: 117–29.
- Felger, I., A. Irion, et al. (1999). "Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania." Trans R Soc Trop Med Hyg **93 Suppl 1**: 3-9.
- Gallup, J. L. and J. D. Sachs (2001). "The economic burden of malaria." Am J Trop Med Hyg **64**(1-2 Suppl): 85-96.
- Gardner, M. J., N. Hall, et al. (2002). "Genome sequence of the human malaria parasite *Plasmodium falciparum*." Nature **419**(6906): 498-511.
- Gibson, G. (1996). "Genetics, ecology and behaviour of anophelines." Ciba Found Symp **200**: 22-37; discussion 37-47.
- Gil, J. P., F. Nogueira, et al. (2003). "Detection of atovaquone and Malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb)." Mol Cell Probes **17**(2-3): 85-9.
- Glynn, J. R. and D. J. Bradley (1995). "Inoculum size, incubation period and severity of malaria. Analysis of data from malaria therapy records." Parasitology **110 (Pt 1)**: 7-19.
- Golenser, J., J. H. Waknine, et al. (2006). "Current perspectives on the mechanism of action of artemisinins." Int J Parasitol **36**(14): 1427-41.
- Gomes, M., I. Ribeiro, et al. (2008). "Rectal artemisinins for malaria: a review of efficacy and safety from individual patient data in clinical studies." BMC Infect Dis **8**: 39.
- Greenhouse, B., A. Myrick, et al. (2006). "Validation of microsatellite markers for use in genotyping polyclonal *Plasmodium falciparum* infections." Am J Trop Med Hyg **75**(5): 836-42.
- Gurkov, R., T. Eshetu, et al. (2008). "Ototoxicity of artemether/lumefantrine in the treatment of *falciparum* malaria: a randomized trial." Malar J **7**: 179.
- Hamer, D. H., M. Ndhlovu, et al. (2007). "Improved diagnostic testing and malaria treatment practices in Zambia." Jama **297**(20): 2227-31.
- Hassan Alin, M., M. Ashton, et al. (1996). "Multiple dose pharmacokinetics of oral artemisinin and comparison of its efficacy with that of oral artesunate in *falciparum* malaria patients." Trans R Soc Trop Med Hyg **90**(1): 61-5.
- Hastings, I. M. and S. A. Ward (2005). "Coartem (Artemether-Lumefantrine) in Africa: The beginning of the end?" Journal of Infectious Diseases **192**(7): 1303-1304.

- Hastings, I. M. and W. M. Watkins (2006). "Tolerance is the key to understanding antimalarial drug resistance." Trends Parasitol **22**(2): 71-7.
- Hatton, C. S., T. E. Peto, et al. (1986). "Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria." Lancet **1**(8478): 411-4.
- Hien, T. T. and N. J. White (1993). "Qinghaosu." Lancet **341**(8845): 603-8.
- Hietala, S. F., A. Bhattarai, et al. (2007). "Population pharmacokinetics of amodiaquine and desethylamodiaquine in pediatric patients with uncomplicated falciparum malaria." J Pharmacokinet Pharmacodyn **34**(5): 669-86.
- Hogh, B., R. Thompson, et al. (1993). "Glutamate rich Plasmodium falciparum antigen (GLURP)." Parasitologia **35 Suppl**: 47-50.
- Holmgren, G., J. P. Gil, et al. (2006). "Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pfcr1 76T and pfmdr1 86Y." Infect Genet Evol **6**(4): 309-14.
- Holmgren, G., J. Hamrin, et al. (2007). "Selection of pfmdr1 mutations after amodiaquine monotherapy and amodiaquine plus artemisinin combination therapy in East Africa." Infect Genet Evol **7**(5): 562-9.
- Hutagalung, R., L. Paiphun, et al. (2005). "A randomized trial of artemether-lumefantrine versus mefloquine-artesunate for the treatment of uncomplicated multi-drug resistant Plasmodium falciparum on the western border of Thailand." Malar J **4**: 46.
- ICH-GCP (1996). "Guideline for Good Clinical Practice." International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use". Document available at: <http://www.ich.org/LOB/media/MEDIA482.pdf>
- Jafari, S., J. Le Bras, et al. (2004). "Plasmodium falciparum clonal population dynamics during malaria treatment." J Infect Dis **189**(2): 195-203.
- Jafari-Guemouri, S., C. Boudin, et al. (2006). "Plasmodium falciparum genotype population dynamics in asymptomatic children from Senegal." Microbes Infect **8**(7): 1663-70.
- Jambou, R., E. Legrand, et al. (2005). "Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6." Lancet **366**(9501): 1960-3.
- Kallander, K., J. Nsungwa-Sabiiti, et al. (2004). "Symptom overlap for malaria and pneumonia--policy implications for home management strategies." Acta Trop **90**(2): 211-4.

- Kanya, M. R., A. Yeka, et al. (2007). "Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment of malaria: a randomized trial." PLoS Clin Trials **2**(5): e20.
- Kitchen, L. W., D. W. Vaughn, et al. (2006). "Role of US military research programs in the development of US Food and Drug Administration--approved antimalarial drugs." Clin Infect Dis **43**(1): 67-71.
- Klayman, D. L. (1985). "Qinghaosu (artemisinin): an antimalarial drug from China." Science **228**(4703): 1049-55.
- Koram, K. A., B. Abuaku, et al. (2005). "Comparative efficacy of antimalarial drugs including ACTs in the treatment of uncomplicated malaria among children under 5 years in Ghana." Acta Trop **95**(3): 194-203.
- Krishna, S., C. J. Woodrow, et al. (2006). "Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance." Trends Mol Med **12**(5): 200-5.
- Krotoski, W. A. (1989). "The hypnozoite and malarial relapse." Prog Clin Parasitol **1**: 1-19.
- Kublin, J. G., J. F. Cortese, et al. (2003). "Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi." J Infect Dis **187**(12): 1870-5.
- Kublin, J. G., F. K. Dzinjalama, et al. (2002). "Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria." J Infect Dis **185**(3): 380-8.
- Kwiek, J. J., A. P. Alker, et al. (2007). "Estimating true antimalarial efficacy by heteroduplex tracking assay in patients with complex *Plasmodium falciparum* infections." Antimicrob Agents Chemother **51**(2): 521-7.
- Lefevre, G., S. Looareesuwan, et al. (2001). "A clinical and pharmacokinetic trial of six doses of artemether-lumefantrine for multidrug-resistant *Plasmodium falciparum* malaria in Thailand." Am J Trop Med Hyg **64**(5-6): 247-56.
- Marsh, K. (1992). "Malaria--a neglected disease?" Parasitology **104** Suppl: S53-69.
- McCall, M. B., A. J. Beynon, et al. (2006). "No hearing loss associated with the use of artemether-lumefantrine to treat experimental human malaria." Trans R Soc Trop Med Hyg **100**(12): 1098-104.
- Metselaar, D. and P. M. Van Thiel (1959). "Classification of malaria." Tropical and Geographic Medicine(11): 157-161.

- Missinou, M. A., J. F. Kun, et al. (2004). "No change in parasite genotype during single episodes of Plasmodium falciparum malaria in Gabonese children." Parasitol Res **93**(4): 322-7.
- MMV/WHO (2008). "Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations." Document available at: http://www.mmv.org/article.php?id_article=472.
- Moody, A. (2002). "Rapid diagnostic tests for malaria parasites." Clin Microbiol Rev **15**(1): 66-78.
- Msellem, I., G. Rotllant, et al. (Submitted). "Rapid malaria diagnostic tests - treatment and health outcome in fever patients, Zanzibar."
- Mubi, M., A. Janson, et al. (Manuscript). "Rapid diagnostic testing by Community Health Workers improves targeting of artemisinin-based combination therapy for malaria: a community based trial in rural Tanzania."
- Mugittu, K., G. Priotto, et al. (2007). "Molecular genotyping in a malaria treatment trial in Uganda - unexpected high rate of new infections within 2 weeks after treatment." Trop Med Int Health **12**(2): 219-23.
- Mullis, K., F. Faloona, et al. (1986). "Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction." Cold Spring Harb Symp Quant Biol **51 Pt 1**: 263-73.
- Mung'Ala-Odera, V., R. W. Snow, et al. (2004). "The burden of the neurocognitive impairment associated with Plasmodium falciparum malaria in sub-saharan Africa." Am J Trop Med Hyg **71**(2 Suppl): 64-70.
- Murray, C. K., D. Bell, et al. (2003). "Rapid diagnostic testing for malaria." Trop Med Int Health **8**(10): 876-83.
- Mutabingwa, T., A. Nzila, et al. (2001). "Chlorproguanil-dapsone for treatment of drug-resistant falciparum malaria in Tanzania." Lancet **358**(9289): 1218-23.
- Mutabingwa, T. K., D. Anthony, et al. (2005). "Amodiaquine alone, amodiaquine+sulfadoxine-pyrimethamine, amodiaquine+artesunate, and artemether-lumefantrine for outpatient treatment of malaria in Tanzanian children: a four-arm randomised effectiveness trial." Lancet **365**(9469): 1474-80.
- Mwangi, J. M., S. A. Omar, et al. (2006). "Comparison of microsatellite and antigen-coding loci for differentiating recrudescing Plasmodium falciparum infections from reinfections in Kenya." Int J Parasitol **36**(3): 329-36.

- Myint, H. Y., P. Tipmanee, et al. (2004). "A systematic overview of published antimalarial drug trials." Trans R Soc Trop Med Hyg **98**(2): 73-81.
- Navaratnam, V., S. M. Mansor, et al. (2000). "Pharmacokinetics of artemisinin-type compounds." Clin Pharmacokinet **39**(4): 255-70.
- Neftel, K. A., W. Woodtly, et al. (1986). "Amodiaquine induced agranulocytosis and liver damage." Br Med J (Clin Res Ed) **292**(6522): 721-3.
- Newton, C. R., T. T. Hien, et al. (2000). "Cerebral malaria." J Neurol Neurosurg Psychiatry **69**(4): 433-41.
- Ngasala, B., M. Mubi, et al. (2008). "Impact of training in clinical and microscopy diagnosis of childhood malaria on antimalarial drug prescription and health outcome at primary health care level in Tanzania: a randomized controlled trial." Malar J **7**: 199.
- Njama-Meya, D., M. R. Kanya, et al. (2004). "Asymptomatic parasitaemia as a risk factor for symptomatic malaria in a cohort of Ugandan children." Trop Med Int Health **9**(8): 862-8.
- Nosten, F., R. McGready, et al. (1999). "Effects of Plasmodium vivax malaria in pregnancy." Lancet **354**(9178): 546-9.
- Nosten, F., M. van Vugt, et al. (1998). "Intrarectal artemisinin derivatives." Med Trop (Mars) **58**(3 Suppl): 63-4.
- Nosten, F. and N. J. White (2007). "Artemisinin-based combination treatment of falciparum malaria." Am J Trop Med Hyg **77**(6 Suppl): 181-92.
- Nsoby, S. L., M. Kiggundu, et al. (2008). "Complexity of Plasmodium falciparum Clinical Samples from Uganda during Short-Term Culture." J Infect Dis.
- Nyachio, A., V. A. N. O. C, et al. (2005). "Plasmodium falciparum genotyping by microsatellites as a method to distinguish between recrudescence and new infections." Am J Trop Med Hyg **73**(1): 210-3.
- O'Dempsey, T. J., T. F. McArdle, et al. (1993). "Overlap in the clinical features of pneumonia and malaria in African children." Trans R Soc Trop Med Hyg **87**(6): 662-5.
- Ohr, C., L. Mirabelli-Primdahl, et al. (1997). "Distinguishing Plasmodium falciparum treatment failures from reinfections by restriction fragment length polymorphism and polymerase chain reaction genotyping." Am J Trop Med Hyg **57**(4): 430-7.
- Olliaro, P., C. Nevill, et al. (1996). "Systematic review of amodiaquine treatment in uncomplicated malaria." Lancet **348**(9036): 1196-201.

- Piola, P., C. Fogg, et al. (2005). "Supervised versus unsupervised intake of six-dose artemether-lumefantrine for treatment of acute, uncomplicated Plasmodium falciparum malaria in Mbarara, Uganda: a randomised trial." Lancet **365**(9469): 1467-73.
- Plowe, C. V., A. Djimde, et al. (1995). "Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa." Am J Trop Med Hyg **52**(6): 565-8.
- Price, R., M. van Vugt, et al. (1999). "Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives." Am J Trop Med Hyg **60**(4): 547-55.
- Price, R. N., F. Nosten, et al. (1996). "Effects of artemisinin derivatives on malaria transmissibility." Lancet **347**(9016): 1654-8.
- Price, R. N., A. C. Uhlemann, et al. (2006). "Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria." Clin Infect Dis **42**(11): 1570-7.
- Reyburn, H., H. Mbakilwa, et al. (2007). "Rapid diagnostic tests compared with malaria microscopy for guiding outpatient treatment of febrile illness in Tanzania: randomised trial." Bmj **334**(7590): 403.
- Ringwald, P. (2004). "Monitoring antimalarial drug efficacy." Clin Infect Dis **38**(8): 1192-3; author reply 1193-4.
- Ringwald, P. and L. K. Basco (1999). "Comparison of in vivo and in vitro tests of resistance in patients treated with chloroquine in Yaounde, Cameroon." Bull World Health Organ **77**(1): 34-43.
- Rodger, A. J., G. S. Cooke, et al. (2008). "Cluster of falciparum malaria cases in UK airport." Emerg Infect Dis **14**(8): 1284-6.
- Rogerson, S. J., V. Mwapasa, et al. (2007). "Malaria in pregnancy: linking immunity and pathogenesis to prevention." Am J Trop Med Hyg **77**(6 Suppl): 14-22.
- Saiki, R. K., D. H. Gelfand, et al. (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science **239**(4839): 487-91.
- Singh, B., L. Kim Sung, et al. (2004). "A large focus of naturally acquired Plasmodium knowlesi infections in human beings." Lancet **363**(9414): 1017-24.

- Sisowath, C., P. E. Ferreira, et al. (2007). "The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa." Trop Med Int Health **12**(6): 736-42.
- Sisowath, C., I. M. Petersen, et al. (In Press). "In vivo selection of *P. falciparum* parasites carrying the chloroquine-sensitive *pfprt* K76 allele upon treatment with artemether-lumefantrine in Africa."
- Sisowath, C., J. Stromberg, et al. (2005). "In vivo selection of *Plasmodium falciparum* *pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem)." J Infect Dis **191**(6): 1014-7.
- Skinner, T. S., L. S. Manning, et al. (1996). "In vitro stage-specific sensitivity of *Plasmodium falciparum* to quinine and artemisinin drugs." Int J Parasitol **26**(5): 519-25.
- Skinner-Adams, T. S., J. S. McCarthy, et al. (2008). "HIV and malaria co-infection: interactions and consequences of chemotherapy." Trends Parasitol **24**(6): 264-71.
- Smythe, J. A., R. L. Coppel, et al. (1991). "Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2." Proc Natl Acad Sci U S A **88**(5): 1751-5.
- Snounou, G. and H. P. Beck (1998). "The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment." Parasitology Today **14**(11): 462-467.
- Snounou, G., X. Zhu, et al. (1999). "Biased distribution of *msh1* and *msh2* allelic variants in *Plasmodium falciparum* populations in Thailand." Trans R Soc Trop Med Hyg **93**(4): 369-74.
- Steketee, R. W., J. J. Wirima, et al. (1996). "The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi." Am J Trop Med Hyg **55**(1 Suppl): 33-41.
- Stepniewska, K., W. R. Taylor, et al. (2004). "In vivo assessment of drug efficacy against *Plasmodium falciparum* malaria: duration of follow-up." Antimicrob Agents Chemother **48**(11): 4271-80.
- Stepniewska, K. and N. J. White (2006). "Some considerations in the design and interpretation of antimalarial drug trials in uncomplicated *falciparum* malaria." Malar J **5**: 127.
- Tanabe, K., M. Mackay, et al. (1987). "Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*." J Mol Biol **195**(2): 273-87.

- Taylor, W. R. and N. J. White (2004). "Antimalarial drug toxicity: a review." Drug Saf **27**(1): 25-61.
- Toovey, S. and A. Jamieson (2004). "Audiometric changes associated with the treatment of uncomplicated falciparum malaria with co-artemether." Trans R Soc Trop Med Hyg **98**(5): 261-7; discussion 268-9.
- Trape, J. F. (2001). "The public health impact of chloroquine resistance in Africa." Am J Trop Med Hyg **64**(1-2 Suppl): 12-7.
- van den Broek, I., C. Kitz, et al. (2006). "Efficacy of three artemisinin combination therapies for the treatment of uncomplicated Plasmodium falciparum malaria in the Republic of Congo." Malar J **5**: 113.
- van Vugt, M., A. Brockman, et al. (1998). "Randomized comparison of artemether-benflumetol and artesunate-mefloquine in treatment of multidrug-resistant falciparum malaria." Antimicrob Agents Chemother **42**(1): 135-9.
- van Vugt, M., F. Ezzet, et al. (1999). "No evidence of cardiotoxicity during antimalarial treatment with artemether-lumefantrine." Am J Trop Med Hyg **61**(6): 964-7.
- van Vugt, M., S. Looareesuwan, et al. (2000). "Artemether-lumefantrine for the treatment of multidrug-resistant falciparum malaria." Trans R Soc Trop Med Hyg **94**(5): 545-8.
- Waller, D., S. Krishna, et al. (1995). "Clinical features and outcome of severe malaria in Gambian children." Clin Infect Dis **21**(3): 577-87.
- Warrell, D. A. (1999). "Management of severe malaria." Parassitologia **41**(1-3): 287-94.
- Warrell, D. A. and H. M. Gilles (2002). Essential Malariology. Fourth Edition.
- Wernsdorfer, W. H. (2004). "Coartemether (artemether and lumefantrine): an oral antimalarial drug." Expert Rev Anti Infect Ther **2**(2): 181-96.
- Wernsdorfer, W. H. and H. Noedl (2003). "Molecular markers for drug resistance in malaria: use in treatment, diagnosis and epidemiology." Curr Opin Infect Dis **16**(6): 553-8.
- White, N. J. (1997). "Assessment of the pharmacodynamic properties of antimalarial drugs in vivo." Antimicrob Agents Chemother **41**(7): 1413-22.
- White, N. J. (1999). "Delaying antimalarial drug resistance with combination chemotherapy." Parassitologia **41**(1-3): 301-8.
- White, N. J. (2008). "How antimalarial drug resistance affects post-treatment prophylaxis." Malar J **7**: 9.

- White, N. J. (2008). "Plasmodium knowlesi: the fifth human malaria parasite." Clin Infect Dis **46**(2): 172-3.
- White, N. J., F. Nosten, et al. (1999). "Averting a malaria disaster." Lancet **353**(9168): 1965-7.
- White, N. J., K. Stepniewska, et al. (2008). "Simplified antimalarial therapeutic monitoring: using the day-7 drug level?" Trends Parasitol **24**(4): 159-63.
- WHO (1986). "WHO Expert Committee on Malaria, Eighteen Report."
- WHO (1996). "Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas of intense transmission" Document WHO/MAL/96.1077.
- WHO (2000a). "Millennium Development Goals."
Document available at : <http://www.un.org/millenniumgoals/>.
- WHO (2000b). "Management of severe malaria: A practical handbook."
Document available at: <http://www.who.int/malaria/docs/hbsm.pdf>.
- WHO (2001). "Monitoring Antimalarial Drug Resistance, Report of a WHO consultation." Document available at:
<http://www.who.int/malaria/whomalariapublications.htm#2001>.
- WHO (2003a). "The Abuja Declaration and the Plan of Action." Document available at: <http://www.who.int/malaria/whomalariapublications.htm#2003>.
- WHO (2003b). "Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria." Document available at:
<http://www.who.int/malaria/docs/ProtocolWHO.pdf>.
- WHO (2005). "Susceptibility of Plasmodium falciparum to antimalarial drugs. Report on global monitoring : 1996-2004." Document available at:
<http://www.who.int/malaria/whomalariapublications.htm#2005>.
- WHO (2006). "Guidelines for the treatment of malaria." Document available at:
<http://www.who.int/malaria/docs/TreatmentGuidelines2006.pdf>.
- WHO (2008a). "THE GLOBAL MALARIA ACTION PLAN - For a malaria free world." Document available at: <http://www.rbm.who.int/gmap/gmap.pdf>.
- WHO (2008b). "Global burden of disease: 2004 update."
Document available at: www.who.int/healthinfo/bodestimates/en/index.html.
- WHO (2008c). "World Malaria Report." Document available at:
<http://www.who.int/malaria/whomalariapublications.htm#2008>.
- WMA (2008). "Declaration of Helsinki." The World Medical Association
Document available at: <http://www.wma.net/e/policy/b3.htm>.

Zongo, I., G. Dorsey, et al. (2007). "Randomized comparison of amodiaquine plus sulfadoxine-pyrimethamine, artemether-lumefantrine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated Plasmodium falciparum malaria in Burkina Faso." Clin Infect Dis **45**(11): 1453-61.