Diagnosis, prognosis and treatment of severe falciparum malaria in African children
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Chapter 1

Introduction and scope of the thesis
Introduction

Africa remains the region with the greatest burden of malaria cases and deaths in the world. The WHO estimated 890,000 malaria deaths in 2004, of which 91% were in Africa and 771,000 (85%) were in children under 5 years of age.2 Efforts to quantify the burden of malaria have shown highly variable results. A recently published paper estimated much higher malaria mortality in children >5 years and adults.3 Despite a global reduction of malarious areas over the last century, the malaria-related mortality has increased substantially in the eighties and nineties, with a peak in 2004.3,4 This increased mortality has been associated with resistance to antimalarial drugs, first chloroquine and later sulfadoxine-pyrimethamine (Fansidar) that originated in southeast Asia and have swept across Africa5,6 and the emerging HIV-1 pandemic in sub-Saharan Africa.7

In sub-Saharan Africa, severe malaria and malaria-attributable mortality are almost exclusively caused by P. falciparum, one of the 5 Plasmodium species known to infect humans.8 Once falciparum malaria takes a complicated course, the mortality is high and many children die before reaching a health facility. Hospital studies report case fatality rates between 10 and 20% despite antimalarial treatment, with the majority of deaths (2/3) occurring within the first 24 hours of admission.9-11 Below follows a brief review of the pathogenesis, diagnosis, host immunity, clinical presentation and treatment of severe falciparum malaria.

Pathogenesis of severe malaria

Plasmodium falciparum infection can result in asymptomatic parasitaemia; clinical malaria (febrile uncomplicated disease) and severe malaria (e.g. complicated disease marked by multi-organ failure, severe anaemia, acidosis) that may subsequently lead to death. The course and clinical outcome of the P. falciparum infection is determined by many factors that include parasite factors (e.g. cytoadherence, sequestration), host factors (malaria-specific immunity, age) and geographical and social aspects (e.g. distance to health facilities, health care seeking behaviour).
The infection

*Plasmodium falciparum* is transmitted via the bite of an infected female *Anopheles* mosquito, which injects the sporozoite form of the parasite when probing for a blood meal. After inoculation the parasite hides and replicates in the liver for an average of 5.5 days, after which $10^5$ to $10^6$ merozoites are released into the bloodstream. The merozoites invade the circulating erythrocytes, where the erythrocytic cycle of the parasite begins. The parasite matures from a small ring form to the pigment (end-product of the haemoglobin digestion) containing trophozoite, which undergoes multiple nuclear divisions, finally developing into the schizont stage. After approximately 48 hours the red blood cell contains 8-32 merozoites and bursts, destroying the red cell. The released merozoites subsequently infect other red blood cells to start a new asexual cycle. This gives an exponential expansion of the infection in the human host, with a multiplication factor of around 10 per cycle, as observed in early studies with *P. falciparum* as a treatment for neurosyphilis. Once the parasite number has increased to around $10^{10}$ parasites (approximately 13 days after inoculation), the patient starts to have fever. In the non-immune patient, the disease can quickly progress into severe disease if untreated, with an increase in parasite burden estimated to average $10^{12}$ till $10^{13}$ in adults. A small portion of the invading merozoites develop into gametocytes, the sexual form of the parasites, which can infect a biting mosquito and continue the transmission cycle.

Cytoadherence and sequestration

During the erythrocytic cycle, the maturing parasite progressively alters the functions and physical characteristics of the host red blood cell. One of these mechanisms is the transport and insertion of parasite proteins into the erythrocyte membrane, most importantly the transmembrane protein *P. falciparum* erythrocyte membrane protein-1 (*PfEMP1*) which is associated with the formation of “knobs” from the erythrocyte membrane. This protein acts as a ligand for attachment of the infected red blood cell to the vascular endothelium. Cytoadherence begins approximately 12-14 hours after merozoite invasion and is progressive in the second two-thirds of the parasite life cycle. Once infected red blood cells adhere, they remain stuck until rupture at schizogony. This process is known as sequestration and predominantly takes place in the venules and capillaries of the vital organs, being greatest in the brain, but also prominent in the heart, eyes, liver, kidneys, intestines and adipose tissue. The heterogeneous pattern of sequestration between and within tissues is associated with differences in the expression of various endothelial receptors, for example ICAM-1. A number of other pathophysiological processes contribute to the microcirculatory obstruction like rosetting.
(infected erythrocytes adhering to uninfected erythrocytes), aggregation (platelet-mediated clumping of infected erythrocytes) and reduced red cell deformability of infected as well as uninfected erythrocytes. The microcirculatory obstruction results in hypoxia, metabolic disturbances, and multi-organ failure. Coma and acidosis are clinical manifestations of this process and are amongst the strongest predictors of death.

**Diagnosis**

**Microscopy**

Microscopy has been the gold standard for malaria diagnosis since the discovery of the intra-erythrocytic malaria parasites by the French malarialogist Alphonse Laveran in 1880. The diagnosis of falciparum malaria relies on microscopic examination of the Field’s or Giemsa stained thin and thick peripheral blood film. Parasite densities can be calculated by counting of the number of infected red blood cells against white blood cells on the thick film or red blood cells on the thin film. In addition, the type and developmental stages of the parasite can be identified on the thin film. Due to sequestration, the late stages of the parasite disappear from the peripheral circulation and therefore these forms are only sparsely detected on the peripheral blood film. When they do appear in significant numbers (>20% of the total parasites) this is a poor prognostic sign representing a large sequestered parasite load. In addition, neutrophils containing malaria pigment (in >5% of the neutrophils) reflect recent schizogony and also have prognostic significance in severe malaria. Reliable microscopy requires the preparation of good quality slides and reagents and the presence of a microscope with an experienced microscopist. The quality of routine microscopy in African settings has been disputed.

**Malaria rapid diagnostic tests**

In recent years, numerous malaria rapid diagnostic tests have been brought onto the market. These tests are based on antibody detection of malaria specific antigens; *Plasmodium falciparum* Histidine-Rich Protein-2 (PfHRP2), *Plasmodium* lactate dehydrogenase (pLDH) and/or aldolase. The histidine-rich proteins were the first plasmodial proteins to be studied in detail. Three types of histidine-rich proteins have been described; PfHRP1 (the knob-associated HRP), PfHRP2 and PfHRP3. PfHRP2 is the most abundant protein, which is water-soluble and predominantly produced by the asexual stages. PfHRP3 is closely related to PfHRP2 and cross reacts with epitopes of the PfHRP2 based antibody tests. The pLDH-
based tests detect the *Plasmodium* intracellular metabolic enzyme LDH, either the pan-plasmodial LDH or the *Plasmodium falciparum* specific form and both antibodies are usually present in rapid diagnostic tests that therefore allow species identification. The *Pf*HRP2 and the pLDH based test have been evaluated most extensively and have similar diagnostic sensitivities to microscopy for the diagnosis of uncomplicated malaria. The *Pf*HRP2-based RDTs have the highest sensitivities, but lower specificities than the pLDH based RDTs. Their specificity is compromised by persistent *Pf*HRP2 antigenicity after parasite clearance with reported duration up to 4 weeks. This can result in false-positive results in patients after a recent malaria attack, which is of particular concern in high transmission settings. In contrast, pLDH has a very short half-life related to the presence of alive malaria parasites and can therefore also be used for treatment control. A general disadvantage of rapid diagnostic tests is the inability to assess parasitaemia and parasite stages.

**Plasma *Pf*HRP2 as a marker of parasite burden**

*Plasmodium falciparum* specific sequestration increasingly renders the most pathogenic parasites from the second half of the erythrocytic cycle invisible to the microscopist observing the peripheral blood film. As a consequence, the peripheral parasite count will underestimate the total number of parasites in the body. This discrepancy increases as the parasite multiplication and malaria infection progresses and therefore peripheral blood parasitaemia is only a weak predictor of mortality in falciparum malaria. *Pf*HRP2 is found inside the parasite’s food vacuole in the infected red blood cell. Its production increases mostly during the development into the trophozoite stage. At the moment of schizont rupture, approximately 90% of the total produced amount per erythrocytic cycle is released into the plasma where it persists for several days. During the acute malaria infection, a large amount of *Pf*HRP2 is located within asexual parasites associated with the exponential rise in parasitaemia that occurs during the development of *P. falciparum* and whole-blood *Pf*HRP2 concentrations therefore correlate with peripheral blood parasitaemia. By contrast, the much smaller amount of *Pf*HRP2 in the plasma provides a summative picture of previous schizont ruptures that reflects the sequestered burden rather than the circulating parasite burden. Quantification of *Pf*HRP2 in plasma has been shown to provide a method of assessing the hidden parasites and the total body parasite burden in Asian adults. The exact role of *Pf*HRP2 has not yet been fully understood, but it has been implicated as a heme polymerase that detoxifies the free heme by its polymerization to the inactive hemozoin. The *Pf*HRP2 gene sequences are highly variable and can even be deleted.
Remarkably, its presence seems not essential to the survival of the malaria parasite, since *Pf*HRP2 gene deletions have been reported in various in-vitro cultured and patient-derived *P. falciparum* strains from the Peruvian Amazon. Very recently, a first report was made of false-negative *Pf*HRP2-based RDT results attributable to *Pf*HRP2 gene deletions in Africa. Simultaneous infection with multiple *P. falciparum* strains is likely to mask the in vivo existence of *Pf*HRP2 deletions. *Pf*HRP2 sequence variations do not appear to affect the sensitivity of *Pf*HRP2-based RDT.

**Host immunity**

Host immunity is an important yet incompletely understood determinant of the outcome of malaria infection. Malaria-specific immunity is dependent on the innate immunity (host genetics, e.g. RBC polymorphisms) and acquired immunity which develops with exposure and is affected by malaria transmission intensity, age, parasite density, pregnancy and HIV infection. From the era of malaria therapy for neurosyphilis it has been known that this acquired immunity is strain-specific. The development of immunity requires repeated exposure to the local malaria parasite strains. Protection against death or severe disease develops already after limited exposure, but more slowly against clinical disease and sterile immunity is probably never achieved. In controlling the acute infection, non-specific host defence mechanisms and the later development of more specific and cell-mediated and humoral responses are both important. The “anti-toxic” immunity includes the down-regulation of non-specific mechanisms like pro-inflammatory cytokine release. Eventually this will lead to “premunition”; a state of tolerance to erythrocytic malaria parasites without fever or symptoms. More specific “anti-parasite” immune responses involve the formation of antibodies to merozoite antigens (e.g. the invasion antigens: MSP1 & 2 and AMA-1) or targets expressed on the infected red blood cell surface (the variant surface antigens (VSA), most importantly *Pf*EMP1). However, these targets are all extremely polymorphic and variant and hence numerous synergistic immune responses are generated. In summary, the immune responses are complex and there are no good markers to quantify the malaria specific immunity. However, an understanding of immunity is important, since it can raise or lower apparent cure rates and therefore is a determinant of antimalarial drug efficacy. HIV infection causes chronic depletion of CD4+ cells and there is evidence that this affects the malaria specific immunity, although much is unknown about the underlying immune mechanisms. Clinical studies have shown that HIV coinfection is associated with a higher incidence of clinical malaria, severe malaria and malaria-related mortality,
particularly in adults with deteriorating immune status as evidenced by declining CD4+ counts.41,55-58 These effects have been less well described in children who experience frequent clinical malaria episodes anyway,59,60 although HIV coinfection seems to increase severe malaria associated mortality.61,62 Conversely, *P. falciparum* infection also accelerates HIV transmission and progression.63,64 The geographical overlap of HIV and malaria and their reciprocal impacts have been postulated to enhance the spread of both diseases.7,65,66

**Clinical presentation of severe malaria**

The clinical features of severe malaria include impaired consciousness or unrousable coma, prostration (generalized weakness), multiple convulsions, acidosis (deep and laboured breathing pattern), severe anaemia, shock, jaundice and haemoglobinuria. The WHO definition includes the following laboratory findings: hypoglycaemia, metabolic acidosis (plasma bicarbonate <15 mmol/L), severe anaemia (haemoglobin <5 g/dL, haematocrit <15%), hyperlactataemia (lactate >5 mmol/L), renal impairment (serum creatinine >265 μmol/L), haemoglobinuria or hyperparasitaemia.67

In African children, the major clinical manifestations are severe anaemia, cerebral malaria and metabolic acidosis. However, the disease pattern and the relative contribution of individual symptoms to mortality differ with transmission intensity and age, among other factors. In high transmission settings, the most common clinical presentation of severe malaria is severe anaemia in infants and young children. In lower transmission areas the most common clinical presentation is cerebral malaria (unrousable coma and/or convulsions in the presence of *Pf* parasitaemia) in older children, and also severe disease in adults. The lower the transmission intensity, the slower the development of malaria-specific immunity and severe disease will occur at older age.68 This age-shift phenomenon of severe disease has also been described in places where malaria transmission has declined over time.69

Irrespective of exposure, age affects the clinical presentation of severe malaria.70 Asian adults have been described with different severe malaria complications and have more pulmonary oedema, liver failure (jaundice) and renal failure (due to tubular necrosis) compared to African children and their mortality increases with age.71,72

Another difference in the severe malaria syndrome between children and adults is the increased susceptibility to bacterial infections in children with malaria. Positive blood cultures have been reported in 4.6 to 12.6% of children with malaria.62,73-77 This is part of a major diagnostic problem in high transmission settings, where asymptomatic parasitaemia is common and invasive bacterial disease can be the cause of illness with
coincidental parasitaemia or be a concomitant disease in “true” severe malaria. In these settings, a positive peripheral blood smear thus lacks specificity and generally blood cultures lack sensitivity. In addition, the clinical symptoms of severe malaria and sepsis or pneumonia are overlapping. A study in Kenya showed that 20% of in-hospital malaria mortality was associated with bacterial disease. Malaria, particularly in association with severe anaemia or HIV-coinfection predisposes to gram-negative bacteraemia, predominantly non-typhi Salmonella infections. It has been postulated that sequestration of the malaria parasites in the gut impairs the normal defences against bacterial invasion. Salmonella infections have been reported to follow a recent malaria episode (evidenced by detectable Plasmodium falciparum histidine-rich protein-2 in the absence of parasitaemia), rather than occur with current malaria. The association between malaria and bacteraemia is further strengthened by observations that declining malaria transmission also reduced all-cause paediatric admissions and mortality.

**Treatment of severe malaria**

**Quinine**

Quinine has remained the most important treatment for severe malaria since the discovery of the Cinchona alkaloids derived from the bark of the Peruvian tree in the 17th century. It became in disuse in the 1950s by the introduction of the synthetic antimalarial chloroquine. However, within 12 years resistance to chloroquine developed in Southeast Asia and South America, subsequently spread to Africa in the 1970s, and fuelled the burden of malaria worldwide. Since then, quinine resumed its primary role as sole treatment for severe malaria. Despite some evidence for declining quinine efficacy in South east Asia in terms of parasite and fever clearance and coma recovery times, there is no evidence that this has translated into a rise of mortality under quinine treatment. There are no convincing reports of high grade quinine resistance in the treatment of severe malaria.

The antimalarial mechanism of action of quinine is unknown, but it acts principally on the mature trophozoite stage of parasite development. It does not prevent sequestration or further development of circulating ring stages and does not kill the pre-erythrocytic or sexual stages of *P. falciparum*. Quinine is usually formulated as dihydrochloride salt for parenteral administration, which is given either by intramuscular injection or intravenous infusion. The WHO recommended dosing includes a loading dose of
20 mg/kg in order to achieve prompt therapeutic plasma concentrations, followed by 10 mg/kg every eight hours. Quinine has a narrow therapeutic range. Intravenous administration requires rate-controlled infusion, which may be challenging in the setting of a busy African hospital. Intramuscular administration is a suitable alternative, resulting in rapid absorption and peak concentrations within 4 hours. However, intramuscular administration is painful, may cause sterile abscesses and predispose to lethal tetanus. Quinine is distributed throughout the body fluids and is highly protein bound, mainly to alpha-1 acid glycoprotein. The binding capacity in plasma is concentration dependent, but also depends on the levels of alpha-1 acid glycoprotein, which are positively associated with severity. Only the unbound quinine fraction is responsible for the therapeutic and toxic actions. Serious toxicity is rare in the treatment of severe malaria.

Artesunate

While the Cinchona alkaloids became widely used antimalarial treatment in Europe and Africa from the 17th century, the extracts of the plant qinghao; Artemesia annua were known to cure fevers in China for over 2 millennia. The specific antimalarial properties of artemisinin were discovered by Chinese scientists in the 1970s. The artemisinins kill nearly all erythrocytic developmental stages of the parasite, and also have a gametocidal effect. Their effect on the young circulating rings results in a rapid reduction in parasitaemia compared with other antimalarials, and prevents the development to the more pathological mature parasites that sequester.

There are various formulations of the artemisinins; the oil-soluble derivatives artemether and artepotil (formerly known as arteether), the water-soluble artesunate and dihydro-artemisinin (DHA). The former 3 are all converted to the biologically active metabolite DHA within the body. In the early 1990s, artemether was strongly supported over artesunate by the WHO because a GMP formulation of the former was being developed, and artemether was considered more practical. This preference was declared before evidence of clinical benefit or data on human pharmacokinetics became available. The first large clinical trials compared intramuscular artemether versus quinine. Artemether was safer and easier to use than quinine, but although overall survival was better, it was not statistically significantly better than quinine. In the subgroup analysis,
artemether did significantly reduce mortality in Southeast Asian adults, but did not in African children.\textsuperscript{103} Unfortunately intramuscular artemether was not the best artemisinin formulation to choose, since it is an oil-based formulation, which may release the drug slowly and erratically from the injection site.\textsuperscript{104,105} In contrast, the water-soluble artesunate can be given intravenously and is instantly bioavailable, and intramuscular artesunate is absorbed reliably and rapidly with peak concentrations occurring within one hour.\textsuperscript{105,106} Moreover, artesunate is a more potent antimalarial than artemether.\textsuperscript{107} To provide conclusive data, a large multinational randomised comparison of parenteral artesunate versus parenteral quinine in Southeast Asian adults with severe malaria (“SEAQUAMAT”) was conducted.\textsuperscript{108} This randomized comparative trial was the largest ever in severe malaria and enrolled 1461 patients, of whom 202 were children. The trial showed that parenteral artesunate compared to quinine reduced the mortality of severe malaria by 35\% (relative reduction) in adults (\(p=0.0002\)). In the subgroup analysis, children treated with artesunate had similar mortality reduction, but this did not reach statistical significance related to the small sample size.\textsuperscript{108} Besides higher antimalarial potency, the advantages of artesunate over quinine are the ease of administration, because it can be given as a bolus intravenous injection or intramuscular injection, and the much broader therapeutic window.\textsuperscript{109} Following the SEAQUAMAT trial, intravenous artesunate became the recommended treatment for severe malaria in adults.\textsuperscript{110} However, malaria experts were not convinced that these results could be translated to African children, based on the results of the earlier artemether-quinine trials\textsuperscript{103} and the more fulminant disease course in African children.
Scope of the thesis

The ongoing high burden of severe falciparum malaria and its mortality in sub-Saharan Africa urges a search for better treatment strategies, that are feasible in resource-limited settings. The overall objectives of the studies presented in this thesis are aimed to improve the diagnosis and identification of patients at highest risk of death of severe malaria, and to study efficacious antimalarial treatment with optimum dosing in the target population.

The “African Quinine versus Artesunate Malaria Trial” (“AQUAMAT”) was the parent study for this thesis; a large multicentre trial comparing artesunate versus quinine for the treatment of severe malaria in African children (Chapter 2). This study is the sister study of the SEAQUAMAT trial in Asia. We hypothesized that artesunate would be a better treatment for severe malaria than quinine, and that its pharmacodynamic advantages and safer and easier drug administration will be translated into reduction of mortality in African children. The study was conducted in 11 sites in 9 countries in Africa, including sites of varying malaria transmission intensities, co-morbidities (including HIV) and levels of care (ranging from academic teaching hospitals to rural district hospitals). The primary outcome of this study was mortality. This trial provided excellent opportunities to study additional aspects about the diagnosis, prognosis and treatment of severe malaria in African children.

The outcome of severe malaria is variable according to age, transmission intensity and clinical presentation and the case fatality usually exceeds 10%. Identification of predictors of mortality among children with severe malaria, independent of study site and population, would be useful to guide triage for clinical management or enrolment into clinical trials. These indicators can also give insight into the pathogenesis and guide the identification of new targets for intervention. We examined the prognostic value of a wide range of admission clinical signs and laboratory parameters in the AQUAMAT trial (Chapter 3).

The currently internationally accepted definition of severe malaria depends on the presence of clinical signs, limited bedside or laboratory investigations and the presence of asexual P. falciparum parasites on the peripheral blood film. In recent years, numerous malaria rapid diagnostic tests have been brought onto the market and been evaluated for the diagnosis of uncomplicated malaria, but not for severe malaria. We compared two types of RDTs, a PfHRP2- and a pLDH-based RDT as an alternative to microscopy.
for the diagnosis of severe malaria in 2 populations in different transmission settings in Africa (Chapter 4).

Parasitological diagnosis by microscopy has an advantage over RDTs, because it allows quantification and the peripheral blood parasitaemia has been very widely used to assess disease severity in malaria. However, the pathogenic sequestered parasites cannot be seen on the peripheral blood film, and therefore peripheral blood parasitaemia is only a weak predictor of mortality in falciparum malaria. We assessed plasma \( PfHRP2 \) as a marker of parasite burden in parasitaemic African children presenting with severe disease. We tested the hypothesis whether plasma \( PfHRP2 \) would be a better prognostic marker than peripheral blood parasitaemia and could distinguish children with “true” severe malaria from those with non-malarial severe febrile illness (Chapter 5).

Following the findings of the study described in Chapter 5, we found that parasitaemic children with clinical severe malaria had an increased risk of death associated with low plasma \( PfHRP2 \) concentrations as well as an increasing risk of death associated with increasing plasma \( PfHRP2 \) concentrations. We hypothesized that children with clinical severe disease but low \( PfHRP2 \) concentrations have coincidental parasitaemia (due to the development of malaria-specific immunity) and have severe non-malarial illness. \( PfHRP2 \) may thus distinguish children with “true” severe malaria from those with alternative diseases and coincidental parasitaemia. We tested this hypothesis by assessing the \( PfHRP2 \) and parasitaemia distributions in healthy and asymptomatic cases, uncomplicated malaria and severe malaria patients, in additional datasets outside the AQUAMAT trial, from a high transmission setting in Tanzania. We aimed to identify plasma \( PfHRP2 \) thresholds that could distinguish asymptomatic parasitaemia from severe disease and a more specific threshold aimed to identify patients with a high probability of having “true” severe malaria (Chapter 6).

Severe malaria with HIV coinfection may be frequently encountered in areas with a high prevalence of both diseases. In Beira, Mozambique, the HIV prevalence was reported to be 30% in sentinel surveys of pregnant women and hospital data indicated an increasing number of admissions and deaths attributable to malaria. We hypothesized that HIV-coinfection is an important determinant of outcome in severe malaria. The diagnosis, clinical presentation and outcome of severe malaria with HIV coinfection were studied in children and adults in Mozambique (Chapter 7).
Optimal treatment strategies include detailed knowledge of the pharmacokinetic (PK) properties of drugs in the target population where the drug is used. Age, disease status and severity may cause differences in the drug absorption, distribution, metabolism and excretion and alter the PK properties of the drug. Children in Africa account for >90% of the malaria case fatality rate worldwide, however antimalarial PK information is often lacking in this group. Dosing regimens for children are often derived from adult studies, which have led to important underdosing. For instance, a PK study on sulfadoxine-pyrimethamine (SP) in African children with uncomplicated falciparum malaria showed that with the usual dose of 25 mg.kg-1/ 1.25 mg.kg-1, the area under the concentration-time curves (AUCs) in children 2 to 5 years old were half of those in adults, which was caused by higher clearance. This might have caused not only antimalarial treatment failure in young children, but also might well have contributed to the spread of resistance. This information has come only decades after introduction of SP. Antimalarial underdosing in severe malaria may have immediate consequences for the patient’s outcome, thus PK studies are of utmost importance in this group.

Conventional PK studies with dense blood sampling is evidently problematic in severely ill children, but the population pharmacokinetic approach can be used, allowing to model sparse, random samples, but from a large number of individuals. The approach considers the typical population profile, rather than the individual as a unit of analysis and allows assessing the quantitative relationships among dose, drug response (PK/PD), patient covariates and variability using a nonlinear mixed-effects modelling.

We have conducted a population pharmacokinetic assessment of quinine (Chapter 8) and artesunate (Chapter 9) in the treatment of paediatric severe falciparum malaria. The model included many co variates to provide insight into the parameters contributing to variation in the PK profile of these antimalarial drugs in African children with severe malaria. Dosing simulations were used to identify practical dosing regimens which may assist in the future deployment of artesunate.
Chapter 1

References

Introduction and scope of the thesis


