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

Ilse C.E. Hendriksen

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

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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. 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.

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. 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 Africa and the emerging HIV-1 pandemic in sub-Saharan Africa.

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. 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.

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 (*Pf*HRP2), *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; *Pf*HRP1 (the knob-associated HRP), *Pf*HRP2 and *Pf*HRP3. *Pf*HRP2 is the most abundant protein, which is water-soluble and predominantly produced by the asexual stages. *Pf*HRP3 is closely related to *Pf*HRP2 and cross reacts with epitopes of the *Pf*HRP2 based antibody tests. The pLDH-
Based tests detect the *Plasmodium* intracellular metabolic enzyme LDH, either the panplasmodial LDH or the *Plasmodium falciparum* specific form and both antibodies are usually present in rapid diagnostic tests that therefore allow species identification. The PfHRP2 and the pLDH based test have been evaluated most extensively and have similar diagnostic sensitivities to microscopy for the diagnosis of uncomplicated malaria. The PfHRP2-based RDTs have the highest sensitivities, but lower specificities than the pLDH based RDTs. Their specificity is compromised by persistent PfHRP2 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 PfHRP2 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. PfHRP2 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 PfHRP2 is located within asexual parasites associated with the exponential rise in parasitaemia that occurs during the development of *P. falciparum* and whole-blood PfHRP2 concentrations therefore correlate with peripheral blood parasitaemia. By contrast, the much smaller amount of PfHRP2 in the plasma provides a summative picture of previous schizont ruptures that reflects the sequestered burden rather than the circulating parasite burden. Quantification of PfHRP2 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 PfHRP2 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 PfHRP2 gene sequences are highly variable and can even be deleted.
Remarkably, its presence seems not essential to the survival of the malaria parasite, since PfHRP2 gene deletions have been reported in various in-vitro cultured and patient-derived P. falciparum strains from the Peruvian Amazon.\textsuperscript{37} Very recently, a first report was made of false-negative PfHRP2-based RDT results attributable to PfHRP2 gene deletions in Africa.\textsuperscript{38} Simultaneous infection with multiple P. falciparum strains is likely to mask the in vivo existence of PfHRP2 deletions. PfHRP2 sequence variations do not appear to affect the sensitivity of PfHRP2-based RDT.\textsuperscript{39}

**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.\textsuperscript{40-43} From the era of malaria therapy for neurosyphilis it has been known that this acquired immunity is strain-specific.\textsuperscript{44} 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.\textsuperscript{45,46} 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.\textsuperscript{47} 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 PfEMP1).\textsuperscript{48-50} 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.\textsuperscript{51} 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.\textsuperscript{52-54} Clinical studies have shown that HIV coinfection is associated with a higher incidence of clinical malaria, severe malaria and malaria-related mortality,
Introduction and scope of the thesis

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 anyway59,60 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 were 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.\textsuperscript{71,87} 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.\textsuperscript{88} However, intramuscular administration is painful, may cause sterile abscesses and predispose to lethal tetanus.\textsuperscript{89} Quinine is distributed throughout the body fluids and is highly protein bound, mainly to alpha-1 acid glycoprotein.\textsuperscript{90} 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.\textsuperscript{91,92} Only the unbound quinine fraction is responsible for the therapeutic and toxic actions. Serious toxicity is rare in the treatment of severe malaria. Quinine exacerbates the malaria-induced orthostatic hypotension and is cardiotoxic as evidenced by a prolongation of the QT interval.\textsuperscript{93,94} Of particular concern in the patient management is the quinine-induced hyperinsulinemia, which can cause life-threatening hypoglycaemia.\textsuperscript{95,96}

\textbf{Artesunate}

While the Cinchona alkaloids became widely used antimalarial treatment in Europe and Africa from the 17\textsuperscript{th} century, the extracts of the plant qinghao; \textit{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.\textsuperscript{97} The artemisinins kill nearly all erythrocytic developmental stages of the parasite, and also have a gametocidal effect.\textsuperscript{85,98} 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.\textsuperscript{99,100} 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.\textsuperscript{101,102} Artemether was safer and easier to use than quinine, but although overall survival was better, it was not statistically significantly better than quinine.\textsuperscript{103} 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.
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Chapter 2

Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial

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Summary

Background Severe malaria is a major cause of childhood death and often the main reason for paediatric hospital admission in sub-Saharan Africa. Quinine is still the established treatment of choice, although evidence from Asia suggests that artesunate is associated with a lower mortality. We compared parenteral treatment with either artesunate or quinine in African children with severe malaria.

Methods This open-label, randomised trial was undertaken in 11 centres in 9 African countries. Children (<15 years) with severe falciparum malaria were randomly assigned to parenteral artesunate or parenteral quinine. Randomisation was in blocks of 20, with study numbers corresponding to treatment allocations kept inside opaque sealed paper envelopes. The trial was open label at each site, and none of the investigators or trialists, apart from for the trial statistician, had access to the summaries of treatment allocations. The primary outcome measure was in-hospital mortality, analysed by intention to treat. This trial is registered, number ISRCTN50258054.

Findings 5425 children were enrolled; 2712 were assigned to artesunate and 2713 to quinine. All patients were analysed for the primary outcome. 230 (8.5%) patients assigned to artesunate treatment died compared with 297 (10.9%) assigned to quinine treatment (odds ratio [OR] stratified for study site 0.75, 95% CI 0.63–0.90; relative reduction 22.5%, 95% CI 8.1–36.9; p=0.0022). Incidence of neurological sequelae did not differ significantly between groups, but the development of coma (65/1832 [3.5%] with artesunate vs 91/1768 [5.1%] with quinine; OR 0.69, 95% CI 0.49–0.95; p=0.0231), convulsions (224/2712 [8.3%] vs 273/2713 [10.1%]; OR 0.80, 0.66–0.97; p=0.0199), and deterioration of the coma score (166/2712 [6.1%] vs 208/2713 [7.7%]; OR 0.78, 0.64–0.97; p=0.0245) were all significantly less frequent in artesunate recipients than in quinine recipients. Post-treatment hypoglycaemia was also less frequent in patients assigned to artesunate than in those assigned to quinine (48/2712 [1.8%] vs 75/2713 [2.8%]; OR 0.63, 0.43–0.91; p=0.0134). Artesunate was well tolerated, with no serious drug-related adverse effects.

Interpretation Artesunate substantially reduces mortality in African children with severe malaria. These data, together with a meta-analysis of all trials comparing artesunate and quinine, strongly suggest that parenteral artesunate should replace quinine as the treatment of choice for severe falciparum malaria worldwide.
Artesunate versus quinine in the treatment of severe falciparum malaria

Introduction

Falciparum malaria is a major contributor to child mortality in Africa and one of the main causes of paediatric hospital admission across sub-Saharan Africa. Many deaths occur in or near the home, but for children who are admitted to hospital with severe malaria and receive parenteral antimalarial treatment, about one in six will die. From the time of its introduction to European medicine in the 1630s until the deployment of parenteral chloroquine in the 1950s, quinine was the mainstay of severe malaria treatment. Resistance to chloroquine emerged in southeast Asia and then spread to Africa at the end of the 1970s. Quinine then resumed its primary role in the treatment of severe malaria. Parenteral quinine has a narrow therapeutic ratio. Intravenous quinine administration needs a constant rate infusion with dosing three times a day. Intramuscular administration is painful, and can cause sterile abscesses and predispose to lethal tetanus. Although blindness and deafness may follow self-poisoning, these side-effects are rare in severe malaria; however, quinine-induced hyperinsulinaemic hypoglycaemia is a particular problem in patient management, especially in pregnant women.

The primacy of quinine in the treatment of severe malaria has been challenged by the introduction of artemisinin derivatives. The first comparative clinical trials were done with intramuscular artemether; a lipophilic derivative of dihydroartemisinin. Artemether proved safer and easier to use than quinine, but did not improve overall survival in an individual patient data meta-analysis of 1919 randomised patients. In a prospectively defined subgroup analysis, artemether reduced mortality significantly in southeast Asian adults, but not in African children. A large multicentre randomised trial (South East Asian Quinine Artesunate Malaria Trial [SEAQUAMAT]), which compared intravenous artesunate with quinine in Asian patients (mainly adults) with severe malaria was then undertaken. It was stopped after enrolment of 1461 patients because of a substantial survival benefit in favour of artesunate. In a meta-analysis of the SEAQUAMAT study and earlier, smaller randomised trials, artesunate reduced the mortality of severe malaria in Asian patients from 23.1% to 14.2%; a relative reduction of 38.6%. The treatment was also highly cost-effective. In 2006, WHO changed its guidelines to recommend artesunate for severe malaria in adults.

The treatment effect in the SEAQUAMAT trial was similar in adults and the 202 children enrolled, but perceived differences in the natural history and drug susceptibility of severe falciparum malaria in African children compared with Asian patients left uncertainty about the optimum treatment for this important patient group. Expert opinion regarded parenteral quinine as a satisfactory treatment, and research priorities focused on
improving other aspects of the care of the sick child. Nowadays quinine remains by far the most widely used treatment of severe malaria in Africa. We undertook a large multicentre randomised trial (African Quinine Artesunate Malaria Trial [AQUAMAT]) that compared parenteral treatment with either artesunate or quinine in African children with severe malaria.

Methods

Study design
This was a multicentre, open-label trial in children admitted to hospital with severe malaria, undertaken between Oct 3, 2005, and July 14, 2010. Eleven centres in 9 countries (Mozambique, The Gambia, Ghana, Kenya, Tanzania, Nigeria, Uganda, Rwanda, and Democratic Republic of the Congo) in Africa participated (Figure S1). The study was coordinated by the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, Thailand, which provided logistic support and data management. All clinicians were familiarised with the severe malaria criteria for enrolment (Panel 1). Most children were managed on general paediatric wards. Children younger than 15 years were included if they had a positive rapid diagnostic test for *Plasmodium falciparum* lactate dehydrogenase (Optimal, Diamed, Cressier, Switzerland) and, in the admitting physician's clinical opinion, they had severe malaria, and they or their attendant relative or guardian gave fully informed written consent. Patients were not included if there was a convincing history of full treatment with parenteral quinine or an artemisinin derivative for more than 24 h before admission. Age criteria varied slightly between sites at the request of the respective ethics review boards (Text S2). In Mozambique, adults were also studied but were analysed separately and will be reported elsewhere. The trial protocol was reviewed and approved by each site’s ethics review board, and by the Oxford Tropical Research Ethics Committee. Permission to investigate HIV status was obtained only from some ethics review boards.

Randomisation and masking
Eligible patients were randomly assigned to treatment with either intravenous or intramuscular artesunate or quinine. Each centre had a policy of using one route of administration. Randomisation was done by people unrelated to the study and provided to the study sites in blocks of 20. Study numbers were kept inside opaque sealed paper envelopes. After full informed written consent was obtained, the next envelope, which
Panel 1: Modified criteria for severe falciparum malaria

At least one of:
- Plasma base excess less than -3.3 mmol/L
- Glasgow coma scale less than 11 of 15, or Blantyre coma scale less than 3 of 5 in preverbal children
- Haemoglobin less than 50 g/L and parasitaemia greater than 100,000 parasites/µL
- Blood urea nitrogen greater than 10 mmol/L.
- Compensated shock; capillary refill ≥3 s or temperature gradient on legs, but no hypotension
- Decompensated shock; systolic blood pressure less than 70 mm Hg and cool peripheries
- Asexual parasitaemia more than 10%
- Visible jaundice and more than 100,000 parasites per µL
- Plasma glucose less than 3 mmol/L
- Respiratory distress, defined as costal indrawing, use of accessory muscles, nasal alar flaring, deep breathing, or severe tachypnoea

Procedures
Artesunate (Guilin Pharmaceutical Factory, Guangxi, China) was given in a dose of 2.4 mg/kg on admission, at 12 h, at 24 h, and thereafter once daily until oral medication could be taken reliably. The contents of each 60 mg vial were dissolved initially in 1 mL 5%
sodium bicarbonate (provided with the drug) and then diluted with 5% dextrose before injection either as a bolus into an indwelling intravenous cannula, or administration by deep intramuscular injection to the anterior thigh. Quinine dihydrochloride (Indus Pharma, Karachi, Pakistan) was given in a 20 mg salt per kg loading dose infused over 4 h (in 5–10 mL/kg of 5% dextrose), followed by a 10 mg salt per kg infusion over 2–8 h three times daily until starting oral therapy. For intramuscular treatment the doses were the same as for intravenous treatment; quinine was diluted in normal saline to a concentration of 60 mg/mL, and injected into the anterior thigh. The loading dose was given as a split dose into each thigh.

When the patient was able to take tablets, but after a minimum of 24 h of parenteral treatment, oral artemether-lumefantrine (Coartem, Novartis, Basel, Switzerland) in a full standard dose (1.5/9 mg/kg twice daily for 3 days with milk or fat) was given to complete the treatment.

A 1 mL blood sample was taken for immediate haematocrit and biochemical analyses with the EC8+ card for a handheld blood analyser (i-STAT, Abbott Laboratories, Abbott Park, IL, USA). This device produced an immediate printed paper report with time of day, which was kept with the case record form. Haemoglobin was reported with the i-STAT result or, when not available, calculated from the measured haematocrit (n=146).10 Thick and thin blood smears were prepared for later malaria parasite counting at the Bangkok reference laboratory. In 109 cases no count was available from the reference laboratory, so the parasitaemia reported by the study site was used. Children were discharged from hospital at the discretion of the physician, and a discharge assessment was completed. Children who had not made a full neurological recovery by discharge were followed up at regular intervals for 12 months or until full recovery. Full neurological assessments were completed at every visit. Training in neurological assessment was provided to all sites by a specialist paediatric neurologist (MO) to ensure uniformity.

Trial sites were monitored regularly by Family Health International, Nairobi, Kenya. Investigators provided reports every 2 weeks to the coordinating centre in Bangkok and met every year to review study progress. Drug content and quality were checked in ampoules taken randomly from the purchase lots. The data and safety monitoring committee made three interim analyses during the trial. Adequacy of randomisation was assessed by checking by centre that randomisation was balanced according to baseline variables, and by allocation time to assess whether the expected random variations were observed.
Study outcomes

The analysis was undertaken according to a prespecified analytical plan. The primary outcome measure was in-hospital mortality compared between treatments on an intention-to-treat basis. Secondary outcome measures were the incidence of severe neurological complications (assessed at 28 days, range 3–8 weeks) and a combined outcome measure of death and severe persistent neurological sequelae. Initially, neurological outcomes were assessed only at discharge from hospital, but this procedure led to substantial overestimation of neurological deficit, especially in young children. The protocol was therefore changed in April 2007, (after 11% of patients had been enrolled) so that children who had not yet fully recovered at discharge were assessed 28 days after enrolment, and active follow-up was instituted. Outcome measures were also assessed per protocol, excluding patients not fulfilling the entry criteria, those with a negative or missing admission blood slide for *P. falciparum*, those dying before receiving the study drug, and those missing a study drug dose on the first day of treatment. Subgroup analyses included stratification into two predefined groups: those who fulfilled the criteria for severe malaria\(^1,2,11\) and those who did not. In sites at which testing was approved, the HIV status of participants was assessed after obtaining a separate informed consent, and the outcomes in HIV-1 infected patients with malaria were compared according to treatment allocation.

Two committees were formed to provide independent expert assessment and classification of outcomes before study unblinding. A neurological outcome committee, comprising two experienced paediatricians (both with neurological expertise and experience of working in Africa) and one clinical malariologist, graded neurological sequelae. These sequelae were assessed in four domains: motor function, visual function, hearing, and speech, and also whether the child had developed epilepsy. Sequelae persisting at follow-up were graded as mild, moderate, or severe in each domain according to the extent of functional impairment (Text S3). The overall grade was combined. If there were sequelae in more than one functional domain the grade was increased. The relation of sequelae to severe malaria was assessed taking into account any pre-existing comorbidity. A separate endpoint review committee, comprising one paediatrician with malaria experience and one clinical malariologist, identified cases in which a pathological change other than malaria or its acute complications was likely to be the main cause of death.
Statistical analysis
Inclusion of more than 5306 children with severe malaria was needed to show, with 80% power and 95% confidence, a 25% reduction in mortality from 8% to 6%. This calculation was based on an estimated severe malaria mortality of 16% with about half the enrolled patients anticipated to fulfil the criteria for severe malaria. Patient data and outcomes were provided to an independent data and safety monitoring committee, who reviewed the trial yearly.

For binary outcomes, the odds ratios (ORs) between treatment groups, stratified by study site, were estimated by the Mantel-Haenszel method. Heterogeneity between sites was examined with the Breslow-Day test. In Rwanda, the two small sites run by the same investigators were pooled. Time to event outcomes were compared with the log-rank test, and hazard ratios (HRs) were estimated by a Cox proportional hazard model, stratified by site. Statistical analyses were done with Stata (version 11.1).

This trial is registered, number ISRCTN50258054.

Role of the funding source
The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Figure 1 shows the trial profile. We recorded no major differences in baseline characteristics between the two treatment groups (Table 1). 5425 patients were recruited (Figure 1), of whom 527 (9.7%) died. 230 of 2712 (8.5%) patients given artesunate died versus 297 of 2713 (10.9%) given quinine (relative risk 0.78, 95% CI 0.66–0.91; OR 0.75, 0.63–0.90, in favour of artesunate; p=0.0022). This represents a relative reduction in mortality of 22.5% (95% CI 8.1–36.9%) and corresponds to an overall number needed to treat to prevent one death of 41 (95% CI 25–112). We recorded no heterogeneity between study sites (p=0.99). Eight patients (5 artesunate, 3 quinine) were taken from hospital against advice and could not be followed up further. They were censored in the survival analysis at the time of discharge. The survival analysis (Figure 2) for overall mortality during admission by antimalarial treatment gave the same result as the Mantel-Haenszel analysis (HR stratified by study site 0.76, 95% CI 0.64–0.91; p=0.0022). Mantel-Haenszel analysis of the predefined subgroups showed no evidence of any differences in odds ratios.
between subgroups (Figure 3), and these results were confirmed by Cox regression (data not shown).

The per-protocol analysis excluded patients who died rapidly before receiving antimalarial treatment (6 artesunate, 22 quinine; p=0.0023), patients with incomplete initial antimalarial treatment with the study drug, and those with negative or missing blood slides for *P. falciparum* (Figure 1), but these omissions did not substantially affect the result (Table 2).

**Figure 1**

![Diagram showing trial profile]

**Trial profile**

- 663 patients in Beira, 442 in Kilifi, 436 in Kumasi, 921 in Muheza, 540 in Korogwe, 502 in Banjul, 450 in Ilorin, 386 in Rwanda, 663 in Mbarara, and 422 in Kinshasa.

- Two patients also had other criteria.
Table 1. Baseline characteristics in the two treatment groups

| Variable                           | Quinine (n=2713) | Artesunate (n=2712) |
|------------------------------------|------------------|---------------------|
| Female                             | 1295 (48%)       | 1315 (48%)          |
| Age (years)                        | 2.9 (1.7–4.3)    | 2.8 (1.6–4.2)       |
| Fever before enrolment (d)         | 3 (2–4)          | 3 (2–4)             |
| Coma before enrolment (h)          | 5.0 (2.5–10)     | 5.0 (2.0–9.5)       |
| **Pre-treatment with antimalarials**|                  |                     |
| None                               | 1270 (47%)       | 1281 (47%)          |
| Ineffective                       | 371 (14%)        | 387 (15%)           |
| Effective                         | 959 (37%)        | 938 (36%)           |
| **Complications on admission**     |                  |                     |
| Coma b                             | 997 (37%)        | 943 (35%)           |
| Convulsions                        | 879 (32%)        | 811 (30%)           |
| Jaundice                           | 59 (2%)          | 55 (2%)             |
| Severe anaemia (haemoglobin<50 g/L)| 693 (29%)        | 738 (30%)           |
| Shock                              | 339 (12%)        | 323 (12%)           |
| Decompensated shock               | 88 (35%)         | 90 (39%)            |
| Severe acidosis (BE <-8 mmol/L)    | 975 (43%)        | 1009 (44%)          |
| Hypoglycaemia (<3 mmol/L)          | 278 (10%)        | 277 (10%)           |
| Respiratory distress c             | 428 (16%)        | 439 (16%)           |
| Severe prostration d               | 1668 (61%)       | 1683 (62%)          |
| Black water fever                  | 116 (4%)         | 121 (4%)            |
| Hyperparasitaemia (>10%)           | 573 (24%)        | 584 (25%)           |
| **Clinical examination**           |                  |                     |
| Weight (kg)                        | 12.6 (4.6)       | 12.4 (4.8)          |
| Temperature (°C)                   | 38.0 (1.1)       | 38.0 (1.1)          |
| Blood pressure (mmHg)              |                  |                     |
| Systolic                           | 95 (16)          | 95 (16)             |
| Diastolic                          | 56 (14)          | 56 (14)             |
| Coma depth (total N, median, [IQR])|                  |                     |
| Blantyre coma scale                | 1704, 4 (2–5)    | 1713, 4 (2–5)       |
| Glasgow coma scale                 | 1005, 11 (8–15)  | 999, 11 (8–15)      |
| **Comorbidity**                    |                  |                     |
| Immune-compromised (from history)  | 49 (2%)          | 45 (2%)             |
| Severe malnutrition                | 43 (2%)          | 54 (2%)             |
### Table 1. Continued

| Variable                        | Quinine (n=2713) | Artesunate (n=2712) |
|---------------------------------|------------------|---------------------|
| Suspected pneumonia             | 223 (8%)         | 227 (8%)            |
| Confirmed by radiograph         | 29 (13%)         | 29 (13%)            |
| Clinical sepsis                 | 355 (13%)        | 302 (11%)           |
| Confirmed by culture            | 33 (9%)          | 32 (11%)            |
| Suspected meningitis            | 166 (6%)         | 169 (6%)            |
| Confirmed meningitis            | 3 (2%)           | 6 (4%)              |
| Other significant comorbidities | 71 (3%)          | 80 (3%)             |

### Laboratory assessments

|                           | Quinine          | Artesunate        |
|---------------------------|------------------|-------------------|
| Parasitaemia (parasites/µL; geometric mean, range) | 49 (0–1858 880) | 47 (0–1 494 640) |
| Sodium (mmol/L)           | 132 (6.5)        | 131 (6.5)         |
| Potassium (mmol/L)        | 4.1 (0.9)        | 4.1 (0.9)         |
| Chloride (mmol/L)         | 105 (10)         | 105 (10)          |
| Blood urea nitrogen (mmol/L) | 6.1 (4.9)    | 6.1 (4.6)         |
| Haemoglobin (g/L)         | 70 (31)          | 68 (29)           |
| pH                        | 7.36 (0.14)      | 7.36 (0.14)       |
| PaCO₂ (mm Hg)             | 28.2 (10.1)      | 27.9 (9.1)        |
| HCO₃ (mmol/L)             | 16.6 (5.7)       | 16.6 (5.6)        |
| Plasma BE (mmol/L)        | -8.6 (7.3)       | -8.5 (7.3)        |
| Anion gap (mmol/L)        | 17.2 (5.0)       | 17.0 (4.9)        |

Data are No. (%) of patients, median (IQR), or mean (SD), unless otherwise indicated.

Abbreviations: BE, base excess; PaCO₂, partial pressure of carbon dioxide; HCO₃, bicarbonate.

a See Text S4 for classification of categories.

b Depth of coma was assessed either by Blantyre coma scale (for preverbal children, n=3417) or Glasgow coma scale (n=2004).

c Respiratory distress was defined as costal indrawing, use of accessory muscles, nasal alar flaring, deep breathing, or severe tachypnoea.

d Severe prostration was defined as inability to breastfeed for children younger than 6 months or inability to sit for older children.

The endpoint review committee identified 16 children (7 artesunate, 9 quinine) in whom death was unlikely to be related to severe malaria. Omission of these cases from the analysis also had no effect on the magnitude of the survival benefit with artesunate (Table 2 and Text S5). In 4618 children fulfilling “strict” criteria for severe malaria (Panel 1), mortality was 9.9% (226/2280) with artesunate and 12.4% (291/2338) with quinine (OR 0.77, 95% CI 0.64-0.93; p=0.0055). Eight fatal cases (5 quinine, 3 artesunate) did not have
an accurate time of death recorded. In the remainder, 345 of 519 deaths occurred within the first 24 h after admission, of which 158 of 2709 (5.8%) were assigned to artesunate treatment and 187 of 2708 (6.9%) to quinine treatment (OR 0.84, 95% CI 0.67–1.04; p=0.109). Of the 174 of 519 deaths that occurred more than 24 h after admission, 69 of 2551 (2.7%) patients were treated with artesunate and 105 of 2521 (4.2%) with quinine (OR 0.63, 95% CI 0.47–0.87; p=0.004). HIV serology was assessed routinely in Beira, Muheza, and Kilifi. Of 2095 patients tested, 125 (6%) were positive (64 artesunate, 61 quinine). Mortality in these patients was high (Table 2).

Figure 2

Kaplan-Meier curves comparing survival in African children with severe falciparum malaria treated with either parenteral artesunate or quinine. The numbers in parentheses are the deaths during the indicated time. In eight patients the exact time of death during the night was missing and was estimated as 23.59 h.
Figure 3

The forest plot shows odds ratios and 95% CIs. The size of the squares is proportional to the size, and therefore weight, of the subgroup. The diamonds show the combined differences. I² denotes the percentage of total variation across subgroups resulting from heterogeneity rather than chance, with the p value of significance. The efficacy of antimalarial pretreatment was classified before study unblinding (Text S4). Hyperparasitaemia means greater than 10% of red cells parasitised. Abbreviations: OR, odds ratio; GCS, Glasgow coma scale; BCS, Blantyre coma scale; BE, base excess.

a Site mortality classified as low if the site mortality rate was lower than the overall study mortality rate, and high if the site mortality rate was higher than the overall study mortality rate.

b Classified according to centre policy (ten sites); classified according to individual data (one site).

c Decompensated or compensated shock.
Table 2. Mortality and complications according to treatment group

|                          | Quinine (n/N, %) | Artesunate (n/N, %) | OR (95% CI) | p value |
|--------------------------|------------------|---------------------|-------------|---------|
| Mortality, ITT analysis  | 297/2713 (10.9%) | 230/2712 (8.5%)     | 0.75        | 0.0022  |
| Mortality, per-protocol analysis | 260/2552 (10.2%) | 208/2563 (8.1%) | 0.78 | 0.0099  |
| Death or sequelae at 28 days | 316/2695 (11.7%) | 253/2689 (9.4%) | 0.78 | 0.0056  |
| Malaria-attributable mortality | 288/2704 (10.7%) | 223/2705 (8.2%) | 0.75 | 0.0025  |
| Mortality in “strictly” defined severe malaria | 291/2338 (12.4%) | 226/2280 (9.9%) | 0.77 | 0.0055  |
| Case fatality in HIV-positive children | 19/61 (31%) | 16/64 (25%) | 0.74 | 0.45    |
| Development of coma | 91/1768 (5.1%) | 65/1832 (3.5%) | 0.69 | 0.0231  |
| Deterioration of coma score | 208/2713 (7.7%) | 166/2712 (6.1%) | 0.78 | 0.0245  |
| Convulsions developing or persisting >6 h after admission | 273/2713 (10.1%) | 224/2712 (8.3%) | 0.80 | 0.0199  |
| Hypoglycaemia | 75/2713 (2.8%) | 48/2712 (1.8%) | 0.63 | 0.0134  |
| Severe anaemia (Hb <50 g/L) after admission | 98/1734 (5.7%) | 78/1696 (4.6%) | 0.81 | 0.18    |
| Blackwater fever | 18/2597 (0.7%) | 30/2591 (1.2%) | 1.69 | 0.076   |

Abbreviations: ITT, intention to treat; Hb, haemoglobin.

* The likelihood that malaria contributed to or directly caused the death was assessed by an independent endpoint review committee blinded to the treatment allocation.

* As defined in Panel 1.

* HIV status was assessed only in Beira, Muheza, and Kilifi (n=2095).

* Development of coma, anaemia, and black water fever was assessed only in patients without these disorders on admission.

497 children (224 artemesunate, 273 quinine) had convulsions that either developed after admission, irrespective of duration, or were present on admission and persisted for more than 6 h, and 156 patients (65 artemesunate, 91 quinine) developed coma or had a deterioration of their coma score after starting antimalarial treatment (Table 2). The development of coma, deterioration in coma score, and convulsions all occurred more frequently in patients who received quinine than in those who received artemesunate (Table 2). In the 4898 survivors, 170 (99 artemesunate, 71 quinine) had not yet made full neurological recoveries at discharge (Figure 4). Of these patients, 129 (76 artemesunate, 53
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quinine) were followed up between 3 and 8 weeks after enrolment. At this first follow-up assessment 68 (53%) had recovered fully, 18 (14%; 11 artesunate, 7 quinine) were mildly or moderately impaired, and 43 (33%; 20 quinine, 23 artesunate) had severe neurological deficits. The overall incidence of any persistent neurological sequelae in assessed survivors at 28 days after cerebral malaria was 3.2% (24/706 artesunate, 23/737 quinine) and of severe neurological sequelae was 2.3% (17/706 artesunate, 17/737 quinine). We recorded no difference between the treatment groups. Of the 14 patients with any neurological sequelae who did not have cerebral malaria initially (10 artesunate, 4 quinine), 7 had multiple convulsions (3 quinine, 4 artesunate) and all had severe prostration on admission.

Figure 4

Neurological sequelae at discharge and after 28 days (range 3–8 weeks) in children with severe falciparum malaria.

*Some patients had severe impairment in more than one domain.
We detected no severe adverse effects that could be attributed directly to drug toxicity. Although one patient treated with artesunate developed a mild urticarial rash, no severe type-1 hypersensitivity reactions were recorded. Another patient treated with artesunate developed peripheral gangrene of toes and fingers, which was attributed to the disease and not the drug. One patient given quinine developed severe stridor after administration of ampicillin, and died. This death was attributed to ampicillin rather than quinine. Hypoglycaemia after starting antimalarial treatment was significantly less frequent in patients who received artesunate than in those who received quinine (Table 2). Blackwater fever was rare in both groups (Table 2).

Blood transfusions were given to 1487/2712 (55%) patients assigned to artesunate and 1495/2713 (55%) assigned to quinine. A fluid bolus at the start of treatment was given to 589/2712 (22%) patients assigned to artesunate and 596/2713 (22%) assigned to quinine. 3259 (60%) children received antibiotics (1606 artesunate, 1653 quinine; p=0.20). In cerebral malaria survivors, the time from randomisation until the child was able to localise a painful stimulus or was able to speak was slightly longer overall in patients treated with artesunate than in those given quinine when compared by survival analysis (Table 3). The times to eat or to sit unsupported did not differ between treatment groups (Table 3).

### Table 3. Recovery times in surviving patients according to treatment group

| Variable                  | Quinine (median, IQR) | n  | Artesunate (median, IQR) | n  | HR          (95% CI) | p value |
|---------------------------|------------------------|----|--------------------------|----|--------------|--------|
| Time to discharge (d)     | 3.0 (2.0–5.0)          | 2412| 3.0 (2.0–5.0)            | 2478| 1.04 (0.99–1.11) | 0.059  |
| Time to eat (h)           | 12 (2–24)              | 2269| 9 (0–24)                 | 2358| 0.99 (0.93–1.06) | 0.74   |
| Time to sit unsupported (h)| 22 (6–44)             | 2312| 18 (6–42)                | 2373| 1.02 (0.95–1.08) | 0.60   |
| Time to localize pain (h) | 12 (6–24)             | 726 | 12 (6–24)                | 698 | 0.87 (0.78–0.98) | 0.0093 |
| Time to speak (h)         | 18 (11–36)            | 695 | 20 (8–42)                | 664 | 0.88 (0.79–0.99) | 0.016  |

*Time to localize pain and time to speak was assessed only for surviving patients with coma on admission (Blantyre coma scale <3 or Glasgow coma scale <11)*
The content of artemisinin in all ampoules tested was within the pharmaceutical content limits of 10% of the stated active ingredient (Text S6). All tested vials of quinine contained between 105% and 112% of the stated content (Text S7).

In a meta-analysis of all severe malaria trials that have compared survival after parenteral artemisinin with that after parenteral quinine, the overall OR was 0.69 (95% CI 0.57–0.84; p<0.00001) in favour of artemisinin (Figure 5). We noted no significant heterogeneity between the results generated in Africa and Asia (Figure 5).

**Figure 5**

[Graph showing meta-analysis results]

Meta-analysis of all randomised controlled trials that have compared mortality of severe malaria in patients treated with parenteral artemisinin versus parenteral quinine12–16

The solid vertical line represents equality of the two groups; the dashed line is the overall treatment difference. The horizontal lines and the width of the diamonds show the CIs for the odds ratios. The size of the squares is proportional to the size, and therefore weight, of the trial. Abbreviations: OR, odds ratio. *99% CIs for totals.

**Discussion**

This large multicentre trial shows that artemisinin substantially reduces the overall mortality of African children diagnosed with severe malaria. We recorded little heterogeneity between treatment centres in the benefit associated with artemisinin, suggesting that the findings are robust. Before this trial, concerns had been raised that artemisinin might not be better than quinine in African children, whereas it clearly was in Asian patients. The concerns arose because of perceived differences in pathology and the prevalence of more quinine-susceptible malaria parasites, and because in the earlier
SEAQUAMAT trial undertaken in Asia, the survival curves did not separate clearly until 48 h after starting treatment, whereas most deaths in African children occurred before this time. Fortunately these concerns were not substantiated. More than 7000 severely ill patients have now been included in randomised comparisons of parenteral artesunate and quinine (Figure 5). Compared with parenteral quinine, artesunate reduced the mortality of severe malaria in African children by 22.5% and in Asian patients by 38.6%. These studies together comprise nearly 80% of all patients ever enrolled in randomised controlled trials of patients admitted with severe malaria, and they provide definitive evidence that artesunate is the most effective available treatment for severe malaria, and that it is safe (Panel 2).

**Panel 2. Research in context**

**Systematic review**
We searched Medline (from January 1966, to September 2010) and Embase (from January 1980, to September 2010) for randomised controlled trials. Search terms used were: “malaria”, “cerebral malaria”, “severe malaria”, “complicated malaria”, “malaria falciparum”, “quinine”, and “cinchona alkaloids” (MeSH/EMTREE terms); and “artemisinin” and “artesunate”. We searched for randomised controlled trials that compared parenteral artesunate with quinine for treatment of severe malaria. We identified six randomised controlled trials, which we have included in the meta-analysis presented in Figure 5.

**Interpretation**
Together these trials provide substantial evidence of the life-saving benefit of artesunate compared with quinine in the treatment of severe falciparum malaria in all age groups worldwide.

This multicentre trial was open label because the substantial differences in the parenteral formulations of the two drugs prevented adequate concealment. Although individual trial site investigators knew the individual treatment allocations, all the other clinical and laboratory investigators were masked to the parenteral treatments given. The similarity of results across the different trial sites suggests that significant bias was unlikely. The great strength of this trial is its unprecedented size, its consistency both internally and with
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previous studies, and therefore the confidence that health-care workers throughout the tropical world can have in its result.

Artesunate prevented death from severe malaria, but not at the expense of an increase in neurological sequelae. Indeed, the overall incidence of confirmed persistent severe sequelae after severe malaria was low.\(^17\) Initially the assessment of neurological deficit was made at discharge from hospital, but many children, especially in the younger age group, had not made a full neurological recovery at discharge. Subsequently, assessments were made 4 weeks later, by which time about half the apparent deficits had resolved fully. This life-saving benefit of artesunate compared with quinine in severe malaria has to derive from its greater intrinsic parasitocidal activity. The principal pharmacodynamic advantage of artesunate is that it has a much broader stage-specificity of action than does quinine.\(^18\) The artemisinins kill circulating ring-stage parasites before they can mature,\(^19,20\) which reduces sequestration of infected erythrocytes in the venules and capillaries of vital organs and thereby prevents potentially lethal microvascular obstruction.\(^21-23\) The large and consistent reduction in mortality associated with artesunate, and the consistent finding that mortality reduction is greatest in hyperparasitaemia,\(^7\) lends support to the central quantitative role of parasitised erythrocyte sequestration in the pathology of malaria.

The benefits of parenteral artesunate compared with quinine were greater than the benefits of intramuscular artemether reported in previous trials.\(^6,7\) In a double-blind trial\(^24\) comparing artesunate and artemether in 370 Vietnamese adults with severe falciparum malaria, 13 patients died in the artesunate group (7%) and 24 in the artemether group (13%). Taken together, these different study results suggest that the life-saving benefit compared with quinine provided by artesunate is roughly twice that provided by artemether. Artesunate and artemether have similar pharmacodynamic properties in vitro, so the most likely explanation for their different efficacies in vivo is the substantial difference in their pharmacokinetic properties. Parenteral artemether is an oil-based formulation given only by intramuscular injection. Absorption is slow and erratic, whereas the water-soluble hemisuccinate artesunate is absorbed rapidly and reliably after intramuscular injection and can be given intravenously.\(^25-30\) Thus the pharmacodynamic advantage of artemether over quinine might have been offset by its poor absorption kinetics. In summary, in the treatment of severe falciparum malaria, it seems that artesunate is better than artemether, which in turn is better than quinine.

The benefits of artesunate (and artemether) compared with quinine were greater in patients from southeast Asia than in African children,\(^6,7\) although the studies were not large enough to show this difference reliably. There are several possible explanations for
this finding. African children usually have some background immunity that assists the therapeutic response, and in particular accelerates circulating parasite clearance. This effect hastens recovery and could reduce the therapeutic advantage of artesunate. The greater quinine susceptibility of \emph{P. falciparum} in Africa is often proposed as contributing to improved therapeutic responses, but the differences are not large, and are unlikely to have a major effect in vivo. Incorrect diagnosis is likely to be a major contributory factor; severe malaria is overdiagnosed in African children, and sepsis is underdiagnosed. Febrile sick children with positive malaria blood smears are usually diagnosed initially as having severe malaria, but the specificity of a positive blood smear is poor in settings in which a high proportion of all children are parasitaemic. Septicaemia and pneumonia are especially difficult to differentiate clinically from severe malaria. Sepsis and severe malaria also commonly coexist in African children. Prompt and appropriate treatment of sepsis should further reduce the mortality of severely ill children with malaria parasitaemia. Parenteral artesunate is simple to administer, is safe, and reduces mortality substantially compared with quinine. No serious adverse effects were identified in this large study or in previous large studies. By contrast, intramuscular quinine is locally toxic (because of its acidity), and intravenous quinine needs a carefully rate-controlled infusion and continuous or three times daily administration to avoid dangerous hypotension. Importantly, quinine is associated with potentially serious hypoglycaemia. In this trial, 22 children died before receiving quinine compared with only six who died before receiving artesunate. This finding is probably indicative of the difficulties in administering parenteral quinine promptly and safely. Any delay in treating severe infection will increase mortality. The ease and safety of parenteral artesunate are important practical advantages. Artesunate is more expensive to buy, but quinine is more expensive to administer. A major factor restricting the deployment of artesunate has been unavailability of a product satisfying international good manufacturing standards. The most widely used product, assessed in this study, does not yet have this certification, which has prevented deployment in some countries. This barrier must be overcome speedily so that parenteral artesunate can be deployed in malaria-endemic areas to save lives. Artesunate should now become the treatment of choice for severe malaria for children and adults worldwide. Malaria causes an estimated 800 000 deaths every year in African children. Severe malaria is often the most common admission diagnosis in febrile children, so a change in treatment policy from quinine to artesunate has the potential to save thousands of children's lives every year. If 4 million African children with severe malaria every year were to receive prompt treatment with parenteral artesunate instead of
Artesunate versus quinine in the treatment of severe falciparum malaria

quinine, and the benefits were similar to those recorded in this trial, then approximately 100,000 lives might be saved per year.

Contributors
The coordinating committee designed the study. All investigators in the trial sites undertook the trial, with support from the team in Bangkok. All investigators and the coordinating committee reviewed and discussed the trial results. The writing committee did the data analysis and prepared the report.

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Chapter 2

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Supporting Information

Figure S1. Enrolment history by site

[Graph showing enrolment history by site, with lines representing different sites such as Beira/Mozambique, Muheza/Tanzania, Mbarara/Uganda, Banjul/The Gambia, Rwanagana/Nyanza/Rwanda, Kilifi/Kenya, Ilorin/Nigeria, Kumasi/Ghana, Kinshasa/DRC. The x-axis represents years from 2005 to 2010, and the y-axis represents the number of patients enrolled, ranging from 0 to 1000.]
| Country   | Study Site | Ethical Review Board | Address of ERB | Reference Number | Document Date   |
|-----------|------------|----------------------|----------------|------------------|-----------------|
| DRC Kinshasa | Kinshasa | ESP UNIKIN Comité d’Éthique | Université de Kinshasa Faculté de Médecine BP 11850 Kinshasa | ESP/CE/050/2009  | Approval 24/09/2009 |
|          |          |                      |                | ESP/CE/050B/2009 | Revision 28/12/2009 |
| Ghana   | Kumasi  | Committee for Human Research | University Office Kumasi Publications and Ethics IRB0001567 Ghana | CHRPE/01/01/06 | Approval 23/01/2006 |
|          |          |                      |                | CHRPE/01/01/06  | Renewal 22/05/2009 |
| Ghana  |        |                      |                | CHRPE/01/01/06  | Approval 21/10/2005 |
|          | Kumasi  |                      |                | CHRPE/01/01/06  | Review 29/07/2008 |
|          | Kumasi  |                      |                | CHRPE/01/01/06  | Approval 06/07/2009 |
|          | Kumasi  |                      |                | CHRPE/01/01/06  | Approval 18/08/2010 |
|          | Kiliﬁ  |                      |                | CHRPE/01/01/06  | Revision 10/07/2008 |
|          | Kiliﬁ  |                      |                | CHRPE/01/01/06  | Approval 21/05/2010 |
| The Gambia  | Banjul  | The Gambia Government/MRC Laboratories Joint Ethics Committee | c/o Laboratories Fajara PO Box 273 Banjul The Gambia | SCC1012 | Approval 30/09/2005 |
### Text S2. Continued

| Country  | Study Site | Ethical Review Board | Address of ERB | Reference Number | Document | Date      |
|----------|------------|----------------------|----------------|-----------------|----------|-----------|
| Rwanda   | Rwamagana  | Rwanda National Ethics Committee (RNEC) IRB00001497/ FWA000001973 | Ministry of Health PO Box 84 Kigali Rwanda | 72/RNEC/2009 | Approval | 03/04/2008 |
| Rwanda   | Rwamagana  |                       |                | 72/RNEC/2009 | Renewal  | 18/06/2009 |
| Rwanda   | Rwamagana  |                       |                | 72/RNEC/2009 | Revision | 02/11/2009 |
| Rwanda   | Nyanza     |                       |                | 127/RNEC/2009 | Approval | 02/11/2009 |
| Nigeria  | Ilorin     | University of Ilorin Teaching Hospital Ethical Review Committee IRB00002974 | PMB 1459 Ilorin Kwara State Nigeria | UITH/CAT/189/10/659 | Approval | 26/10/2007 |
| Nigeria  | Ilorin     |                       |                | UITH/CAT/189/10/659 | Revision | 14/02/2010 |
| Mozambique | Beira       | Comité Nacional de Bioética para a Saúde IRB 00002657 | Ministério da Saúde C Postal 264 Av Eduardo Mondlane/ Salvador Allende Maputo Moçambique | 52/CNBS/05 | Approval | 23/06/2005 |
| Mozambique | Beira       |                       |                | 105/CNBS/07 | Revision | 04/06/2007 |
| Tanzania | Korogwe/ Muheza | Tanzania Medical Research Coordinating Committee (MRCC) | National Institute for Medical Research P O Box 9653 Dar es Salaam Tanzania | NIMR/HQ/R 8a/ Vol IX/435 | Approval | 29/05/2006 |
| Tanzania | Korogwe/ Muheza |                       |                | NIMR/HQ/R 8c/ Vol IX/527 | Renewal | 26/02/2007 |
| Tanzania | Korogwe/ Muheza |                       |                | NIMR/HQ/R 8c/ Vol I/22 | Revision | 20/04/2007 |
| Country   | Study Site   | Ethical Review Board                                      | Address of ERB                      | Reference Number | Document | Date       |
|-----------|--------------|----------------------------------------------------------|-------------------------------------|-------------------|----------|------------|
| Tanzania  | Korogwe/ Muheza | NIMR/HQ/R 8c/ Vol I/60                                   | Revision                           | 15/08/2008        |          |            |
| Uganda    | Mbarara     | Uganda National Council for Science and Technology Plot 6 | HS 349                             | Approval          | 26/09/2007 |            |
|           |             | Kimera Road PO Box 6884 Kampala Uganda                   |                                     |                   |          |            |
| Uganda    | Mbarara     | HS 349                                                   | Renewal                            | 05/09/2008        |          |            |
| Uganda    | Mbarara     | HS 349                                                   | Renewal                            | 31/08/2009        |          |            |
| Uganda    | Mbarara     | HS 349                                                   | Revision                           | 04/01/2010        |          |            |
| UK        | Oxford      | University of Oxford OXTREC                               | 034-02                             | Approval          | 24/05/2005 |            |
|           |             | The John Radcliffe Oxford OX3 9DZ United Kingdom          |                                     |                   |          |            |
| UK        | Oxford      | 034-02                                                   | Renewal                            | 03/10/2007        |          |            |
| UK        | Oxford      | 034-02                                                   | Renewal                            | 02/06/2008        |          |            |
| UK        | Oxford      | 034-02                                                   | Revision                           | 11/08/2008        |          |            |
| UK        | Oxford      | 034-02                                                   | Revision                           | 16/02/2009        |          |            |
| UK        | Oxford      | 034-02                                                   | Revision                           | 09/03/2009        |          |            |
| UK        | Oxford      | 034-02                                                   | Revision                           | 18/03/2009        |          |            |
| UK        | Oxford      | 034-02                                                   | Renewal                            | 08/06/2009        |          |            |
| UK        | Oxford      | 034-02                                                   | Renewal                            | 29/09/2009        |          |            |
| UK        | Oxford      | 034-02                                                   | Renewal                            | 02/02/2010        |          |            |
Text S3. Methods Neurological Endpoint Committee

Neurological sequelae were divided into 4 functional domains or systems, including motor deficits, vision deficits, hearing and speech deficits and persisting seizures. Severity of the deficits was graded according to the tables below. In case a patient had a pathological entry in 2 or more functional domains, the deficit with the most severe grade was moved up one “severity grade” if the “severe” grade had not yet been reached (Example 1: cerebellar ataxia (severe) + speech difficulties (mild) = total grade is severe. Example 2: Facial nerve palsy (moderate) + speech difficulties (mild) = total grade is severe). In case the clinical record form mentioned a pre-existing neurological problem and there was no significant deterioration of symptoms during the malaria episode, the neurological problems were not considered as being sequelae of the acute disease episode.

FUNCTIONAL SYSTEMS TABLES
1) MOTOR

| CRF Entry                  | MILD | MODERATE | SEVERE |
|----------------------------|------|----------|--------|
| Monoparesis                | X    |          |        |
| Hemiplegia/paresis         |      | X        |        |
| Quadripareis               |      | X        |        |
| Continued posturing        |      | X        |        |
| Hypotonia                  |      |          | X      |
| Extrapyramidal rigidity    |      | X        |        |
| Cerebellar Ataxia          |      | X        |        |
| Gait* unsteady             |      |          | X      |
| Gait* hemiplegic/ataxic    |      |          | X      |
| Gait* unable to walk       |      |          | X      |
| Cranial nerve palsies      |      |          | X      |
| Facial nerve palsy         |      |          | X      |

* Gait was considered only in children >18 months

IF there is more than one pathological entry within the motor system; the most severe grading prevails.
2) VISION

| CRF entry                      | MILD | MODERATE | SEVERE |
|-------------------------------|------|----------|--------|
| Blindness bilateral           |      |          | X      |
| Blindness unilateral          |      | X        |        |
| Some impairment bilateral     |      | X        |        |
| Some impairment unilateral    |      |          | X      |

3) HEARING and SPEECH

| CRF entry                      | MILD | MODERATE | SEVERE |
|-------------------------------|------|----------|--------|
| Deafness bilateral            |      |          | X      |
| Deafness unilateral           |      | X        |        |
| Possible impairment bilateral |      |          | X      |
| Possible impairment unilateral|      | X        |        |
| Speech difficulties*          |      |          | X      |
| Unable to speak*              |      |          |        |

*Speech was assessed only in children >18 months

4) SEIZURES

In patients with no previous history of seizures:

Any seizures – moderate
Text S4. Assessing pretreatment: classification of the efficacy of antimalarial drugs

Classification of antimalarials according to likely efficacy for the treatment of uncomplicated falciparum malaria in West Africa (Ghana, The Gambia, Nigeria) or the regions corresponding to the other AQUAMAT study sites. In the main paper, intermediate- efficacy and ineffective antimalarial drugs are grouped together as one category.

| Efficacy of pretreatment: yes (y), no (n), intermediate (i) | Ghana, Gambia or Nigeria | Other study sites |
|------------------------------------------------------------|--------------------------|------------------|
| quinine injection                                          | y                        | y                |
| artemether injection                                       | y                        | y                |
| artesunate/artemether tabs                                 | y                        | y                |
| sulphadoxin-pyrimethamine (SP)                             | i                        | n                |
| SP-amodiaquine                                             | y                        | i                |
| chloroquine                                                | n                        | n                |
| amodiaquine                                                | y                        | i                |
| artemether-lumefantrine                                     | y                        | y                |
| artesunate suppository                                      | y                        | y                |
| artesunate-amodiaquine                                      | y                        | y                |
| artemether-amodiaquine                                      | y                        | y                |
| artemether-quineine                                         | y                        | y                |
| dihydroartemisinin (DHA)                                   | y                        | y                |
| DHA-amodiaquine                                             | y                        | y                |
| SP-artemether-lumefantrine                                 | y                        | y                |
| pyrimethamine-sulphamethopirazine                           | i                        | n                |
Text S5. Methods and findings Mortality Endpoint Review Committee

Methods The end-point review committee included one paediatrician with malaria experience and one clinical malariologist. The reviewers assessed the fatal cases independently from the trial investigators, and were blinded towards the study drug treatment allocations. All clinical and laboratory data (including those from the parasitology reference laboratory) were reviewed, along with the “Severe Adverse Events” forms. Cases were only defined when both reviewers independently judged that a pathology other than malaria or its acute complications was the main cause of death. Cases with pathology other than malaria (or its acute complications) as a likely cause of death.

| Age | Gender | Relevant details |
|-----|--------|------------------|
| 6 y | F      | Sudden onset of abdominal pain without fever. Death occurred within three hours of symptom onset. Parasitaemia 5/1000 RBC (no pretreatment) |
| 22 m | M     | Death followed return of consciousness and defervescence |
| 3 y | F      | Death followed return of consciousness and defervescence |
| 7 m | M      | History of diarrhoea, vomiting and generalized rash. Clinical diagnosis of measles. Parasitaemia 8/200 WC (one dose of sulfadoxine-pyrimethamine pretreatment) |
| 3 y | M      | Clinical diagnosis of tetanus. Parasitaemia 3/1000 RBC (no pretreatment) |
| 11 m | F     | Death followed return of consciousness and defervescence |
| 21 m | M     | Death followed return of consciousness and defervescence |
| 5 y | F      | Suspected meningitis. Haemophilus influenzae type b isolated from blood culture. Parasitaemia 2/1000 RBC (pyrimethamine-sulfadoxine pretreatment) |
| 6 y | F      | Clinical diagnosis of tetanus. Parasitaemia 3/1000 RBC (no pretreatment) |
| 3 m | M      | Clinical diagnosis of mastoiditis. Group A streptococcus isolated from blood culture. Parasitaemia 13/1000 RBC (no pretreatment) |
| 4 y | M      | Clinical diagnosis of left-sided pneumonia. Haemophilus influenzae type b isolated from blood culture. Parasitaemia 81/200 WC (two doses of quinine pretreatment) |
| 22 m | M     | Clinical diagnosis of myositis. Group A streptococcus isolated from blood culture. Parasitaemia 2/200 WC (three doses of quinine pretreatment) |
| 14 m | F     | Clinical diagnosis of severe acute malnutrition. Salmonella spp. isolated from blood culture. Parasitaemia 1/200 WC (no pretreatment) |
| 6 m | M      | Clinical diagnosis of severe cellulitis. Group A streptococcus isolated from blood culture. Parasitaemia 1/500 WC (six doses of artemether-lumefantrine pretreatment) |
| 16 m | M     | Clinical observation of pustular rash and impetigo (no coma). Staphylococcus aureus isolated from blood culture. Parasitaemia 3/200 WC (one dose of amodiaquine pretreatment) |
| 14 m | M     | Death followed return of consciousness and defervescence |
**Text S6. Quality assessment of artesunate batches used in the trial**

**LC-MS/MS Analysis of the artesunate content in vials for injection**

**Methods** From each batch, 3 vials were selected for testing. Each vial's content was quantified using 3 replicate measurements. Samples were quantified using a standard curve constructed from 3 replicate samples at each calibration level (concentrations 19.2, 24.0, 28.8 ng/ml). Results for each vial and a batch average were summarized.

**Sample preparation** The content of each vial to be tested was reconstituted in 1.0–1.5 mL ethanol. The whole amount was transferred into a 250 ml volumetric flask thereby diluting the solution to 240 µg/ml (assuming initial content as stated; 60 mg). This solution was further diluted using volumetric flasks to a final approximate concentration of 24 ng/ml.

**Quantification** Samples were quantified using an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, USA). The final sample was injected into the LC-MS/MS system equipped with a TurboV ionization source (TIS) interface operated in the positive ion mode. Quantification was performed using selected reaction monitoring (SRM) for the transition m/z 402 – 163.

**Results** The following results were obtained for the different batches.

**Conclusion** All tested vials’ contents come within GMP specification of +/- 10% of stated content.

**Reference**

1. Hanpithakpong W, Kamanikom B, Dondorp AM, Singhasivanon P, White NJ, Day NP, Lindegardh N. A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 876: 61-8.
## Table. Artesunate quality

| Site      | Batch   | Expire  | pha ID | Artesunate (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|-----------|---------|---------|--------|----------------------|------------|-------------|-------------------|
| **Mozambique** | 60109   | Dec-08  | A1     | 57.00                | 95.00      | 95.28       | 95.28             |
|           |         |         | A1 2   | 57.25                | 95.42      |             |                   |
|           |         |         | A1 3   | 57.25                | 95.42      |             |                   |
|           |         |         |        | ZA070701 Jun-10      |            |             |                   |
|           |         |         | B1 1   | 58.00                | 96.67      | **96.94**   | 93.79             |
|           |         |         | B1 2   | 58.25                | 97.08      |             |                   |
|           |         |         | B1 3   | 58.25                | 97.08      |             |                   |
|           |         |         | B2 1   | 54.00                | 90.00      | **90.00**   |                   |
|           |         |         | B2 2   | 54.25                | 90.42      |             |                   |
|           |         |         | B2 3   | 53.75                | 89.58      |             |                   |
|           |         |         | B3 1   | 56.75                | 94.58      | **94.44**   |                   |
|           |         |         | B3 2   | 56.25                | 93.75      |             |                   |
|           |         |         | B3 3   | 57.00                | 95.00      |             |                   |
| **Kenya** | ZA060903| Aug-09  | C1 1   | 55.50                | 92.50      | **92.50**   | 93.84             |
|           |         |         | C1 2   | 55.25                | 92.08      |             |                   |
|           |         |         | C1 3   | 55.75                | 92.92      |             |                   |
|           |         |         | C2 1   | 57.50                | 95.83      | **95.28**   |                   |
|           |         |         | C2 2   | 56.75                | 94.58      |             |                   |
|           |         |         | C2 3   | 57.25                | 95.42      |             |                   |
|           |         |         | C3 1   | 56.00                | 93.33      | **93.75**   |                   |
|           |         |         | C3 2   | 56.25                | 93.75      |             |                   |
|           |         |         | C3 3   | 56.50                | 94.17      |             |                   |
| **Tanzania** | ZA070406| Mar-10  | D1 1   | 56.25                | 93.75      | **92.36**   | 91.53             |
|           |         |         | D1 2   | 54.75                | 91.25      |             |                   |
|           |         |         | D1 3   | 55.25                | 92.08      |             |                   |
|           |         |         | D2 1   | 54.25                | 90.42      | **90.55**   |                   |
|           |         |         | D2 2   | 54.25                | 90.42      |             |                   |
|           |         |         | D2 3   | 54.50                | 90.83      |             |                   |
|           |         |         | D3 1   | 55.50                | 92.50      | **91.67**   |                   |
|           |         |         | D3 2   | 54.50                | 90.83      |             |                   |
|           |         |         | D3 3   | 55.00                | 91.67      |             |                   |
| **Tanzania** | ZA070406| Mar-10  | E1 1   | 62.25                | 103.75     | **102.78**  | 98.15             |
|           |         |         | E1 2   | 61.25                | 102.08     |             |                   |
|           |         |         | E1 3   | 61.50                | 102.50     |             |                   |
Table. Artesunate quality (Continued)

| Site   | Batch  | Expire | pha ID | Artesunate (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|--------|--------|--------|--------|-----------------------|------------|-------------|------------------|
|        |        |        | E2 1   | 55.25                 | 92.08      |             | 91.80            |
|        |        |        | E2 2   | 54.50                 | 90.83      |             |                  |
|        |        |        | E2 3   | 55.50                 | 92.50      |             |                  |
|        |        |        | E3 1   | 60.00                 | 100.00     | 99.86       |                  |
|        |        |        | E3 2   | 60.00                 | 100.00     |             |                  |
|        |        |        | E3 3   | 59.75                 | 99.58      |             |                  |
| The Gambia | ZA060203 | Jan-09 | F1 1   | 58.75                 | 97.92      | 97.78       | 96.76            |
|         |        |        | F1 2   | 58.25                 | 97.08      |             |                  |
|         |        |        | F1 3   | 59.00                 | 98.33      |             |                  |
|         |        |        | F2 1   | 59.75                 | 99.58      | 100.00      |                  |
|         |        |        | F2 2   | 59.75                 | 99.58      |             |                  |
|         |        |        | F2 3   | 60.50                 | 100.83     |             |                  |
|         |        |        | F3 1   | 55.50                 | 92.50      | 92.50       |                  |
|         |        |        | F3 2   | 55.25                 | 92.08      |             |                  |
|         |        |        | F3 3   | 55.75                 | 92.92      |             |                  |
| Uganda | ZA070406 | Mar-10 | G1 1   | 59.75                 | 99.58      | 100.14      | 92.50            |
|         |        |        | G1 2   | 60.25                 | 100.42     |             |                  |
|         |        |        | G1 3   | 60.25                 | 100.42     |             |                  |
|         |        |        | G2 1   | 55.25                 | 92.08      | 92.08       |                  |
|         |        |        | G2 2   | 55.50                 | 92.50      |             |                  |
|         |        |        | G2 3   | 55.00                 | 91.67      |             |                  |
|         |        |        | G3 1   | 51.25                 | 85.42      | 85.28       |                  |
|         |        |        | G3 2   | 50.75                 | 84.58      |             |                  |
|         |        |        | G3 3   | 51.50                 | 85.83      |             |                  |
| Nigeria | ZA070701 | Jun-10 | H1 1   | 54.75                 | 91.25      | 91.39       | 92.31            |
|         |        |        | H1 2   | 54.50                 | 90.83      |             |                  |
|         |        |        | H1 3   | 55.25                 | 92.08      |             |                  |
|         |        |        | H2 1   | 57.50                 | 95.83      | 96.39       |                  |
|         |        |        | H2 2   | 58.00                 | 96.67      |             |                  |
|         |        |        | H2 3   | 58.00                 | 96.67      |             |                  |
|         |        |        | H3 1   | 53.75                 | 89.58      | 89.17       |                  |
|         |        |        | H3 2   | 53.25                 | 88.75      |             |                  |
|         |        |        | H3 3   | 53.50                 | 89.17      |             |                  |
Table. Artesunate quality (Continued)

| Site  | Batch | Expiry Date | pha ID | Artesunate (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|-------|-------|-------------|--------|----------------------|------------|-------------|------------------|
| Rwanda | ZA070701 | Jun-10 | I1 1 | 58.75 | 97.92 | **97.64** | 95.00 |
|       |       |     | I1 2 | 58.25 | 97.08 |           |      |
|       |       |     | I1 3 | 58.75 | 97.92 |           |      |
|       |       |     | I2 1 | 55.00 | 91.67 | **92.50** |      |
|       |       |     | I2 2 | 55.50 | 92.50 |           |      |
|       |       |     | I2 3 | 56.00 | 93.33 |           |      |
|       |       |     | I3 1 | 56.75 | 94.58 | **94.86** |      |
|       |       |     | I3 2 | 57.25 | 95.42 |           |      |
|       |       |     | I3 3 | 56.75 | 94.58 |           |      |
| DRC   | LA091001 | 10-08-12 | J1 1 | 57.25 | 95.42 | **95.00** | 95.88 |
|       |       |     | J1 2 | 57.25 | 95.42 |           |      |
|       |       |     | J1 3 | 56.50 | 94.17 |           |      |
|       |       |     | J2 1 | 57.50 | 95.83 | **96.25** |      |
|       |       |     | J2 2 | 58.00 | 96.67 |           |      |
|       |       |     | J2 3 | 57.75 | 96.25 |           |      |
|       |       |     | J3 1 | 57.50 | 95.83 | **96.39** |      |
Text S7. Quality assessment of quinine batches used in the trial

LC-UV Analysis of quinine content in vials for injection

**Methods** (modified from\(^1\)): From each batch, 3 vials were selected for testing. Each vial's content was quantified using 3 replicate measurements. Samples were quantified using a standard curve constructed from 3 replicate samples at each calibration level (concentrations 15.7, 19.6 and 23.4 µg/ml). Results for each vial and a batch average were summarized.

**Sample preparation** The content of each vial to be tested was transferred into a 100 ml volumetric flask thereby diluting the solution to 6.00 mg/ml (assuming initial content 600 mg). Exactly 1000 µl of this solution was further diluted using a 250 ml volumetric flask to a final approximate concentration of 19.6 µg/ml.

**Quantification** Samples were quantified using a LaChrom Elite LC-UV system (Hitachi, Tokyo, Japan). Quantification was performed at the wavelength 250 nm.

**Results** The following results were obtained for the different batches.

**Conclusion** All tested vials contained between 105-112% of stated content.

**Reference**

1. Newton PN, Keeratithakul D, Teja-Isavadharm P, Pukrittayakamee S, Kyle D, White NJ. Pharmacokinetics of quinine and 3-hydroxyquinine in severe falciparum malaria with acute renal failure. Trans R Soc Trop Med Hyg. 1999; 93:69–72.
Table. Quinine quality

| Site     | Batch | Expire | pha ID | Quinine (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|----------|-------|--------|--------|------------------|------------|-------------|-------------------|
| Mozambique | 396 mrt-10 | A1 1 | 627.56 | 104.59 | 105.42 | 106.06 |
|          |       | A1 2 | 634.11 | 105.68 |          |            |
|          |       | A1 3 | 635.94 | 105.99 |          |            |
|          |       | A2 1 | 633.62 | 105.60 | 106.58 |          |
|          |       | A2 2 | 643.05 | 107.17 |          |            |
|          |       | A2 3 | 641.76 | 106.96 |          |            |
|          |       | A3 1 | 631.20 | 105.20 | 106.16 |          |
|          |       | A3 2 | 633.65 | 105.61 |          |            |
|          |       | A3 3 | 646.11 | 107.68 |          |            |
|          | 397 sep-10 | B1 1 | 641.64 | 106.94 | 108.58 | 108.71 |
|          |       | B1 2 | 667.79 | 111.30 |          |            |
|          |       | B1 3 | 645.01 | 107.50 |          |            |
|          |       | B2 1 | 635.42 | 105.90 | 107.38 |          |
|          |       | B2 2 | 641.58 | 106.93 |          |            |
|          |       | B2 3 | 655.88 | 109.31 |          |            |
|          |       | B3 1 | 651.68 | 108.61 | 110.17 |          |
|          |       | B3 2 | 672.84 | 112.14 |          |            |
|          |       | B3 3 | 658.60 | 109.77 |          |            |
| Kenya    | 397 sep-10 | C1 1 | 639.47 | 106.58 | 108.21 | 108.50 |
|          |       | C1 2 | 650.55 | 108.42 |          |            |
|          |       | C1 3 | 657.74 | 109.62 |          |            |
|          |       | C2 1 | 646.35 | 107.73 | 108.98 |          |
|          |       | C2 2 | 654.01 | 109.00 |          |            |
|          |       | C2 3 | 661.30 | 110.22 |          |            |
|          |       | C3 1 | 643.29 | 107.22 | 108.30 |          |
|          |       | C3 2 | 658.85 | 109.81 |          |            |
|          |       | C3 3 | 647.33 | 107.89 |          |            |
| Korogwe  | 396 mrt-10 | D1 1 | 626.94 | 104.49 | 105.60 | 105.13 |
|          |       | D1 2 | 636.01 | 106.00 |          |            |
|          |       | D1 3 | 637.81 | 106.30 |          |            |
|          |       | D2 1 | 620.57 | 103.43 | 104.62 |          |
**Table. Quinine quality (Continued)**

| Site      | Batch | Expire | pha ID | Quinine (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|-----------|-------|--------|--------|-------------------|------------|-------------|-------------------|
|           | D2 2  |        |        | 624,74            | 104,12     |             |                   |
|           | D2 3  |        |        | 637,93            | 106,32     |             |                   |
|           | D3 1  |        |        | 625,14            | 104,19     | 105,18      |                   |
|           | D3 2  |        |        | 635,06            | 105,84     |             |                   |
|           | D3 3  |        |        | 632,97            | 105,50     |             |                   |
| Muheza    | 396   | mrt-10 | E1 1   | 638,67            | 106,44     | 107,56      | 106,11            |
|           |       |        | E1 2   | 643,41            | 107,24     |             |                   |
|           |       |        | E1 3   | 647,27            | 107,88     |             |                   |
|           |       |        | E2 1   | 632,42            | 105,40     | 106,11      |                   |
|           |       |        | E2 2   | 638,76            | 106,46     |             |                   |
|           |       |        | E2 3   | 638,85            | 106,48     |             |                   |
|           |       |        | E3 1   | 627,80            | 104,63     | 105,04      |                   |
|           |       |        | E3 2   | 634,23            | 105,70     |             |                   |
|           |       |        | E3 3   | 628,72            | 104,79     |             |                   |
|           | 398   | sep-10 | F1 1   | 663,25            | 110,54     | 111,24      | 111,00            |
|           |       |        | F1 2   | 673,82            | 112,30     |             |                   |
|           |       |        | F1 3   | 665,31            | 110,88     |             |                   |
|           |       |        | F2 1   | 654,80            | 109,13     | 110,55      |                   |
|           |       |        | F2 2   | 672,38            | 112,06     |             |                   |
|           |       |        | F2 3   | 662,80            | 110,47     |             |                   |
|           |       |        | F3 1   | 660,68            | 110,11     | 111,20      |                   |
|           |       |        | F3 2   | 666,26            | 111,04     |             |                   |
|           |       |        | F3 3   | 674,71            | 112,45     |             |                   |
| The Gambia| 396   | mrt-10 | G1 1   | 661,39            | 110,23     | 112,04      | 107,14            |
|           |       |        | G1 2   | 676,70            | 112,78     |             |                   |
|           |       |        | G1 3   | 678,56            | 113,09     |             |                   |
|           |       |        | G2 1   | 620,76            | 103,46     | 104,40      |                   |
|           |       |        | G2 2   | 622,04            | 103,67     |             |                   |
|           |       |        | G2 3   | 636,37            | 106,06     |             |                   |
|           |       |        | G3 1   | 624,25            | 104,04     | 104,99      |                   |
|           |       |        | G3 2   | 627,28            | 104,55     |             |                   |
|           |       |        | G3 3   | 638,33            | 106,39     |             |                   |
| Site    | Batch | Expire | pha ID | Quinine (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|---------|-------|--------|--------|-------------------|------------|------------|------------------|
| Uganda  | 396   | mrt-10 | H1 1   | 628,72            | 104,79     | 106,29     | 105,96           |
|         |       |        | H1 2   | 631,81            | 105,30     |            |                  |
|         |       |        | H1 3   | 652,75            | 108,79     |            |                  |
|         |       |        | H2 1   | 628,50            | 104,75     | 105,30     |                  |
|         |       |        | H2 2   | 629,70            | 104,95     |            |                  |
|         |       |        | H2 3   | 637,26            | 106,21     |            |                  |
|         |       |        | H3 1   | 625,23            | 104,20     | 106,30     |                  |
|         |       |        | H3 2   | 649,94            | 108,32     |            |                  |
|         |       |        | H3 3   | 638,15            | 106,36     |            |                  |
|         | 399   | sep-10 | I1 1   | 658,82            | 109,80     | 110,60     | 111,91           |
|         |       |        | I1 2   | 663,38            | 110,56     |            |                  |
|         |       |        | I1 3   | 668,64            | 111,44     |            |                  |
|         |       |        | I2 1   | 658,66            | 109,78     | 111,64     |                  |
|         |       |        | I2 2   | 664,91            | 110,82     |            |                  |
|         |       |        | I2 3   | 685,88            | 114,31     |            |                  |
|         |       |        | I3 1   | 672,41            | 112,07     | 113,49     |                  |
|         |       |        | I3 2   | 676,39            | 112,73     |            |                  |
|         |       |        | I3 3   | 694,09            | 115,68     |            |                  |
| Nigeria | 396   | mrt-10 | J1 1   | 627,28            | 104,55     | 105,63     | 105,34           |
|         |       |        | J1 2   | 638,18            | 106,36     |            |                  |
|         |       |        | J1 3   | 635,85            | 105,98     |            |                  |
|         |       |        | J2 1   | 628,78            | 104,80     | 105,86     |                  |
|         |       |        | J2 2   | 628,84            | 104,81     |            |                  |
|         |       |        | J2 3   | 647,92            | 107,99     |            |                  |
|         |       |        | J3 1   | 620,05            | 103,34     | 104,52     |                  |
|         |       |        | J3 2   | 627,49            | 104,58     |            |                  |
|         |       |        | J3 3   | 633,77            | 105,63     |            |                  |
| Rwanda  | 396   | mrt-10 | K1 1   | 620,12            | 103,35     | 104,12     | 104,03           |
|         |       |        | K1 2   | 622,17            | 103,69     |            |                  |
|         |       |        | K1 3   | 631,87            | 105,31     |            |                  |
|         |       |        | K2 1   | 614,24            | 102,37     | 103,55     |                  |
|         |       |        | K2 2   | 620,85            | 103,48     |            |                  |
|         |       |        | K2 3   | 628,78            | 104,80     |            |                  |
|         |       |        | K3 1   | 619,29            | 103,21     | 104,43     |                  |
Table. Quinine quality (Continued)

| Site | Batch | Expire | pha ID | Quinine (mg/vial) | Amount (%) | Average (%) | Batch average (%) |
|------|-------|--------|--------|-------------------|------------|-------------|------------------|
|      |       |        |        | K3 2              | 627,59     | 104,60      |                  |
|      |       |        |        | K3 3              | 632,82     | 105,47      |                  |
|      | 397   | sep-10 | L1     | 1                 | 639,59     | 106,60      | 107,30           | 107,26           |
|      |       |        |        | L1 2              | 636,53     | 106,09      |                  |
|      |       |        |        | L1 3              | 655,20     | 109,20      |                  |
|      |       |        |        | L2 1              | 635,09     | 105,85      | 107,00           |                  |
|      |       |        |        | L2 2              | 639,13     | 106,52      |                  |
|      |       |        |        | L2 3              | 651,80     | 108,63      |                  |
|      |       |        |        | L3 1              | 636,46     | 106,08      | 107,49           |                  |
|      |       |        |        | L3 2              | 645,34     | 107,56      |                  |
|      |       |        |        | L3 3              | 653,03     | 108,84      |                  |
Chapter 3

Predicting the clinical outcome of severe falciparum malaria in African children: findings from a large randomised trial

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Summary

Background Data from the largest randomised, controlled trial for the treatment of children hospitalized with severe malaria were used to identify predictors of a poor outcome from severe malaria.

Methods African children (<15 years) with severe malaria participated in a randomised comparison of parenteral artesunate and parenteral quinine in 9 African countries. Detailed clinical assessment was performed on admission. Parasite densities were assessed in a reference laboratory. Predictors of death were examined using a multivariate logistic regression model.

Findings Twenty indicators of disease severity were assessed, out of which 5 (base deficit, impaired consciousness, convulsions, elevated blood urea, and underlying chronic illness) were associated independently with death. Tachypnoea, respiratory distress, deep breathing, shock, prostration, low pH, hyperparasitaemia, severe anaemia, and jaundice were statistically significant indicators of death in the univariate analysis but not in the multivariate model. Age, glucose levels, axillary temperature, parasite density, heart rate, blood pressure, and blackwater fever were not related to death in univariate models.

Conclusions Acidosis, cerebral involvement, renal impairment, and chronic illness are key independent predictors for a poor outcome in African children with severe malaria. Mortality is markedly increased in cerebral malaria combined with acidosis.
Introduction

Falciparum malaria can progress rapidly from an uncomplicated febrile illness which usually resolves with oral medication to a potentially lethal multisystem disease, despite in-hospital parenteral treatment. The mortality risk in uncomplicated falciparum malaria is thought to be under 0.1%, rising towards 1% as treatments fail in the context of antimalarial drug resistance. The mortality due to severe malaria in young children usually exceeds 10% and increases with age. Several predictive factors for death in severe childhood malaria have been identified; among them, coma, convulsions, acidosis, respiratory distress, hypoglycaemia, retinal changes, increased concentrations of lactate in blood and cerebrospinal fluid, increased concentrations of tumor necrosis factor, and the presence of malarial pigment in 0.5% or more of peripheral blood neutrophils. Combinations of prognostic indicators or scoring systems have also been evaluated to identify patients who have an increased risk of death. Outcomes in severe falciparum malaria depend on the nature and degree of vital organ dysfunction. This differs between adults and children. For example, severe malaria with renal failure is an important cause of death in adults, but acute renal failure from malaria-induced acute tubular necrosis is rare in children from any cause and is very rarely reported in African children with severe malaria. In contrast, severe anaemia is a very common presentation of severe malaria in young children in high-transmission settings, but is relatively unusual in adults. Universal indicators can be used in clinical trials to define severe malaria, and can also direct studies of the pathogenesis of severe malaria and guide the identification of new targets for intervention. Using data from the largest severe malaria treatment trial to date, we analysed the relationship between clinical and laboratory characteristics collected on admission and the risk of death.

Methods

This analysis is based on a randomised clinical trial comparing parenteral artesunate with parenteral quinine in African children with severe falciparum malaria. The trial was conducted between 2005 and 2010 in 11 participating centres in 9 African countries (Table 1). The spectrum of participating hospitals ranged from university hospitals and research institutes, to rural hospitals without prior research experience. Human immunodeficiency virus (HIV) testing was approved at 4 sites (Beira, Muheza,
Rwamagana/Nyanza and Kilifi). The reported HIV prevalence (Table 1) relied on historical data from adults.74

Table 1. Age distribution, case fatality rate, and adult HIV prevalence by study centre

| Study centre                        | Number of children | Median age (IQR), years | Mortality (%) | HIV/AIDS adult prevalence rate (%) |
|-------------------------------------|--------------------|-------------------------|---------------|------------------------------------|
| Banjul, The Gambia                  | 502                | 4.4 (3.0, 6.1)          | 55 (11%)      | 1                                  |
| Beira, Mozambique                   | 664                | 3.8 (2.5, 5.3)          | 75 (11%)      | 12                                 |
| Ilorin, Nigeria                     | 450                | 2.8 (1.8, 4.2)          | 41 (9%)       | 3                                  |
| Rwamagana and Nyanza, Rwanda        | 271 and 115        | 3.3 (1.7, 5.1)          | 20 (5%)       | 2                                  |
| Kilifi, Kenya                       | 442                | 3.3 (2.3, 4.8)          | 44 (10%)      | 8                                  |
| Kinshasa, DRC                       | 422                | 2.4 (1.6, 4.2)          | 18 (4%)       | 4                                  |
| Korogwe, Tanzania                   | 540                | 2.3 (1.3, 3.5)          | 80 (15%)      | 8                                  |
| Kumasi, Ghana                       | 436                | 3.0 (2.0, 4.3)          | 21 (5%)       | 1                                  |
| Mbarara, Uganda                     | 663                | 1.8 (1.0, 2.8)          | 42 (6%)       | 4                                  |
| Muheza, Tanzania                    | 921                | 2.2 (1.2, 3.3)          | 131 (14%)     | 8                                  |
| Total                               | 5426               | 2.8 (1.7, 4.3)          | 527 (10%)     | 6a                                 |

Abbreviations: DRC, Democratic Republic of the Congo; HIV, human immunodeficiency virus; IQR, interquartile range. a Weighted mean

The methods and the outcome of the trial have been reported in detail elsewhere.73 Febrile children less than 15 years of age with a positive *Plasmodium* lacate dehydrogenase-based rapid test (OptiMAL-IT) for falciparum malaria were eligible for enrolment based on a clinical judgment of severe malaria, which included at least 1 of the following conditions: coma, defined by a Blantyre coma scale (BCS) ≤2 for children less than 2 years of age or Glasgow coma scale (GCS) ≤10 for older children; prostration, defined as the inability to sit unsupported (for children over 6 months of age) or the inability to drink or breastfeed in younger children; convulsions with a duration longer than 30 minutes or a frequency of 2 or more in the 24 hours preceding admission; compensated shock, defined as a peripheral capillary refill time ≥3 seconds or the presence of a temperature gradient with a normal systolic BP (≥70 mmHg); decompensated shock defined as a systolic blood pressure <70 mmHg; severe respiratory distress defined as nasal alar flaring, costal indrawing, or use of accessory muscles, severe tachypnoea, or deep breathing; hypoglycaemia, defined as a blood glucose <3 mmol/L or clinical improvement in the level of consciousness immediately after administration of 10% dextrose; severe symptomatic anaemia, defined
as severe pallor combined with respiratory distress; black water fever; clinical jaundice; hyperparasitaemia, defined as a asexual parasitaemia above 10%. Patients were enrolled after a relative or guardian gave informed written consent.

**Definitions of clinical features on presentation**
Seizures were defined as a history of convulsion of 30 minutes or longer reported by the carer or observed by the health care provider. On admission, the axillary temperature was measured with a digital thermometer, and the respiratory rate was recorded. Coma, respiratory distress, deep breathing, and shock were defined as stated in the inclusion criteria.\(^5^6\) Blood pressure was measured using a digital sphygmomanometer. The presence of lymphadenopathy, candidiasis, nuchal rigidity, visible severe wasting and oedematous malnutrition were noted following standard guidelines.\(^7^5\)

**Baseline laboratory results**
A venous blood sample was collected for malaria rapid diagnostic tests, thin and thick blood films, and a point-of-care assessment of standard base excess (BE), negative logarithm of the blood hydrogen ion concentration (pH), glucose, blood urea nitrogen (BUN), haematocrit (Hct), and haemoglobin (Hb), using an automated handheld analyser (i-STAT, Abbott Laboratories, Abbott Park, IL, USA). In total, 419 i-STAT results were excluded from the analysis because of instrument malfunction. The blood films were read at the reference laboratory at the Mahidol-Oxford Research Unit in Bangkok. Malaria parasites were quantified using the readings from the thin film, or if not available, calculated from the thick blood film (40xcount/200 white blood cells).

**Statistical analysis**
The univariate association between various clinical indictors and mortality from severe malaria was initially examined visually using box plots and histograms. Factors that were considered potential independent predictors of mortality were recorded at admission, and are included (Table 2). Lymphadenopathy, malnutrition, candidiasis, severe visible wasting, and desquamation were combined into a single variable as an indicator of chronic disease, which was also examined as a risk factor. Compensated shock and decompensated shock were also combined into a single variable. The BCS scores (used for preverbal children; \(n=3417\)) and the GCS scores \(n=2004\) were combined into a single coma score (1-5). In Rwanda, 2 smaller sites (Rwamagana and Nyanza), run by the same investigators, were combined. The p values for the univariate analysis were derived by logistic regression analysis, stratified by site, and adjusted for treatment. Correlations
between variables were evaluated using Spearman’s rank correlation coefficients or the 2-sample Wilcoxon rank-sum (Mann-Whitney) test.

Table 2. Indicators evaluateda

|   | Description                                                                 |
|---|-----------------------------------------------------------------------------|
| 15 | Age (years)                                                                 |
| 16 | Base excess (BE, mmol/L)                                                    |
| 17 | Blackwater fever/dark urine                                                  |
| 18 | Coma score based on Blantyre coma scale (BCS; 0 to 5) or Glasgow coma scale (GCS; 3 to 15) |
| 19 | Blood urea nitrogen (BUN, mg/dL)                                            |
| 20 | Chronic disease (candidiasis, lymphadenopathy, malnutrition, severe visible wasting, desquamation of skin) |
| 21 | Convulsions (30 minutes or longer or ≥2 convulsions in 24 h preceding admission) |
| 22 | Glucose (mg/dL)                                                             |
| 23 | Haemoglobin (g/dL)                                                          |
| 24 | Heart rate (beats/min)                                                      |
| 25 | Log parasitaemia (parasites/uL)                                             |
| 26 | pH                                                                          |
| 27 | Prostration (unable to sit unsupported; if under 6 months old, unable to breastfeed) |
| 28 | Respiratory distress (severe tachypnoea, nasal alar flaring, costal indrawing or use of accessory muscles) |
| 29 | Deep breathing (labored breathing pattern with abnormally deep chest excursions) |
| 30 | Respiratory rate (breaths/min)                                              |
| 31 | Sex                                                                         |
| 32 | Shock (compensated and decompensated)                                       |
| 33 | Systolic blood pressure (mmHg),                                              |
| 34 | Axillary temperature (°C),                                                  |
| 35 | Visible jaundice                                                            |

aAlphabetical order

The prognostic importance of these admission variables was assessed in a multivariate logistic regression model. All clinical indicators that were significant in the univariate analysis were included in the model, with death as the dependent variable. A priori specified interactions between respiratory rate and shock, respiratory rate and age, respiratory rate and base excess, and base excess and age were assessed. A visual examination of the univariate associations between the clinical factors and death indicated possible non-linear associations with some of the continuous variables (e.g. glucose, log parasitaemia,
and respiratory rate; Figure 1). Therefore, multivariable fractional polynomials were used in conjunction with logistic regression to identify the model that would best predict the outcome. Fractional polynomials indicated that the best fitting final model was achieved with linear values. Because a large set of potential predictors were compared, there was a risk that at least 1 indicator would reach the 0.05 level of significance purely by chance. Hence, only variables with a p value less than 0.01 were retained in the final model, which was also adjusted for treatment (artesunate or quinine) and stratified by site. Receiver operating characteristic (ROC) analysis was used to evaluate the prediction ability of the final model. The logistic regression models made use of continuous variables. The fit of multivariate regression models was compared using likelihood ratios and Wald tests. In the Venn diagram approach, children with a combined coma score <3 were considered comatose, with a BE <-8 mmol/L as acidotic, and with a BUN ≥20 mg/dL as uremic. All analyses were performed using Stata software, version 11 (StataCorp, College Station, TX, USA).

**Ethical review**

The trial protocol was approved by each local ethical review board, the Oxford Tropical Research Ethics committee, and the Institutional Review Board of the London School of Hygiene and Tropical Medicine.

**Results**

There were 5426 children <15 years of age that participated in the study at 11 sites, of whom 9.7% (527) died (Table 1). Thirteen of the 21 variables examined were significantly associated with death in univariate models and were included in the multivariate model (Tables 3 and 4, Figure 1). The final model was fitted with data from 4089 children. The case fatality rate of children included and excluded from the model was 9.8%. There were 5 highly significant independent predictors of mortality from severe malaria in children, including base deficit (adjusted odds ratio [AOR] 1.12, 95% confidence interval [CI] 1.10–1.13), coma score (AOR 1.40, 95% CI 1.34–1.45), convulsions (AOR 1.72, 95% CI 1.30–2.30), BUN (AOR 1.02, 95% CI 1.02–1.03), and chronic illness (AOR 2.12, 95% CI 1.25–3.58). The area under the ROC curve indicated good ability of the model to predict mortality (area = 85%, 95% CI 83–87). None of the investigated interaction terms (respiratory rate and shock, respiratory rate and age, respiratory rate and base excess or base excess and age) were statistically significant. One or more of the independently
Table 3. Association between childhood severe malaria clinical markers with death: continuous variables\(^a\)

| Risk factor                     | Died                        | Survived                     |
|---------------------------------|-----------------------------|------------------------------|
|                                 | n                           | Mean (SD) or median (IQR)    | n                           | Mean (SD) or median (IQR)    | p value\(^c\)   |
| BE, mmol/L                      | 413                         | -15.6 (8.1)                  | 3784                        | -7.80 (6.5)                  | <0.001           |
| pH                              | 413                         | 7.25 (0.19)                  | 3782                        | 7.38 (0.11)                  | <0.001           |
| Coma score\(^d\)                | 525                         | 2.2 (1.6)                    | 4897                        | 3.7 (1.5)                    | <0.001           |
| BUN, mg/dL                      | 409                         | 23.9 (17.2)                  | 3739                        | 14.9 (11.6)                  | <0.001           |
| Respiratory rate, breaths/min   | 517                         | 52.6 (14.6)                  | 4879                        | 46.8 (14.1)                  | <0.001           |
| Haemoglobin, mg/dL              | 449                         | 6.6 (3.0)                    | 4052                        | 7.0 (2.9)                    | <0.001           |
| Heart rate, beats/min           | 515                         | 148.5 (32.2)                 | 4878                        | 144.3 (28.1)                 | 0.03             |
| Age, years (median)             | 527                         | 2.5 (1.5-4.1)                | 4899                        | 2.8 (1.7-4.3)                | 0.06             |
| Axillary temperature, °C        | 521                         | 38.1 (1.2)                   | 4885                        | 38.0 (1.1)                   | 0.24             |
| Glucose, mg/dL                  | 444                         | 117 (97.4)                   | 3994                        | 120 (71.1)                   | 0.35             |
| Systolic blood pressure, mmHg   | 403                         | 94.5 (17.3)                  | 3886                        | 94.8 (15.5)                  | 0.67             |
| Parasite density/µL\(^e\)       | 450                         | 45.533 (16-1 858 880)        | 4321                        | 45 232 (16-251 227)          | 0.82             |

Abbreviations: BE, base excess; BUN, blood urea nitrogen; IQR, interquartile range; SD, standard deviation.
\(^a\) Sorted by p value
\(^b\) Unless otherwise specified
\(^c\) From logistic regression analysis, stratified by site and adjusted for treatment
\(^d\) Blantyre coma scale and Glasgow coma scale were combined into 1 variable for analysis
\(^e\) Geometric mean (range)

significant variables from the final model were present at admission in 391/401 (98%) of children who died. There were 9 of 1065 (1%) of children without any of potential risk factors on admission who died, in contrast to 66 of 124 (53%) children with 4 predictors (Table 5). In a Venn diagram that incorporates the 3 most frequent, independently statistically significant predictors, acidosis (BE <-8 mmol/L), impaired consciousness (combined coma score <3) or convulsions, and elevated blood urea (≥20 mg/dL), the estimated mortality in children with all 3 predictors was 43% (Figure 2). In children with acidosis and impaired consciousness, the mortality was 23%. The sensitivity of acidosis as a prognostic indicator for death in this cohort was 78% (specificity 96%), impaired consciousness was 66% (specificity 95%), and elevated blood urea was 53% (specificity 95%). If 2 of the 3 prognostic signs (acidosis, impaired consciousness, or elevated blood urea) were detected, the sensitivity increased to 94% (specificity 98%).
Table 4. The association between childhood severe malaria clinical markers with death: categorical variables

| Risk factor         | n     | Died (%) | P       |
|--------------------|-------|----------|---------|
| Convulsions        |       |          |         |
| Yes                | 1692  | 242 (14) | <0.001  |
| No                 | 3734  | 285 (8)  | ...     |
| Prostration        |       |          |         |
| Yes                | 2974  | 142 (5)  | <0.001  |
| No                 | 2452  | 385 (16) | ...     |
| Shock\(^b\)        |       |          |         |
| Yes                | 663   | 123 (19) | <0.001  |
| No                 | 4763  | 404 (9)  | ...     |
| Respiratory distress |     |          |         |
| Yes                | 867   | 145 (17) | <0.001  |
| No                 | 4559  | 382 (8)  | ...     |
| Deep breathing     |       |          |         |
| Yes                | 938   | 218 (23) | <0.001  |
| No                 | 4488  | 309 (7)  | ...     |
| Jaundice           |       |          |         |
| Yes                | 114   | 22 (19)  | <0.001  |
| No                 | 5312  | 505 (10) | ...     |
| Chronic disease\(^c\) |     |          |         |
| Yes                | 189   | 34 (18)  | <0.001  |
| No                 | 5190  | 486 (9)  | ...     |
| Sex                |       |          |         |
| Male               | 2815  | 275 (10)| 0.74    |
| Female             | 2611  | 252 (10)| ...     |
| Blackwater fever   |       |          |         |
| Yes                | 237   | 22 (9)   | 0.96    |
| No                 | 5189  | 505 (10)| ...     |

\(^a\) From logistic regression analysis, stratified by site and adjusted for treatment.
\(^b\) Compensated and decompensated shock combined.
\(^c\) Lymphadenopathy, malnutrition, candidiasis, severe visible wasting and desquamation combined as an indicator for chronic disease.

Table 5. Score based on 5 independently significant variables: base excess (<-8mmol), blood urea nitrogen (≥20 mg/dL), combined coma score <3, chronic disease, and convulsions\(^a\)

| Score | Survived | Died | Total |
|-------|----------|------|-------|
| 0     | 1056     | 9 (1)| 1065  |
| 1     | 1339     | 75 (5)| 1414  |
| 2     | 923      | 118 (11)| 1041  |
| 3     | 311      | 131 (30)| 442   |
| 4     | 58       | 66 (53)| 124   |
| 5     | 1        | 2 (67)| 3     |
| Total | 3688     | 402 (10)| 4089  |

\(^a\) The presence of each variable provides one point to the score.
Figure 1. Baseline distribution of continuous variables in relation to outcome: survival or death.
Figure 1. Continued
Specific predictors

Acidosis
Increasing levels of acidosis were associated with increasing mortality (Table 3). The mean (SD) BE on admission among the 413 children who died was -16 mmol/L (8.1) compared with -8 mmol/L (6.5) among the 3784 survivors (p<0.001). Similarly, there was a statistically significant difference between the mean pH (SD) of the 413 children who died (pH=7.25, 0.19) and the 3,782 children who survived (pH=7.38, 0.11; p<0.001). When BE and pH were included in multivariate models, BE but not pH remained an independent and statistically significant covariable.

Impaired consciousness and cerebral malaria
Mortality increased with decreasing coma scores. Of the children with a combined coma score <3, 21% (347/1645) died in contrast to 5% (178/3777) of the remaining children (P p<0.001). Of the children who experienced convulsions, 14% (242/1692) died in contrast to 8% (285/3734) of the remaining children (p<0.001). The coma score retained statistical significance in the multivariate model.

Blood urea nitrogen
Mortality increased with increasing BUN in the study population. The median BUN was 24 mg/dL among the 409 children who died and 15 mg/dL among the 3,739 children who survived (p<0.001). There was a statistically significant but weak correlation between increasing age of children and increasing BUN (Spearman ρ=0.08; p<0.001).

Underlying chronic illnesses
Clinical signs of an underlying chronic illness were observed in 189 (4%) of the 5379 children participating in the study. Lymphadenopathy on its own was associated with high mortality (21%; 17/82 compared to 9%; 504/5312; p<0.001) but was not statistically significant in multivariate models. A combined variable representing signs of chronic illness was associated with a high mortality of 18% (34/189) compared with 9% (486/5190; p<0.001) for separate variables, and the combined variable retained statistical significance in the multivariate model.

Respiration
The respiratory rate was significantly higher (Figure 1), and respiratory distress (Table 4) and deep breathing were significantly more frequently encountered in children who died than in children who survived. In a multivariate model, which included a base deficit,
none of the clinical signs related to respiration were independently significant. There was a significant correlation between respiratory distress ($p<0.001$), respiratory rate ($\rho=-0.3; p<0.001$), deep breathing ($p<0.001$), and base deficit. In a model without base deficit, deep breathing and respiratory rate were significant; however, the model that included base deficit had a significantly better fit (likelihood ratio test $p<0.0001$).

**Figure 2**

Venn diagram illustrating the combinations of presentations and associated mortality

**Discussion**

In this study, 4 predictors were independently associated with an increased risk of death: acidosis indicated by a large base deficit, cerebral manifestations of malaria (coma and/or convulsions), an elevated blood urea nitrogen, or signs of chronic illness on admission. The standard base deficit was found to be the single most relevant predictor of death in this series, which confirms findings from Malawian children$^{30}$ and adult Asians$^{43}$ with severe malaria. In our study, the specificity of impaired consciousness and acidosis were similar (95% versus 96%), but the sensitivity of acidosis as a prognostic marker was 78% compared with a sensitivity of 66% for impaired consciousness. Deep breathing and
respiratory distress also had high specificities (93% and 92%), but the sensitivity of deep breathing was only 41% and that of respiratory distress was only 28%.

In severe malaria, sequestration of red blood cells containing the mature parasites leads to tissue ischaemia. Reduced red cell deformability, the clumping of uninfected to infected red cells (rosetting), and the clumping between infected red cells (auto-agglutination) further contribute to impairment of microcirculatory flow, causing tissue hypoxia and a shift from aerobic to anaerobic metabolism. With anaerobic metabolism, pyruvate is converted into lactic acid, an important but not exclusive contributor to acidosis.44,78 A venous blood base deficit had a stronger prognostic value than pH, which has been confirmed in other studies.43 This is not surprising, because a base deficit better reflects metabolic acidosis, whereas the pH starts to drop only when respiratory compensation is insufficient and the blood's buffering capacity becomes exhausted.78 Because respiratory signs in severe malaria are a response to acidosis, it should also not be surprising that respiratory signs are not independently significant predictors in models that include a base deficit. The sequestration of infected red blood cells in the cerebral microvasculature is thought to be the central pathological process preceding the cerebral manifestations of severe malaria.18,21 Coma and convulsions suggest advanced cerebral involvement, and deep coma can progress to central respiratory depression followed by death.

The association of an elevated BUN with poor outcome is of interest, as renal involvement is rarely reported in children13,35,72 in contrast to adults with severe malaria.2,53,71 The findings from this study suggest that renal involvement could be frequently overlooked in children with severe malaria. A number of factors can contribute to an increased BUN, including hypovolaemia and increased protein breakdown. More research is required to explore the significance of elevated BUN in children with severe malaria.

Overall, 4% of the study participants were diagnosed with chronic debilitating illness, the causes of which include a diverse spectrum of pathologies, including malnutrition and HIV infection. It is likely that a proportion of the chronically debilitated children were suffering from AIDS, but only 4 sites conducted diagnostic tests for it. Increased morbidity and mortality from severe malaria in HIV-infected children has been reported previously.79,80

Independent of other factors, there was no association of severe anaemia with death in our study. This may be because life-threatening severe anaemia is accompanied by metabolic acidosis and prostration. Furthermore, early transfusion in life-threatening severe anaemia in paediatric falciparum malaria can prevent death.81 In the current series, only those children with extremely low haemoglobin concentrations (<3 g/dL) were at an increased risk of dying. Similarly, hypoglycaemia can be reversed when detected early,
and as a severity sign, it is, again, not independent of other severity symptoms, including metabolic acidosis.

Hyperparasitaemia has been associated with a poor outcome in severe malaria. In our cohort, only extremely high parasite densities above 440 000/µL carried an increased risk of death. There was a small group of 25 severely ill children who presented with very low parasite densities, and the mortality in this group was also high (20%). It is possible that the malaria in these children was not the primary cause of illness, but was instead a coincidental coinfection. Very high mortality in patients with malaria and bacteraemia coinfections has been documented.

We observed a lower mortality in children with prostration (5%) compared to children without prostration (16%). Cerebral impairment caused by malaria exhibits a wide spectrum that consists of the relatively mild presentation of prostration as compared with convulsions and coma, which lie on the other end of this spectrum. Children whose presentation includes prostration have a lower mortality compared to children with more advanced impairment of their central nervous system, but have an increased mortality compared to children with uncomplicated malaria.

While the inclusion of a very large cohort from highly heterogeneous settings assures the generalizability of the findings, there is a risk that high interobserver variability occurred. This could explain, at least in part, the relatively poor performance of several clinical signs such as shock, prostration, and respiratory distress as indicators for severity in this study. It is reassuring that coma scores turned out to be valid prognostic indicators. A limitation of our study is the fact that not all participants in the trial had all test results assessed. The major reason for missing results was the unavailability of i-STAT results due to a malfunctioning analyser or an interrupted supply of test cards.

Robust and locally affordable point-of-care tests to measure any base deficit would be highly desirable for the management of severe malaria patients in sub-Saharan Africa. Until such diagnostic tests are widely available, clinical signs will have to suffice to guide clinicians. In the absence of a diagnostic test for respiratory acidosis, deep breathing signs can be a valuable marker for high risk patients in the hands of skilled observers. For large, multicentre studies, which employ observers with variable levels of training and skills, a standardized test for acidosis seems preferable.
Contributors
RO, IH, OTA, TA, NA, SBN, KB, KDC, JE, EG, WBRJ, CK, EK, KM, OAM, GM, JMA, BN, MN, MAO, AS, AKT, NU, AU, and LVS managed the study participants. LVS and SL did the analyses. LVS, SL, IH and AMD wrote the report. NJW, NPJD and AMD designed the trial. KS oversaw the laboratory. WPN managed the data. CF coordinated the trial and TS managed the study logistics. All authors read and approved the final manuscript.

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Chapter 4

Evaluation of a PfHRP2 and a pLDH-based rapid diagnostic test for the diagnosis of severe malaria in 2 populations of African children

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Summary

Background Rapid diagnostic tests (RDTs) now play an important role in the diagnosis of falciparum malaria in many countries where the disease is endemic. Although these tests have been extensively evaluated in uncomplicated falciparum malaria, reliable data on their performance for diagnosing potentially lethal severe malaria is lacking.

Methods We compared a Plasmodium falciparum histidine-rich protein-2 (PfHRP2)-based RDT and a Plasmodium lactate dehydrogenase (pLDH)-based RDT with routine microscopy of a peripheral blood slide and expert microscopy as a reference standard for the diagnosis of severe malaria in 1898 children who presented with severe febrile illness at two centres in Mozambique and Tanzania.

Results The overall sensitivity, specificity, positive predictive value, and negative predictive values of the PfHRP2-based test were respectively 94.0%, 70.9%, 85.4% and 86.8%, respectively, and for the pLDH-based test, the values were 88.0%, 88.3%, 93.2%, and 80.3%, respectively. At parasite counts <1000 parasites/μl (n=173), sensitivity of the pLDH-based test was low (45.7%) compared with that of the PfHRP2-based test (69.9%). Both RDTs performed better than did the routine slide reading in a clinical laboratory as assessed in 1 of the centres.

Conclusion The evaluated PfHRP2-based RDT is an acceptable alternative to routine microscopy for diagnosing severe malaria in African children and performed better than the evaluated pLDH-based RDT.
Introduction

The clinical diagnosis of severe malaria is unreliable, because signs and symptoms overlap with other life-threatening febrile illnesses, including pneumonia, meningitis and bacterial sepsis.1-3 Because severe malaria kills rapidly, prompt diagnosis and management are vital.4,5 On the other hand, overdiagnosis of severe malaria in African children is common and diverts attention from other infectious causes, which has been shown to contribute to mortality.6 A rapid and reliable parasitological diagnosis of severe malaria is thus essential for proper management of patients with severe febrile illness. Microscopy remains the reference standard,7 but this requires the availability of a good microscope, significant technical skills, good-quality reagents, and clean slides. The diagnostic quality of microscopy is highly variable in routine hospital settings in sub-Saharan Africa.8,9 Compared with microscopy, malaria rapid diagnostic tests (RDTs) do not require extensive training or well-maintained equipment. They are increasingly used for malaria diagnosis. Malaria RDTs are immunochromatographic tests that identify malaria antigens, most commonly Plasmodium falciparum histidine-rich protein 2 (PfHRP2) or Plasmodium lactate dehydrogenase (pLDH). Disadvantages are that test results are qualitative and do not provide prognostic information such as parasite staging and neutrophil pigment.10,11 RDTs have been evaluated extensively for the diagnosis of uncomplicated malaria but not for severe malaria,12 and diagnostic test requirements are different in severe disease; e.g. a high sensitivity is of utmost importance, because missing a case may result in inappropriate treatment and death. We therefore compared the diagnostic performance of a commonly used PfHRP2-based RDT (Paracheck; Orchid Biomedical) and a pLDH-based RDT (OptiMAL-IT; DiaMed) with that of expert microscopy, which was used as the reference standard, for the diagnosis of severe malaria in children with severe febrile illness who were admitted to 2 African hospitals in areas with different malaria transmission intensities.

Methods

The study sites were in Teule Hospital in Muheza, Tanzania and Hospital Central da Beira in Beira, Mozambique. The sites were chosen because of their different transmission dynamics, because this determines the a priori probability of the diagnosis, which influences test performance. The study was part of a large multicentre clinical trial that compared quinine and artesunate for the treatment of severe malaria.5 Ethical approval
was obtained from Comité Nacional de Bioética para a Saúde in Mozambique and the Tanzania Medical Research Coordinating Committee. Written informed consent for enrolled patients was obtained from attending relatives.

Teule Hospital is a rural 300-bed district hospital in Muheza in North-Eastern Tanzania. Malaria transmission is high, with an estimated Entomological Inoculation Rate (EIR) of 149 in 2000. In-patient paediatric human immunodeficiency virus (HIV) prevalence in children presenting with febrile illnesses was reported as 3.9%. Beira Central Hospital is an 800-bed tertiary referral hospital in Beira in Central Mozambique. EIR has not been documented here, but the observed age-distribution, including older children and occasionally including adults, suggests significantly lower transmission intensity than in Muheza. HIV prevalence is high and was reported to be 16% in adults in 2005. Children (weight ≥5 kg; age <15 years) who presented with severe febrile illness according to modified World Health Organization (WHO) clinical criteria for severe malaria, were screened using 2 malaria RDTs and a peripheral blood slide. Severity criteria included decreased consciousness (coma or severe prostration), convulsions, respiratory distress or acidic breathing, shock, severe symptomatic anaemia, hypoglycaemia, haemoglobinuria, or severe jaundice. Health care workers were trained to recognize the criteria for severe febrile illness, perform the RDTs, and prepare a peripheral blood slide, and they were supervised by the study site coordinator. In Tanzania, health workers were clinical officers and nurses who were employed for the research project. In Mozambique, hospital nurses performed the screening as part of routine care.

**Definitions**

Fever was defined as an axillary temperature ≥37.5°C or by a history of recent fever. Coma was defined as a Blantyre coma scale ≤2 for children <2 years of age or a Glasgow coma scale ≤10 for older children. Prostration was defined as the inability to sit unsupported (for children >6 months of age) or the inability to drink or breast-feed in younger children. Convulsions were recorded in cases in which the duration was >30 min or the frequency ≥2 within the 24 h preceding hospital admission. Compensated shock was defined as a peripheral capillary refill time ≥3 sec or the presence of a temperature gradient with a normal systolic blood pressure (≥70 mmHg). Decompensated shock was defined as a systolic blood pressure <70 mmHg. Severe respiratory distress was defined as nasal alar flaring, costal indrawing or recession or the use of accessory muscles, or severe tachypnoea, whereas severe acidosis was suspected if deep breathing was present. A blood glucose level <3 mmol/L or clinical improvement in the level of consciousness immediately after administration of 10% dextrose was regarded as hypoglycaemia. Anaemia was defined
as severe pallor combined with respiratory distress. Haemoglobinuria was assessed by
carer's history or observation of dark or black discoloration of the urine. Jaundice was
assessed by clinical examination.

**Sampling and slide reading**
Paracheck test (Orchid Biomedical; Mumbai, India; 0.65 USD/test), OptiMAL-IT test
(DiaMed AG; Cressier, Switzerland; 1.70 USD/test) and peripheral blood slides were
prepared from blood taken by finger prick. Thin and thick blood films were stained with
5% Giemsa for 20 min. These slides were read by local laboratory technicians (routine
practice slide reading), with later assessment by expert microscopy at the reference
laboratory at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, Thailand.
In Beira, the slides were read by microscopists working in the routine hospital laboratory.
These microscopists were blinded to the RDT results and reported thick film results
using a semi-quantitative method with a 5-point scale. In Muheza, microscopists were
unblended, and hence their findings were not included in the analysis.
Microscopists in the reference laboratory were blinded to the RDT results. The slide
findings were reported as negative if no parasites were encountered per 500 white blood
cells (WBCs) counted. Parasitaemia was quantified in the thick film if <1 parasite was
encountered per 1000 red blood cells (RBCs) in the thin film, using the formula parasites/
μL = (parasites/200 WBC) x 40, assuming a WBC count of 8000 WBCs/μL. Parasitaemia
was quantified in the thin film if >5 parasites were seen per field in the thick film, using
the formula parasites/μL = (parasites/1000 RBCs) x 30 x 125.6, assuming a haematocrit
of 30%. Slides with gametocytes but no asexual parasites were scored as negative.
Haemoglobin testing, HIV testing and blood cultures were not performed routinely.

**Data management and analysis**
Data were double-entered using Access database software (Microsoft) and analyzed
using Stata, version 10.0 (StataCorp, Texas, USA). Sensitivity, specificity, and positive
and negative predictive values were calculated using expert laboratory microscopy as
the reference standard. Categorical variables were compared using the Chi squared
or Fisher’s exact test, and continuous variables were compared using Student’s t test
or Mann-Whitney U test, depending on the distribution of the data. Sensitivities and
specificities between methods were compared using McNemar’s test. To determine
the prognostic significance of the clinical signs and symptoms, a logistic regression model was
constructed with the positive malaria slide by the reference laboratory as the dependent
variable and the age group and signs and symptoms of severe disease as independent
variables. Age groups <3 and ≥3 yrs were chosen on the basis of the age distribution within sites. Using a backwards stepwise approach, only variables with \( p<0.05 \) were retained in the final model.

**Results**

Between July 2005 through April 2009, 2190 patients were screened (Figure 1). Paired \( PfHRP2 \) and pLDH tests and slide results were available for 1898 patients, after excluding 40 patients (2%) who did not fulfill clinical severity criteria, 235 patients (10.7%) lacked an evaluable slide for the reference laboratory, and 17 (0.8%) patients who were without a valid \( PfHRP2 \)- or pLDH-based test result. Excluded patients did not differ regarding age, sex or severity criteria.

**Figure 1**

**MUHEZA, TANZANIA**

- Children screened for severe malaria \( n=1100 \)
- Fulfilling criteria for severe disease \( n=1092 \)
- Expert slide results \( n=1032 \)
  - Unreadable slide (\( n=47 \))
  - Missing slide (\( n=13 \))
- \( PfHRP2 \) and pLDH & microscopy \( n=1024 \)

**BEIRA, MOZAMBIQUE**

- Children screened for severe malaria \( n=1090 \)
- Fulfilling criteria for severe disease \( n=1058 \)
- Expert slide results \( n=883 \)
  - Unreadable slide (\( n=105 \))
  - Missing slide (\( n=73 \))
- \( PfHRP2 \) and pLDH & microscopy \( n=874 \)

Study profile comparing 2 different rapid diagnostic tests with expert microscopy in African children presenting with severe febrile illness. \( PfHRP2 \), *Plasmodium falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase.
Patients differed in age and clinical signs and symptoms between sites (Table 1). In Muheza, related to the higher malaria transmission rate, children were younger, and severe respiratory distress and prostration were the most frequent presenting symptoms. Severe anaemia with respiratory distress was more prevalent at the Muheza site than it was at the Beira site. In Beira, the most common presenting symptoms were convulsions and coma. Haemoglobinuria and severe jaundice were rare at both sites. Peripheral blood parasitaemia at hospital admission did not differ significantly between sites (p=0.711).

**Table 1.** Baseline characteristics of African paediatric patients with severe febrile illness screened for severe malaria, by study site

| Variable                                                                 | Muheza (n=1024) | Beira (n=874) | p value |
|--------------------------------------------------------------------------|-----------------|---------------|---------|
| Data collection period                                                   | Dec 2006–Mar 2008 | Jul 2005–Apr 2009 |         |
| Male sex                                                                 | 527 (51.5)      | 463 (53.0)    | 0.512   |
| Age, median months (IQR)                                                 | 18 (9–35)       | 36 (24–65)    | <0.001  |
| Presenting symptoms                                                      |                 |               |         |
| Severity criteria per patient, median N of criteria (range)              | 2 (1–7)         | 2 (1–6)       | 0.006   |
| Coma                                                                     | 177 (17)        | 417 (48)      | <0.001  |
| Prostration                                                              | 412 (40)        | 338 (39)      | 0.488   |
| Convulsions                                                              | 211 (21)        | 724 (83)      | <0.001  |
| Decompensated shock                                                      | 16 (2)          | 10 (1)        | 0.434   |
| Compensated shock                                                        | 76 (7)          | NAa           |         |
| Severe respiratory distress and/or acidotic breathing                    | 636 (62)        | 27 (3)        | <0.001  |
| Hypoglycaemia                                                            | 109 (11)        | 36 (4)        | <0.001  |
| Severe anaemia with respiratory distress                                 | 156 (15)        | 85 (10)       | <0.001  |
| Haemoglobinuria                                                          | 3 (<1)          | 16 (2)        | 0.001   |
| Severe jaundice                                                          | 12 (1)          | 17 (2)        | 0.171   |
| Slide results/parasitaemia                                               |                 |               |         |
| Slide negative                                                           | 409 (40)        | 268 (31)      | <0.001  |
| Slide positive                                                           | 530 (52)        | 550 (63)      |         |
| Slide positive, but no Pf count                                          | 85 (8)          | 56 (6)        |         |
| Geometric mean parasitaemia/μL[^b^] (95% CI)                             | 35 196 (27 533–44 990) | 32 974 (25 841–42 076) | 0.711 |

Data are No. (%) of patients, unless otherwise indicated.

Abbreviations: CI, confidence interval; IQR, interquartile range; NA, not available; Pf, *Plasmodium falciparum.*

[^a^] Compensated shock was not recorded at the moment of screening in Beira.

[^b^] From slide-positive patients with Pf count.
One slide from the Muheza site showed mixed infection of *P. falciparum* and *P. ovale*. In 141 slides (7.4%), *P. falciparum* asexual parasites were detected but could not be quantified because of poor slide quality, most was commonly caused by precipitations of the Giemsa stain.

A total of 345 patients had disconcordant results between the 3 diagnostic tests (Table 2). Most frequent were the combinations of a positive PfHRP2 test result and negative slide findings (in 197 [10.4%] of 1898 patients) and a negative pLDH test result with positive slide findings (147 [7.7%] of 1898). Patients with a positive peripheral blood slide finding and a negative PfHRP2-based test all also had negative pLDH-based test results.

| Table 2. Rapid diagnostic test results compared with expert microscopy findings |
|---------------------------------------------------------------|
| Slide positive (n=1221)       | Slide negative (n=677)       |
| pLDH positive | pLDH negative | pLDH positive | pLDH negative |
| PfHRP2 positive | 1074 | 74 | 78 | 119 |
| PfHRP2 negative | 0 | 73 | 1 | 479 |

Abbreviations: PfHRP2, *Plasmodium falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase.

A small number of the patients with false-positive RDT results (5 [2.5%] of 197 PfHRP2-based test results and [1.3%] of 79 pLDH-based test results) showed *P. falciparum* gametocytes on the slide. The presence of gametocytes in the patients with negative blood slide findings was associated with a positive PfHRP2 test result (5 of 197 with false-positive results versus 1 of 480 with true negative results; p=0.009).

The PfHRP2-based test was more sensitive than the pLDH-based test (94.0% vs 88.0%; p<0.001), but the pLDH test was more specific (88.3% versus 70.9%; p=0.001). This difference in sensitivity and specificity was observed at both sites. Both RDTs performed better at the Muheza site than at the Beira site (Table 3).

RDT sensitivity correlated positively with peripheral blood parasitaemia, as shown in Figure 2. The sensitivity of both tests was <50% with parasite counts <100 parasites/μl. For the PfHRP2-based test, sensitivity increased substantially with higher parasite densities (85% at parasite counts of 100-1000 parasites/μl and >90% at parasite counts >1000 parasites/μl). With the pLDH-based test, the sensitivity increased >90% only at parasite densities above 10 000 parasites/μl.

The peripheral blood slides assessed by hospital microscopists in Beira were compared with slide readings of the reference laboratory. Results were available for 861 of 874...
patients. Using expert microscopy as the reference standard, the sensitivity of routine slide reading was significantly less than of both RDTs: 78.0% (95% confidence interval [CI] 74.4%–81.2%) versus 92.2% for the PfHRP2-based test and 84.8% for the pLDH-based test (p<0.001 for both). The specificity of routine slide reading was 84.0% (95% CI 79.0–88.2%), which is higher than that of the PfHRP2 test (64.9%; p<0.001) and the same as that of the pLDH test (82.5%; p=0.552). The positive and negative predictive values of routine slide reading using expert microscopy as a reference test were 91.8% (95% CI 89.0%–94.0%) and 62.5% (95% CI 57.2%–67.6%), respectively.

Table 3. Comparison of the performance of 2 malaria rapid tests for the diagnosis of paediatric severe falciparum malaria compared with expert microscopy as the reference standard.

| Variable                      | Muheza (n=1024) | Beira (n=874) | Combined (n=1898) |
|-------------------------------|-----------------|---------------|------------------|
| Slide positive, n (%)         | 615 (60)        | 606 (69)      | 1221 (64)        |
| **PfHRP2-based test**         |                 |               |                  |
| PfHRP2-based test positive    | 692 (68)        | 653 (75)      | 1345 (71)        |
| Sensitivity, % (95% CI)       | 95.8 (93.9–97.2)| 92.2 (89.8–94.3)| 94.0 (92.5–95.3)|
| Specificity, % (95% CI)       | 74.8 (70.3–79.0)| 64.9 (58.9–70.6)| 70.9 (67.3–74.3)|
| Positive predictive value, % (95% CI) | 85.1 (82.2–87.7) | 85.6 (82.7–88.2) | 85.4 (83.4–87.2) |
| Negative predictive value, % (95% CI) | 92.2 (88.7–94.8) | 78.7 (72.7–83.9) | 86.8 (83.7–89.6) |
| **pLDH-based test**           |                 |               |                  |
| pLDH-based test positive, n (%) | 592 (58)       | 561 (64)      | 1153 (61)        |
| Sensitivity, % (95% CI)       | 91.1 (88.5–93.2)| 84.8 (81.7–87.6)| 88.0 (86.0–89.7)|
| Specificity, % (95% CI)       | 92.2 (89.1–94.6)| 82.5 (77.4–86.8)| 88.3 (85.7–90.7)|
| Positive predictive value, % (95% CI) | 94.6 (92.5–96.3) | 91.6 (89.0–93.8) | 93.2% (91.5–94.5) |
| Negative predictive value, % (95% CI) | 87.3 (83.8–90.3) | 70.6 (65.2–75.6) | 80.3 (77.2–83.1) |

*Abbreviations: CI, confidence interval; PfHRP2, *Plasmodium falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase.*

The sensitivity of the RDTs improved with increasing numbers of presenting signs and symptoms of severe disease (data not shown). The sensitivity of both RDT was highest in patients presenting with reduced consciousness, severe anaemia with respiratory distress, and hypoglycaemia (Table 4).
Table 4. Parasitaemia and rapid diagnostic test performance by presenting clinical signs

| Variable                                      | Total, No. (%) of patients | Positive slide results, No. (%) of patients | Parasite count, geometric mean parasites/μL (95% CI) | Sensitivity, % (95% CI) | Specificity, % (95% CI) | Sensitivity, % (95% CI) | Specificity, % (95% CI) |
|-----------------------------------------------|-----------------------------|---------------------------------------------|------------------------------------------------------|-------------------------|--------------------------|-------------------------|--------------------------|
| Clinical signs                                |                             |                                             |                                                      |                         |                          |                         |                          |
| Reduced consciousness<sup>a</sup>             | 1344 (71)                   | 999 (74)                                    | 42 730 (35 607–51 279)                                | 96.1 (94.7–97.2)        | 58.6 (53.2–63.8)          | 91.1 (89.2–92.8)         | 79.7 (75.1–83.8)          |
| Convulsions                                   | 935 (49)                    | 669 (72)                                    | 34 972 (27 744–44 082)                                | 94.3 (92.3–96.0)        | 61.3 (55.1–67.2)          | 87.7 (85.0–90.1)         | 80.5 (75.2–85.0)          |
| Shock<sup>b</sup>                             | 102 (5)                     | 72 (71)                                     | 50 789 (24 707–104 403)                               | 97.2 (90.3–99.7)        | 53.3 (34.3–71.7)          | 90.3 (81.0–96.0)         | 80.0 (61.4–92.3)          |
| Severe respiratory distress and/or acidotic breathing | 663 (35)                   | 354 (53)                                    | 41 675 (30 271–57 377)                               | 93.8 (90.7–96.1)        | 80.9 (76.1–85.1)          | 90.1 (86.5–93.0)         | 94.5 (91.3–96.8)          |
| Hypoglycaemia                                 | 145 (8)                     | 111 (77)                                    | 132 645 (78 968–222 809)                              | 98.2 (93.6–99.8)        | 50.0 (32.4–67.6)          | 98.2 (93.6–99.8)         | 73.5 (55.6–87.1)          |
| Severe anaemia with respiratory distress      | 241 (13)                    | 207 (86)                                    | 76 895 (53 10–110 294)                                | 98.1 (95.1–99.5)        | 35.3 (19.8–53.5)          | 96.6 (93.2–98.6)         | 61.8 (43.6–77.8)          |
| Haemoglobinuria                               | 19 (1)                      | 17 (90)                                     | 29 713 (6255–141 151)                                 | 100.0 (80.5–100.0)      | 50.0 (1.3–98.7)           | 100.0 (80.5–100.0)       | 50.0 (1.3–98.7)           |
| Severe jaundice                               | 29 (2)                      | 20 (69)                                     | 92 446 (31 960–267 405)                                | 100.0 (83.2–100.0)      | 88.9 (51.8–99.7)          | 100.0 (83.2–100.0)       | 88.9 (51.8–99.7)          |

Abbreviations: CI, confidence interval, PfHRP2, *Plasmodium falciparum* histidine-rich-protein-2; pLDH, *Plasmodium* lactate dehydrogenase.

<sup>a</sup> Reduced consciousness: coma or prostration.

<sup>b</sup> Shock included compensated and decompensated shock.
RDT sensitivity according to the level of peripheral blood parasitaemia (expressed as log_{10} parasites/µL) from n=1080 patients with positive slide and parasite count

Closed circles: *Plasmodium* lactate dehydrogenase (pLDH)-based test; open circles: *Plasmodium falciparum* histidine-rich protein-2 (PfHRP2)-based test. Bars represent 95% confidence intervals.

A logistic regression model was used to identify independent predictors of slide positivity on the basis of clinical parameters and age group (<3 or ≥3 years of age). Reduced consciousness (adjusted odds ratio [AOR] 4.0, 95% CI 3.2–5.1; p<0.001), and convulsions (AOR 1.7, 95% CI 1.3–2.3; p<0.001) were associated with slide positivity in the final model, adjusted for site. There was a significant interaction between age and severe anaemia (p=0.024), indicating a higher risk of severe anaemia with younger age (for the <3 year-old age group: AOR 5.8, 95% CI 3.6–9.4; p=0.001; for the ≥3 year-old age group: AOR 2.2, 95% CI 1.1–4.4; p=0.024). Shock, severe respiratory distress, hypoglycaemia, haemoglobinuria, and severe jaundice were not independent predictors of slide positivity.

**Discussion**

This is a large comparative study of RDTs for diagnosing severe malaria in severely ill children presenting to African hospitals. In our evaluation, the *Pf*HRP2-based test was a reliable alternative to routine microscopy for the diagnosis of paediatric severe malaria.
and was more sensitive than the pLDH-based RDT, but this was at the expense of a lower specificity. The PfHRP2-based test sensitivity of 96.9% (95% CI 95.7%–97.9%) for parasite densities >100 parasites/μL, is above the WHO-recommended threshold of 95%.20 The pLDH-based test had a sensitivity of 91.2% (95% CI 89.3%–92.9%) for parasite densities >100 parasites/μL. Severe malaria requires a diagnostic test with high sensitivity, because missing the diagnosis and withholding treatment may well cause death. Conversely, suboptimal specificity leads to underdiagnosis of other severe infections.6,21

It should be reminded that, even with slide-proven severe malaria, a substantial proportion of children have concomitant invasive bacterial infections that warrant antimicrobial treatment.14,22

The a priori probability of severe malaria depends on malaria transmission intensity and the prevalence of alternative diseases causing severe febrile illnesses, notably HIV/AIDS. Because HIV/AIDS prevalence is high in sub-Saharan Africa, including Mozambique,16 and with decreasing malaria transmission in several African countries,23 alternative diagnoses will become increasingly prevalent. In our study, all children had at least 1 of the WHO-defined criteria for severe malaria,17 but these clinical signs did not have a strong predictive value for peripheral blood parasitaemia, confirming the findings from earlier studies;6 only reduced consciousness had a predictive value for the diagnosis. Severe anaemia was more common in the younger age group, as reported in other studies.24,25

We identified only 2 small studies that evaluated RDT performance for the diagnosis of severe or cerebral malaria,12,26 whereas numerous studies have compared the performance of various PfHRP2-based and pLDH-based RDTs in the laboratory or for diagnosis of uncomplicated malaria in the field. The WHO and the Foundation for Innovative New Diagnostics (FIND) evaluated 68 RDTs, including both RDTs used in our study.10,11 Although assessed under laboratory conditions, the FIND evaluation confirmed that, with parasite counts <200 parasites/μL, detection rates dropped substantially for most RDTs. The WHO/FIND recommendation for RDT procurement requires a minimum detection score of 50% at a P. falciparum parasite count of 200 parasites/μL.27 The PfHRP2-based test evaluated in our study complied with this, whereas the pLDH-based test did not.

In field studies evaluating PfHRP2- and pLDH-based RDTs for the diagnosis of uncomplicated malaria, some studies reported sensitivities >95% for both RDTs,28–31 but most studies that directly compared PfHRP2 with pLDH-based tests confirm a higher sensitivity (and lower specificity) for RDTs detecting PfHRP2.32 For example, a study by Hopkins et al33 found a sensitivity of 92% for a PfHRP2-based test and 85% for a pLDH-
based test, which was mainly attributable to a better performance of the PfHRP2-based test at low parasite densities. Although, in our evaluation both RDTs performed poorly at very low parasite densities, even at very low parasite densities, the sensitivity of the PfHRP2-based test was significantly better, and this better performance was apparent at parasite counts up to 100 000/μl (Figure 2).

The persistence of PfHRP2 in the bloodstream for an extended period of up to 1 month following successful malaria parasite clearance is well documented.\(^{34-36}\) This is in contrast with the kinetics of pLDH, in which enzyme activity is no longer detectible after parasite clearance,\(^{37}\) which contributes to the higher specificity of the pLDH test. In addition, gametocytes are known to produce PfHRP2, which contributes to false-positive PfHRP2 test results.\(^{38,39}\) Indeed, in patients with negative slide findings, the presence of gametocytes was associated with false-positive PfHRP2-based test results.

False-negative PfHRP2-based test results occurred in 1% of patients with parasite counts >100 000 parasites/μl, including 1 patient with a parasite count of 1 073 880 parasites/μl. This could be related to the so-called prozone effect that has been reported for PfHRP2-based RDTs, which is the phenomenon that an excess of either antigen or antibodies can cause a false-negative test result. The prozone effect has not been observed in association with pLDH-based tests.\(^{40}\) Alternative explanations for false-negative RDT results are PfHRP2 gene polymorphisms that potentially change the antigenicity of PfHRP2.\(^{41-43}\) This has also been postulated for the gene encoding pLDH, but to date this has not been substantiated.

RDT performance was better at the site in Tanzania than at the site in Mozambique. Most likely, these differences are explained by operator-related issues, because in Tanzania the tests were operated by research staff. A contributing factor could have been that the pLDH-based test was perceived as more complicated to operate, because it requires multiple steps.\(^{10,11,31}\) Additionally, weak positive results are easier to detect with the PfHRP2-based test, in which a thin test band can be observed, than with the pLDH-based test, in which a faint test band indicates a weak positive result.

A limitation of our study was the presence of only a single-observer to report the RDT result and the absence of batch or stability testing. However, the RDTs were shipped to the study sites by cold-chain transport, and the study site coordinator and the independent monitor assured that batches were used within the expiration date, as well as stored in air-conditioned, temperature-controlled rooms. The PfHRP2-based tests are relatively heat-stable, but some of the pLDH based tests, particularly the one evaluated in this study, are
known to be heat-unstable. At both study sites, the staff was regularly trained and supervised in slide preparation and RDT operation. RDTs have mainly been promoted for outpatient management of uncomplicated malaria, but the challenges of microscopy in sub-Saharan Africa are likely to extend their use to inpatient settings. At the study site in Mozambique, which is a tertiary care centre with experienced microscopists, the sensitivity of routine microscopy was significantly lower than that of both RDTs, despite the provision of good quality reagents and training. Where the current WHO guidelines leave uncertainty about the best method for a parasitological diagnosis of severe malaria in young children, the findings of our study suggest that a PfHRP2-based RDT is considerably better than routine microscopy. To optimize the diagnosis of severe malaria and severe illness, a diagnostic algorithm could be employed in which only negative RDT results should be confirmed by reliable microscopy. A reduction in the workload of the hospital laboratory could improve microscopy quality. A negative RDT result should trigger contemplation of an alternative diagnosis. A positive RDT does not exclude co-existing bacterial infections, and antimicrobial treatment is recommended.

In conclusion, this study shows that the PfHRP2-based RDT is a reliable and easy-to-perform alternative to routine microscopy for the diagnosis of severe malaria in African children and performs better than a pLDH-based test.

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RDTs to diagnose severe malaria

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Chapter 5

Diagnosing severe falciparum malaria in parasitaemic African children; a prospective evaluation of plasma PfHRP2 measurement

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Summary

Background
In African children, distinguishing severe falciparum malaria from other severe febrile illnesses with coincidental *Plasmodium falciparum* parasiteamia is a major challenge. *P. falciparum* histidine-rich protein-2 (*Pf*HRP2) is released by mature sequestered parasites and can be used to estimate the total parasite burden. We investigated the prognostic significance of plasma *Pf*HRP2 and used it to estimate the malaria-attributable fraction in African children diagnosed with severe malaria.

Methods and findings
Admission plasma *Pf*HRP2 was measured prospectively in African children (from Mozambique, The Gambia, Kenya, Tanzania, Uganda, Rwanda, and the Democratic Republic of the Congo) aged 1 month to 15 years with severe febrile illness and a positive *Plasmodium* lactate dehydrogenase (pLDH)-based rapid test in a clinical trial comparing parenteral artesunate versus quinine (the AQUAMAT trial, ISRCTN 50258054). In 3826 severely ill children, plasma *Pf*HRP2 was higher in patients with coma (p=0.0209), acidosis (p<0.0001), and severe anaemia (p<0.0001). Admission geometric mean (95% CI) plasma *Pf*HRP2 was 1611 (1350-1922) ng/mL in fatal cases (n=381) versus 1046 (991-1104) ng/mL in survivors (n=3445; p<0.0001), without differences in parasitaemia as assessed by microscopy. There was a U-shaped association between log10 plasma *Pf*HRP2 and risk of death. Mortality increased 20% per log10 increase in *Pf*HRP2 above 174 ng/mL (adjusted odds ratio [AOR] 1.21, 95% CI 1.05-1.39; p=0.009). A mechanistic model assuming a *Pf*HRP2 independent risk of death in non-malaria illness closely fitted the observed data and showed malaria-attributable mortality less than 50% with plasma *Pf*HRP2 ≤174 ng/mL. The odds ratio (OR) for death in artesunate versus quinine-treated patients was 0.61 (95% CI 0.44-0.83; p=0.0018) in the highest *Pf*HRP2 tertile, whereas there was no difference in the lowest tertile (OR 1.05, 95% CI 0.69-1.61; p=0.82). A limitation of the study is that some conclusions are drawn from a mechanistic model, which is inherently dependent on certain assumptions. However, a sensitivity analysis of the model indicated that the results were robust to a plausible range of parameter estimates. Further studies are needed to validate our findings.

Interpretation
Plasma *Pf*HRP2 has prognostic significance in African children with severe falciparum malaria and provides a tool to stratify the risk of “true” severe malaria-attributable disease as opposed to other severe illnesses in parasitaemic African children.
Introduction

Severe falciparum malaria in children presents a major diagnostic challenge in malaria-endemic countries where a high proportion of children is parasitaemic at any time. A positive malaria blood smear is therefore not specific for severe malaria, and neither are clinical signs which are similar to those of other severe childhood infections.\textsuperscript{1-3} Overdiagnosis of falciparum malaria in severely ill children is an important problem in sub-Saharan Africa.\textsuperscript{4,5} Misdiagnosis is associated with increased mortality.\textsuperscript{6} Autopsy studies in children dying with “slide-positive” cerebral malaria show an alternative diagnosis in up to 23\% of cases.\textsuperscript{4} The central pathological process in severe falciparum malaria is sequestration of trophozoite- and schizont-stage infected erythrocytes in venules and capillaries, which compromise microcirculatory flow to vital organs.\textsuperscript{7} The circulating young ring-form parasites do not sequester and therefore do not reflect accurately the sequestered parasite burden. Thus peripheral parasite counts have weak prognostic significance,\textsuperscript{8,9} although this can be improved by assessing the stage of development of these peripheral blood parasites or counting the numbers of malaria pigment-containing neutrophils which reflects recent schizogony.\textsuperscript{10,11}

*Plasmodium falciparum* histidine-rich protein-2 (*PfHRP2*) is a water-soluble protein found inside the malaria parasite and host erythrocyte, and that circulates free or bound to proteins or antibodies in the plasma compartment.\textsuperscript{12,13} *PfHRP2* production peaks during the trophozoite stage, and approximately 90\% is released during schizont rupture.\textsuperscript{14} Since released *PfHRP2* is distributed through the total plasma volume, plasma *PfHRP2* can be considered a measure of total parasite burden of the preceding 48-hour asexual parasite life cycle.\textsuperscript{14,15} Studies in Asian adults have shown a strong correlation between plasma *PfHRP2*, disease severity and outcome.\textsuperscript{15,16}

In the current study we assessed the prognostic significance of plasma *PfHRP2* in African children with severe malaria and tested the hypothesis that its assessment could distinguish children with “true” severe malaria, in need of urgent antimalarial treatment, from those with non-malarial severe febrile illness and coincidental peripheral blood parasitaemia, in whom alternative diagnoses and additional treatment need to be considered.
Methods

The study was part of a large multinational trial comparing quinine and artesunate for the treatment of severe malaria in African children (“AQUAMAT”, ISRCTN 50258054), undertaken between October 2005 and July 2010. Ethics approval was granted by the Oxford Tropical Research Ethics Committee and the countries’ ethics review boards. Full details of this trial have been described elsewhere. In brief, children with signs of severe malaria confirmed by a positive *P. falciparum* lactate dehydrogenase (pLDH)-based rapid diagnostic test were included, provided their parents or carers gave full written informed consent. Severity was defined by clinical criteria (see Text S1). Patients were excluded if treated parenterally for >24 hours before admission. Patients were randomised to treatment with either parenteral artesunate or quinine. A venous blood sample was taken for peripheral blood slide, haematocrit (Hct), *Pf*HRP2, biochemistry, and acid-base parameters (EC8+ cartridge for i-STAT handheld blood analyser). Slide reading was performed by expert microscopists at the Mahidol-Oxford Tropical Medicine Research Unit, and parasites/µL was calculated from thin film (count/1000 RBC x 125.6 x Hct) or thick film (count/200 WBC x 40). Plasma *Pf*HRP2 was assessed blinded to patient outcomes from freeze-thawed EDTA plasma samples by a commercial sandwich ELISA kit (Celisa, Cellabs, Sydney, Australia), according to the manufacturer’s instructions with minor modifications. Pooled reference plasma from 20 subjects with *P. falciparum* parasitaemia >200 0000/µl was calibrated with recombinant *Pf*HRP2 standard (kindly provided by D. Sullivan, John Hopkins School of Public Health, Baltimore, USA) and used to construct standard curves. Concentrations in duplicate plasma dilutions (1/25 to 1/3125 in PBS/0.01%Tween) were determined according to the linear segment of the standard curve, with re-assay in cases where duplicates differed by more than 50%. Plasma samples for *Pf*HRP2 were received from 9 of the 11 AQUAMAT research sites in 7 countries (Mozambique, The Gambia, Kenya, Tanzania, Uganda, Rwanda and the Democratic Republic of the Congo). The study site in Ghana did not collect samples and the samples from Nigeria defrosted during transportation.

**Individual patient estimation of parasite burden**

Estimation of the total body parasite burden from plasma *Pf*HRP2 has been described in detail in Asian adults with severe malaria and requires incorporation of an elimination half-life estimate.
This was assessed separately in African children because clearance might be dependent on immunity (antibodies against PfHRP2), which is greater in high transmission settings, and PfHRP2 production is parasite strain dependent. Plasma PfHRP2 half-life was assessed in 30 patients from Tanzania from samples taken on admission and after 3 and 7 days following treatment. Separate ethical approval for this sub-study was obtained from the Ethics Committee of the National Institute for Medical Research, Tanzania (NIMR/HQ/R.8c/Vol.I/60). These data were analysed using WinNonlin statistical package (Pharsight, Mountain View, California, USA). Individual PfHRP2 concentration-time curves were fitted according to a first-order elimination model. From this, a mean (95% CI) plasma elimination half-life ($t_{1/2}$) was estimated as 1.10 (0.91–1.29) days, or 0.55 erythrocytic cycles. Half-life was not significantly different between treatment arms, and was not correlated with renal function (estimated by blood urea nitrogen). A parasite multiplication factor of 3 immediately before peak parasitaemia was assumed, based on in-vitro and Saimiri monkey studies of African parasite strains causing severe malaria. Higher multiplication rates were explored in a sensitivity analysis. The formula for total parasite burden is: $P_{\text{tot}} = 7.3 \times \text{PfHRP2} \times (1 - \text{Hct}) \times \text{body weight} \times 10^{13}$, with PfHRP2 in g/L. The differences in the current formula with the one used earlier in adult Asian patients result from the different estimates for plasma PfHRP2 half-life and parasite multiplication rates. The circulating parasite burden was calculated from the peripheral blood parasites/µL x 10⁶ x blood volume (=0.08 x weight [kg]). The sequestration index was calculated as total parasite burden/circulating burden.

**Statistical analysis**

Data were analysed with STATA, version 10 (StataCorp, TX, USA). Categorical variables were compared between survivors and fatal cases with $\chi^2$ or Fisher’s exact test. Normally distributed or log$_{10}$-normalized variables were compared using a Student’s $t$ test, the remainder by Wilcoxon rank-sum test. For lowest, middle, and highest tertiles of plasma PfHRP2, comparisons were made between peripheral blood parasitaemia, sequestration index, and treatment effect (mortality) following artesunate versus quinine treatment. To determine the prognostic significance of plasma PfHRP2, a logistic regression model was constructed with in-hospital death as the dependant variable and PfHRP2 as the independent variable. Since the risk of death showed a non-linear association with log$_{10}$ PfHRP2 (Figure 2A), both first- and second- degree fractional polynomial functions were explored to find the optimal fit. A quadratic polynomial function provided the best fit using the likelihood ratio test and by comparison of the areas under the curve (AUCs). The regression model was stratified for study site and adjusted for treatment and other
established predictors of death, including coma, convulsions, prostration, hypoglycaemia, respiratory distress, shock (combined compensated and decompensated), parasitaemia (parasites/µL), haemoglobin (Hb; g/dl), blood urea nitrogen (BUN; mg/dL), and base excess (BE; mmol/L). Using a stepwise approach, only covariates that were significant at \( P < 0.01 \) were retained in the final model. Fit of the final logistic regression model was confirmed using the Hosmer-Lemeshow goodness-of-fit test after ordering the data on predicted probabilities and then regrouping the data into 10 nearly equal-sized groups. Any interaction by transmission intensity regarding associations between plasma \( PfHRP2 \) and survival was checked and accounted for, if significant. Study sites in Mozambique and The Gambia were defined as low transmission; Rwanda, Tanzania, and Kenya as intermediate; and study sites in Uganda and the Democratic Republic of the Congo as high transmission.

**Modelling malaria-attributable mortality based on plasma \( PfHRP2 \)**

A mechanistic model was constructed to describe the observed U-shaped relationship between \( PfHRP2 \) strata and probability of in-hospital death (Figure 2A) making the following assumptions: (1) an exponential increase of malaria-attributable mortality with plasma \( PfHRP2 \), which describes the right side of the curve in Figure 2A:

\[
\Pr_{\text{death|malaria}} = 1 - \exp (k_1 \log PfHRP2^{k_2});
\]

(2) a probability of severe febrile illness due to non-malaria which decreased exponentially with increasing \( \log PfHRP2 \):

\[
\Pr_{\text{non-malaria}} = \exp (-k_4 \log PfHRP2);
\]

(3) a risk of death in patients with non-malaria infection equal to 0.3, independent of plasma \( PfHRP2 \):

\[
\Pr_{\text{death|non-malaria}} = 0.3;5,27
\]

and (4) that 20% of all deaths were due to non-malaria illness:

\[
\text{Death}_{\text{non-malaria}} / \text{Death}_{\text{total}} = 0.2.4
\]

The number of non-malarial deaths according to \( PfHRP2 \) stratum is then given by:

\[
\text{Death}_{\text{non-malaria}} = \Pr_{\text{death|non-malaria}} \times \Pr_{\text{non-malaria}} \times \text{Cases}_{\text{total}}
\]

and the number of deaths due to malaria by:

\[
\text{Death}_{\text{malaria}} = \Pr_{\text{death|malaria}} \times (1 - \Pr_{\text{non-malaria}}) \times \text{Cases}_{\text{total}}.
\]

For more details, see Text S2. The effects of assumptions 3 and 4 were explored in a sensitivity analysis.

**Results**

**Patient characteristics**

Of the 5425 children with pLDH-based rapid diagnostic test (RDT) confirmed falciparum malaria included in the AQUAMAT trial, plasma \( PfHRP2 \) was measured in 3826 patients. \( PfHRP2 \) could not be measured in 1600 (29%) patients either because the sample was either not collected or not received in optimal condition. Patients without \( PfHRP2 \)
data did not differ from the remainder regarding malaria slide positivity rate, geometric mean parasitaemia, or case fatality rate. Baseline clinical and laboratory characteristics according to outcome are summarized in Table 1. Although many clinical and laboratory variables associated with severity differed between survivors and fatal cases, admission parasitaemia did not.

Table 1. Demographic, clinical and laboratory characteristics of children diagnosed with severe falciparum malaria according to outcome

| Characteristic                        | Survivors (n=3445) | Fatal cases (n=381) | p value |
|--------------------------------------|--------------------|--------------------|---------|
| Female sex                           | 1692 (49%)         | 188 (49%)          | 0.93    |
| Age, y                               | 2.7 (1.5–4)        | 2.3 (1.4–4)        | 0.055   |
| Fever before enrolment, d            | 3 (2–4)            | 3 (2–4)            | 0.54    |
| Coma before enrolment, h             | 4 (2–8)            | 5 (3–8)            | 0.020   |
| **Complications on admission**       |                    |                    |         |
| Coma (GCS ≤10 or BCS ≤2)             | 983 (29%)          | 247 (65%)          | <0.0001 |
| Convulsions                          | 1176 (34%)         | 186 (49%)          | <0.0001 |
| Severe acidosis (BE < -8 mmol/L)     | 1132 (41%)         | 251 (80%)          | <0.0001 |
| Severe anaemia (Hb < 5 g/dl)         | 841 (29%)          | 117 (34%)          | 0.030   |
| Hypoglycaemia                        | 317 (9%)           | 136 (36%)          | <0.0001 |
| Respiratory distress                 | 466 (14%)          | 103 (27%)          | <0.0001 |
| Shock (combined)                     | 470 (14%)          | 100 (26%)          | <0.0001 |
| Black Water Fever                    | 126 (4%)           | 18 (5%)            | 0.30    |
| Jaundice                             | 75 (2%)            | 16 (4%)            | 0.014   |
| Hyperparasitaemia                    | 778 (25%)          | 101 (30%)          | 0.046   |
| **Laboratory assessments**           |                    |                    |         |
| *P. falciparum* slide positive       | 99%                | 98%                | 0.088   |
| Parasitaemia, geometric mean (range) | 45 008 (0-1 858 880) | 39 589 (0-1 252 227) | 0.33    |
| Blood urea nitrogen (mg/dL)          | 15 (11)            | 23 (16)            | 0.0001  |
| Haemoglobin g/dL                     | 6.9 (2.8)          | 6.5 (2.9)          | 0.015   |
| pH                                   | 7.38 (0.11)        | 7.24 (0.19)        | <0.0001 |
| HCO₃ (mmol/L)                        | 17.0 (5.4)         | 11.3 (5.8)         | <0.0001 |
| Base excess (mmol/L)                 | -8 (7)             | -16 (8)            | 0.0001  |

Data are No. (%) of patients, median (IQR), or mean (SD), unless otherwise indicated.

Abbreviations: BCS, Blantyre coma scale; BE, base excess; GCS, Glasgow coma scale; Hb, haemoglobin
Plasma *Pf*HRP2 in relation to disease severity

*Pf*HRP2 was detectable in 3800/3826 (99%) patients with severe malaria. A detectable plasma *Pf*HRP2 (geometric mean 450 ng/mL, 95% CI 209–966 ng/mL) with a negative blood slide result (but positive malaria RDT) was found in 36 (0.9%) children. Geometric mean plasma *Pf*HRP2 (95% CI) in survivors was 1046 ng/mL (991–1104 ng/mL) versus 1611 ng/mL (1350–1922 ng/mL) in fatal cases (p<0.0001; Table 2). There was no heterogeneity by stratification for transmission intensity in the difference of plasma *Pf*HRP2 concentrations between survivors and fatal cases (p=0.1). Plasma *Pf*HRP2 concentrations in relation to established features of severe falciparum malaria are summarized in Table 2. Plasma *Pf*HRP2 was significantly higher in patients with coma, acidosis, and severe anaemia but not in those with shock.

### Table 2. Plasma *Pf*HRP2 according to clinical and laboratory features of severe malaria

| Parameter          | Trait          | n   | plasma *Pf*HRP2<sup>a</sup> | p value |
|--------------------|----------------|-----|-----------------------------|---------|
| Outcome            | Fatal          | 381 | 1611 (1350–1922)            | <0.0001 |
|                    | Surviving      | 3445| 1046 (991–1104)             |         |
| Coma (GCS ≤10 or BCS ≤2) | Yes          | 1230| 1193 (1079–1320)           | 0.0209  |
|                    | No             | 2596| 1047 (986–1111)            |         |
| Acidosis (BE <−8mmol/L)<sup>b</sup> | Yes         | 1383| 1494 (1382–1614)           | <0.0001 |
|                    | No             | 1692| 969 (896–1047)             |         |
| Severe anaemia (Hb <5 g/dL)<sup>b</sup> | Yes         | 958 | 1585 (1458–1722)           | <0.0001 |
|                    | No             | 2306| 1044 (975–1118)            |         |
| Shock<sup>c</sup>  | Yes            | 570 | 1193 (1051–1355)           | 0.16    |
|                    | No             | 3256| 1075 (1016–1138)           |         |

Abbreviations: BCS, Blantyre coma scale; BE, base excess; GCS, Glasgow coma scale; Hb, haemoglobin

<sup>a</sup> Data are geometric mean (95% CI)

<sup>b</sup> BE available for n=3075 and Hb available for n=3264 due to missing i-STAT values

<sup>c</sup> Compensated and decompensated shock combined

Estimated total body parasite burden

Geometric mean (95% CI) *Pf*HRP2-derived total parasite burden was 7.5x10<sup>11</sup> (7.2x10<sup>11</sup> to 7.9x10<sup>11</sup>) parasites/body (n=3800); this was greater in fatal cases (1.2x10<sup>12</sup> [1.0x10<sup>12</sup>–1.5x10<sup>12</sup>], n=327) than in survivors (7.2x10<sup>11</sup> [6.8x10<sup>11</sup>–7.6x10<sup>11</sup>], n=3070; p<0.0001; Figure 1). In contrast, the total circulating peripheral blood parasite burden did not differ significantly between survivors and fatal cases (p=0.66). The geometric mean (95% CI)
calculated sequestration index, the ratio of total parasitaemia to circulating parasitaemia was 17 (15–18) in survivors, versus 30 (23-40) in fatal cases (p=0.0001). The sequestered parasite burden, calculated by subtracting the circulating parasite burden from the total parasite burden, gave a negative result in 296/3397 (8.7%) patients. Excluding these patients, the geometric mean (95% CI) total sequestered parasite burden was $7.7 \times 10^{11}$ parasites/body ($7.3 \times 10^{11}$-$8.2 \times 10^{11}$, n=3101). A sensitivity analysis varying the multiplication factor and PfHRP2 plasma half-life is shown in Text S3.

Comparison of circulating parasite burden and total parasite burden between surviving (circles, n=3070) and fatal (squares, n=327) cases. Circulating parasite burden was calculated from the peripheral blood parasitaemia and the total parasite burden was estimated from plasma PfHRP2, including 3397 patients with both detectable PfHRP2 and malaria parasites on the peripheral blood smear.

**Plasma PfHRP2 and risk of death**
There was a U-shaped association between plasma PfHRP2 and risk of death with a nadir in case fatality rate at a logPfHRP2 of 2.24 (=174 ng/mL; Figure 2A). In an adjusted logistic regression model, stratified by study site, plasma PfHRP2 was a strong independent predictor of death. Odds for death were 20% higher per unit increase in logPfHRP2 (adjusted odds ratio [AOR] 1.21, 95% CI 1.05-1.39; p=0.009) above
a threshold $\log \text{PfHRP2}$ value of 2.24 (=174 ng/ml). Below this concentration, risk of death increased with decreasing plasma $\log \text{PfHRP2}$ (AOR 2.3, 95% CI 1.1-5.0; p=0.03). The final model was adjusted for plasma BE, BUN, coma, convulsions, hypoglycaemia, peripheral blood parasitaemia and antimalarial treatment (Hosmer-Lemeshow $\rho$-value for goodness-of-fit=0.35).

**Figure 2**

2A

2B
Figure 2. Continued

Observed and modelled malaria-attributable mortality and morbidity according to plasma PfHRP2 concentrations

2A. Observed number of patients (grey bars, n=3826) and observed probability of death (squares with 95% CI error bars, n=381) according to PfHRP2 half-log_{10} strata. The statistical polynomial regression model (dashed line) and the mechanistic model (black line) show the probability of death according to PfHRP2 half-log_{10} strata. For a detailed description of the mechanistic model see Text S2.

2B. Malaria-attributable mortality and morbidity according to plasma PfHRP2 concentrations. The curve derived from the mechanistic model (Figure 2A) describing the relationship between log_{10} plasma PfHRP2 concentration and probability of death has been deconvoluted in two separate functions: (1) Non-malaria-attributable probability of death (dotted line, left axis), which describes the negative exponential probability of dying from non-malaria illness with increasing plasma PfHRP2 concentrations, at a constant PfHRP2 independent case fatality rate of 30%.

(2) Malaria-attributable probability of death (thin solid line, left axis), which describes the exponential increase in the probability of death with increasing plasma PfHRP2 concentration, a measure of total parasite burden, in the patient population with “true” severe malaria. From these deconvoluted functions the proportion of the total number of deaths attributable to “true” severe malaria was derived according to PfHRP2 half-log_{10} strata (diamonds and heavy solid line, malaria-attributable deaths, right axis). Using the “true” severe malaria case fatality rates per PfHRP2 half-log_{10} strata, the proportion of “true” severe malaria-attributable cases according to PfHRP2 half-log_{10} strata was derived (circles and dashed line, malaria-attributable cases).

Distinguishing death attributable to severe malaria from death attributable to other causes

High mortality rates were associated with either low or very high values of plasma PfHRP2 (Figure 2A), with the former presumably resulting from a disease other than malaria (including sepsis). The observed case fatalities in the lowest PfHRP2 half log stratum and the higher PfHRP2 strata of ≥3.5–4.0 were both over 15%. A mechanistic model describing the U-shaped correlation between log PfHRP2 stratum and risk of death showed a good fit with the observed data and the statistical model (Figure 2A). This model was deconvoluted into 2 separate functions corresponding to non-malaria- and malaria-attributable case fatality rates (Figure 2B). The model showed that below a plasma logPfHRP2 value of 2.24 (=174 ng/mL) (derived from the nadir in the polynomial logistic regression model), the probability that death resulted from malaria fell below
50%, corresponding to overall proportions of malaria-attributable severe disease <90% (Figure 2B). In the logPfHRP2 stratum of 3 to 3.5 (1000–3162 ng/mL) and above, the absolute risk of death due to malaria exceeded 8% with a probability of “true” severe malaria >95% and a probability that a death was caused by severe malaria >85% (Figure 2B). For a sensitivity analysis of the mechanistic model see Text S2.

In patients within the highest PfHRP2 tertile, corresponding to logPfHRP2 ≥3.4 (2300 ng/ml), the odds ratio (OR) for death in patients treated with artesunate versus quinine was 0.61 (95% CI 0.44–0.83; p=0.0018). In patients in the lowest PfHRP2 tertile, there was no difference in mortalities with an odds ratio for death of 1.05 (95% CI 0.69–1.61; p=0.82; Figure 3). The geometric mean (95% CI) sequestration index, the ratio of total to circulating parasite numbers was 69.8 (60.8–80.1) in patients in the highest and 4.6 (4.0–5.3) in the lowest PfHRP2 tertile (Table 3).

**Figure 3**

Treatment effect, as odds ratio for death, of artesunate versus quinine according to plasma PfHRP2 tertiles and compared to the overall treatment effect observed in the AQUAMAT trial in 5425 African children and in the similar SEAQUAMAT trial in 1461 (predominantly) adults in Asia.
Table 3. Parasite density, sequestration index and treatment effect of artesunate versus quinine according to PfHRP2 tertiles

| PfHRP2 tertiles | Low (n=1115) | Middle (n=1154) | High (n=1128) |
|-----------------|-------------|----------------|--------------|
| Plasma PfHRP2 (n=3397) | 0–829 | 830–2298 | 2299–78 848 |
| (range, geometric mean, 95% CI) | 218 (201–236) | 1401 (1379–1424) | 4762 (4598–4932) |
| Parasitaemia (n=3397) | 32 934 | 60 864 | 50 597 |
| (geometric mean, 95% CI) | (28 993–37 410) | (53 924–68 698) | (44 463–57 577) |
| p<0.0001 | p=0.041 |
| Sequestration Index (n=3397) | 4.6 | 16.9 | 69.8 |
| (geometric mean, 95% CI) | (4.0–5.3) | (15.0–19.2) | (60.8–80.1) |
| p<0.0001 | p<0.0001 |
| OR (95% CI) for fatal outcome | 1.05 | 0.81 | 0.61 |
| artesunate versus quinine (n=3826) | (0.69–1.61) | (0.54–1.22) | (0.44–0.83) |
| p=0.82 | p=0.32 | p=0.0018 |

*Tertiles derived from complete PfHRP2 data set (n=3826)*

Discussion

This very large prospective study in African children with severe falciparum malaria shows the strong and independent prognostic value of admission plasma PfHRP2 concentration, but not the conventional peripheral blood malaria parasite count. In addition, plasma PfHRP2 was found to be the best immediate measure available to distinguish severe disease caused by malaria from severe febrile illness resulting from another disease with incidental *P. falciparum* parasitaemia. Since PfHRP2 is a measure of total parasite burden, this suggests a critical pathophysiological role played by sequestered parasites in severe falciparum malaria. This is supported by studies which have correlated obstruction of microcirculatory flow in the rectal and retinal circulations to disease severity and outcome, the strong prognostic value of metabolic acidosis in severe malaria, and autopsy studies showing intense sequestration in vital organs.8,25,28-31

These results suggest that in areas of moderate or high malaria transmission where a high proportion of children are parasitaemic, admission plasma PfHRP2 can differentiate children at highest risk of death of severe falciparum malaria from those with likely alternative causes of severe febrile illness. These findings are supported by several observations.

Firstly, plasma PfHRP2 derived total parasite numbers (geometric mean 7.5x10^{11}/body) are biologically plausible, and were significantly higher in fatal cases. In contrast, less pathogenic circulating peripheral blood parasite numbers were not correlated with a fatal
outcome. The calculated sequestration index was 17 in surviving patients and 30 in non-survivors, which is similar to the median (IQR) sequestration index of 40 (9.9–273.8) calculated directly from post-mortem blood vessel counts in 50 Thai and Vietnamese adults who died from cerebral malaria.25

Second, the U-shaped curve with a nadir at 174 ng/mL describing the relationship between PfHRP2 and risk of death fits with the assumption that with low PfHRP2 death is caused by non-malarial febrile illness (including sepsis) which are independent of the low parasite burden, whereas in patients with plasma PfHRP2 above this nadir the probability of death increases with PfHRP2, representing “true” severe malaria with increasing sequestered parasite burdens. The mechanistic model based on these assumptions had a close fit with the observed data. An alternative explanation could be the presence of highly virulent parasite strains causing severe disease independent of a high total parasite burden. However, this would result in a PfHRP2-independent mortality at the left side of the curve and cannot explain the U-shape that was actually observed. Assumptions in constructing the mechanistic model included an alternative cause of death in 20% of patients and a risk of death in non-malaria disease of 30%, based on published autopsy and clinical microbiology data.4,27 The conclusions were robust to the plausible ranges of values defined for the sensitivity analysis.

Third, the treatment benefit of artesunate over quinine was absent in patients in the lowest PfHRP2 tertile, and strongest in the highest tertile (OR 0.61, 95% CI 0.44–0.83; p=0.0018). Since injectable artesunate can benefit only patients with “true” severe malaria, this provides strong supportive evidence that patients with high PfHRP2 do represent this group, and patients with low PfHRP2 do not. The OR of 0.61 in the highest PfHRP2 tertile is remarkably close to the OR of 0.60 (95% CI 0.45–0.79) reported in the large SEQUAMAT trial comparing artesunate with quinine in the treatment of severe falciparum malaria in 1461 patients in low-transmission settings in Asia.32 In these epidemiological settings incidental peripheral blood malaria parasitaemia is rare. The diagnosis of severe malaria based on a peripheral blood slide is therefore highly specific, and so the treatment effect of artesunate over quinine is undiluted by non-malarial disease.

Identification of children with slide-positive severe febrile illness but who do not have severe malaria is very important for patient management, since overdiagnosis of severe malaria is associated with increased mortality.6 A low plasma PfHRP2 should prompt investigation of alternative diagnoses including septicaemia, early administration of parenteral broad spectrum antibiotics (if not already routine), and intensive monitoring. Often antibiotics are given only after a disappointing clinical response to antimalarials,
which may be too late. High plasma PfHRP2 concentrations should not discourage antibiotic treatment combined with antimalarial treatment, because of the high proportion of concomitant invasive bacterial disease. Patients with high plasma PfHRP2, which indicates “true” severe malaria with a poor prognosis, should be monitored closely, preferentially in a high-dependency or intensive care unit. As a tool in the design of clinical trials, plasma PfHRP2 is substantially better than peripheral blood parasitaemia in assessing the malaria-attributable fractions and defining the group of patients with “true” severe malaria and a high risk of death (Figure S3 in Text S4 and 33).

An alternative tool is the presence of malaria retinopathy, which has been shown to be highly specific for cerebral malaria as confirmed by post-mortem autopsy, although this tool does require training and skilled ophthalmoscopy. It has been evaluated for cerebral malaria, whereas many patients with severe falciparum malaria presents with other syndromes. PfHRP2 can be used in both cerebral and severe non-cerebral malaria. A direct comparison between the two methods is currently underway. Development of a semi-quantitative rapid test for the detection of plasma PfHRP2 with carefully chosen thresholds could be a valuable tool in high transmission settings to distinguish “true” severe malaria from severe non-malarial febrile illness. For example, a plasma PfHRP2 concentration >1000 ng/mL (62.1% of cases in our cohort) denotes a probability >95% of “true” severe malaria with an overall case fatality rate of 11.6% (95% CI, 10.3–12.9). Defining populations with “true” severe malaria and high mortality is thus critical information for clinicians as well as researchers. In contrast, a plasma PfHRP2 concentration <100 ng/mL (8.1% of cases in our cohort) denotes a probability >15% that severe non-malarial illness is the cause of illness, warranting additional investigations.

Limitations of this study include the inherent dependency of the models on certain assumptions. Estimating the total parasite burden from PfHRP2 is sensitive to the assumed parasite multiplication factor. In the current study the multiplication rate was assumed to be 3, based on in vitro data comparing multiplication rates and multiplication potency of parasites obtained from African children compared to Asian adults. The multiplication rate of 8 used in the original model in Asian adults was based on non-immune adult patient data from the era of malaria therapy of neurosyphilis, and comparable information is obviously not available for our patient group. Applying this higher multiplication rate in this study results in an implausibly high total parasite burden. In addition to differences in parasite multiplication rates, the calculated total parasite burden is dependent on the assumed half-life of plasma PfHRP2 which can vary between patients, and on the amount of PfHRP2 released per parasite per cycle, which can vary between strains. A sensitivity analysis of these parameters is shown
in Figure S2 in Text S3. The half-life of plasma \( PfHRP2 \) in the current study was shorter than observed in adult patients in Southeast Asia (mean 1.1 versus 3.7 days). This is presumably related to the African setting where malaria transmission is high and immunological factors including high \( PfHRP2 \) antibody titers could increase plasma clearance of \( PfHRP2 \). Since variations in the model parameter estimates are applied to the entire patient group, the model renders either pathophysiological implausible upper (more parasites than the number of circulating red cells) or lower limits (less total parasites than the calculated circulating parasitaemia). Actual total parasite numbers can thus be slightly different from the model estimates. However, differences in the calculated total parasite burdens between subgroups do not depend on the choice of these variables, since these variables will affect this value by the same factor in all subgroups. A recent study in Papuan children with falciparum malaria did not show a correlation between \( PfHRP2 \) and disease severity. However, children (n=220) in this study diagnosed with severe malaria appeared to be only moderately ill as reflected by the <1% case fatality rate and low plasma \( PfHRP2 \) values (median 456 ng/mL), whereas patients in that study considered to have uncomplicated malaria had lower plasma bicarbonate concentrations as a measure of acidosis than those with severe malaria. In the present study, <1% cases had undetectable plasma \( PfHRP2 \) concentrations, despite presence of \( P. falciparum \) on the blood slide. This could have been caused by genetic variation in \( PfHRP2 \), although this polymorphism is thought not to affect the ELISA assay. Deletions of the \( PfHRP2 \) gene have been reported in field isolates from the Amazon region and in a single report from sub-Saharan Africa. However, the incidence of this genotype is thought to be low in parasites causing severe malaria related to reduced parasite fitness. A study sequencing the \( PfHRP2 \) gene in parasites from all patients in the current study who had low plasma \( PfHRP2 \) concentrations is underway.

In conclusion, admission plasma \( PfHRP2 \) provides a tool in areas of moderate and high malaria transmission to distinguish “true” severe falciparum malaria from severe febrile illness with incidental malaria parasitaemia. Plasma \( PfHRP2 \) concentrations are a valuable prognosticator in African children with severe falciparum malaria.
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Supporting information

Text S1. Description of enrolment criteria for severe falciparum malaria

Patients needed to fulfill at least one severity criterion of malaria:

Coma
- Blantyre coma scale ≤2 for preverbal children, or Glasgow coma scale ≤10 for older children.

Prostration
- Inability to sit unsupported (for children over 6 months of age) or the inability to drink or breast-feed in younger children.

Convulsions
- A duration >30 minutes or a ≥2 in the 24 h preceding admission.

Compensated shock
- Peripheral capillary refill time ≥3 sec and/or the presence of a temperature gradient with a systolic blood pressure ≥70mmHg.

Decompensated shock
- Systolic blood pressure <70 mmHg.

Severe respiratory distress
- Nasal alar flaring, costal indrawing/recession, use of accessory muscles, or severe tachypnoea.

Severe acidosis
- Presence of deep breathing.

Hypoglycaemia
- Blood glucose <3 mmol/L, or clinical improvement in the level of consciousness immediately after administration of 10% dextrose.

Anaemia
- Severe pallor combined with respiratory distress.

Blackwater fever
- By carer’s history or observation of dark or black urine.

Jaundice
- Yellow discoloration of the sclera and skin.

Hyperparasitaemia
- Asexual parasitaemia above 10%.
**Text S2. The mechanistic model and sensitivity analysis**

**Description of the mechanistic model**
The mechanistic model of PfHRP2 describing the relationship between plasma PfHRP2 and probability of death caused by “true” severe malaria and probability of “true” severe malaria in patients diagnosed with severe malaria.

The observed data show a U-shaped relationship between the probability of death and plasma PfHRP2 strata (Figure 2A, nadir log$_{10}$ 2.24 (=174 ng/mL). It was assumed that this U-shaped relationship is a composite of two intersecting curves:

1. The right end of the curve at high PfHRP2 concentrations represents cases with “true” severe malaria with an exponential increase of the risk of death with increasing plasma PfHRP2 concentrations. Two functional forms for this curve were evaluated: a simple exponential function (exp(k$_1$h)-1) and an exponential of a power of h (exp(k$_1$h$^k_2$)-1). The latter choice provided a better fit with the observed data and was included in the model, given as:

   $Pr_{death|malaria} = -1 + \exp(k_1 \log PfHRP2^{k_2}) \quad (eq. 1)$
   
   with the total number of “true” malaria cases ($d_m$) defined as:

   $d_m = [-1 + \exp(k_1 \log PfHRP2^{k_2})] \times S$, with S the total number of cases (eq. 2).

2. The left end of the curve represents cases dying from non-malaria illnesses, which is composed of a probability of non-malaria illness decreasing exponentially with increasing plasma PfHRP2 concentration and a fixed probability of death in cases with non-malaria disease. The exponential form was the most simple decay function that provided the closest fit with the observed data, given as:

   $Pr_{non-malaria} = \exp(-k_4 \log PfHRP2) \quad (eq. 3)$
   
   with a total number of non-malaria cases ($d_0$) defined as:

   $d_0 = \exp(-k_4 \log PfHRP2) \times S$, with S the total number of cases (eq. 4),

The probability to die given that the patient has a non-malaria illness is independent of plasma PfHRP2:

   $Pr_{death|non-malaria} = k_3 \quad (eq. 5)$

From eq. 3 and 5 it follows that:

   $d_0 = \exp(-k_4 \log PfHRP2) \times k_3 \times S$, (eq. 6)
This explains why the left side of the curve in Figure 2B declines with increasing PfHRP2, because the proportion of patients with a different disease than severe malaria (who have a PfHRP2 independent risk of death) declines with increasing PfHRP2 levels, so that the risk of death declines too.

Since the total number of deaths $D = d_0 + d_m = S \times [-1 + exp(k_1 \log PfHRP2^{k2}) + \exp(-k_4 \log PfHRP2) \times k_3]$, this represents the overall number of deaths and can be used to fit the relationship between PfHRP2 and observed number of deaths. These equations have 4 parameters to estimate ($k_1, k_2, k_3, k_4$).

To reduce the number of possible estimates, it was assumed for the model that $k_3 = 0.3$ and that the total number of deaths caused by non-malaria disease is 20% of the total number of deaths in all PfHRP2 strata (eq. 7). These proportions are based on published literature, but were further explored in the sensitivity analysis within a plausible range according to the consensus of the investigators.

$\frac{Death_{non-malaria}}{Death_{total}} = k_5 = 0.2$ (eq. 7)

Fitting the parameters $k1-k5$ is done by maximizing the log-likelihood (LL) defined as:

$$LL = \sum N \left( \frac{D}{S}, \frac{d_0 + d_m}{S}, \sigma \right) + nN \left( \frac{\sum d}{\sum D}, k_5, \sigma \right)$$

With $n$ the number of PfHRP2 strata and $N(x, \mu, s)$ being the probability density function of the normal distribution, with mean $\mu$ and standard deviation $s$, evaluated at $x$. The fit of the mechanistic model with the statistical model (adjusted logistical regression model, stratified by study site, see methods section) was confirmed by comparing the predicted probability of death from both models. The mean difference in the predicted probabilities (for $n=3024$, due to missing values from variables in the statistical model) was 0.96% (95% CI 0.49 – 1.43).

In the sensitivity analysis, $k3$ was fixed to be in the set {0.3, 0.4} and the proportion of all deaths cases that are not malaria, $k_5$, was fixed to be in the set {0.15, 0.2, 0.25}. The six possible combinations were used in the sensitivity analysis and the model was then refit for every pair of $k3$ and $k5$.

The sensitivity analysis

A sensitivity analysis including the main model assumptions was conducted for the mechanistic model describing the relationship between PfHRP2 stratum and malaria-attributable disease and mortality. The non-PfHRP2 dependent risk of death in non-malarial illness was tested for the values of 0.3 and 0.4. In addition, the total proportion
of death caused by non-malarial disease was varied from 0.15 to 0.30. Overall the conclusions derived from the model regarding malaria-attributable disease and mortality were robust within the chosen range of values. The variation in the nadir, describing the PfHRP2 value where the risk of death caused by malaria and non-malaria is equal, is within half a log_{10} value of PfHRP2 (Figure S1-A). Taking the most conservative approximation, the risk of death due to malaria falls below 50% with PfHRP2 <100 ng/ml. In the log PfHRP2 stratum of 3 to 3.5 (1000-3162 ng/ml) and above, the probability of “true” severe malaria varied between 93% and 97% and the probability that death was caused by severe malaria varied between 83% and 93% (Figure S1-B).
PfHRP2 in severe *P. falciparum* malaria

**Figure S1-A**

**Figure S1-B**
Text S3. Sensitivity analysis of the estimated total parasite burden as a function of parasite multiplication factor and PfHRP2 half-life

The partial rank correlation coefficient between each parameter and the calculated total parasite burden according to the model were 0.57, -0.27 and -0.44 for the parasite multiplication factor, the PfHRP2 half-life and the amount of PfHRP2 secreted per erythrocytic cycle. This indicated that the multiplication factor was the most influential factor affecting the total parasite burden estimate, followed by the amount of PfHRP2 secreted per cycle and the variations in PfHRP2 half-life respectively.

The figures below show the impact of the multiplication factor and the PfHRP2 half-life on the total parasite burden for a patient with a plasma PfHRP2 concentration of 1000 ng/mL, a (population median) haematocrit of 19% and a (population median) bodyweight of 11.2 kg.

**Figure S2-A and S2-B.** Estimated total parasite burden as a function of the parasite multiplication factor or the PfHRP2 half-life using the model as described in the methods. Values chosen for the model parameters were PfHRP2 concentration of 1000 ng/mL, Haematocrit of 19% and bodyweight of 11.2 kg.
Text S4. Plasma PfHRP2 and parasitaemia by outcome

Figure S3. Scatter plot of parasitaemia and plasma PfHRP2 in surviving (blue dots, n=3070) and fatal (red squares, n=327) cases in patients with both detectable plasma PfHRP2 and malaria parasites seen on the peripheral blood smear.

* Bejon P, Berkley JA, Mwangi T, Ogada E, Mwangi I, et al. Defining childhood severe falciparum malaria for intervention studies. PLoS Med. 2007; 4: e251.
Chapter 6

Defining falciparum malaria-attributable severe febrile illness in moderate to high transmission settings based on plasma PfHRP2

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Summary

Background In malaria-endemic settings, asymptomatic parasitaemia complicates the diagnosis of malaria. Histidine-rich protein-2 is produced by *Plasmodium falciparum* (*Pf*HRP2), and its plasma concentration reflects the total body parasite burden. We aimed to define the malaria-attributable fraction of severe febrile illness using plasma *Pf*HRP2 distributions from parasitaemic children with different clinical presentations.

Methods Plasma samples and peripheral blood slides were collected from 1435 children aged 6–60 months in communities and a nearby hospital in Northeastern Tanzania. The study population included children with severe or uncomplicated malaria, asymptomatic carriers and healthy RDT-negative controls. *Pf*HRP2 distributions in the different groups were used to model severe malaria-attributable disease.

Results Plasma *Pf*HRP2 showed a close correlation with the severity of infection. *Pf*HRP2 concentrations above 1000 ng/ml denoted a malaria-attributable fraction of 99% (95% CI 96–100%) with a sensitivity of 74% (95% CI 72–77%), whereas a concentration below 200 ng/mL denoted a proportion of >10% (95% CI 3–27%) of patients with severe febrile illness of an alternative diagnosis. Bacteraemia was more common in patients within the lowest and highest *Pf*HRP2 quintiles.

Conclusions Plasma *Pf*HRP2 concentration defines malaria-attributable disease and distinguishes severe malaria from coincidental parasitaemia in African children in a moderate to high transmission setting.
**Introduction**

Children under 5 years carry the highest burden of malaria and malaria-associated mortality in sub-Saharan Africa.\(^1\)-\(^4\) In these moderate to high transmission areas, the diagnosis of severe malaria is challenging. Parasitaemic children with severe febrile illness can suffer from severe malaria, but can also have coincidental parasitaemia with an alternative illness causing severe disease. Host partial immunity develops early in life in highly malaria-endemic regions, and malaria parasites can be tolerated without development of symptoms.\(^5\),\(^6\) Community-based cross sectional studies conducted in these settings typically show that over 10% of children under 5 years are parasitaemic by microscopy yet symptom-free, with prevalence varying by age, exposure to infection, transmission season, amongst other factors.\(^7\)-\(^10\) Commonly used malaria case definitions rely on the presence of fever and malaria parasites on peripheral blood films and thus lack specificity. In addition, symptoms of severe malaria are non-specific and can have different etiologies.\(^11\)-\(^13\)

More accurate case definitions for clinical or severe malaria are required for clinical management and research purposes. Specificity of a malaria case definition can be improved by applying a parasite density threshold based on peripheral blood parasitaemia.\(^7\),\(^14\),\(^15\) This approach is useful as an epidemiological tool, but lacks accuracy for clinical management. Peripheral blood parasitaemia does not represent the sequestered parasite burden, which is pivotal to the pathophysiology of severe falciparum malaria. Asexual parasites in the second half of the erythrocytic life-cycle effectively adhere to the endothelial lining of the microcirculation, which prevents their detection in peripheral blood films.\(^16\)

*Plasmodium falciparum* histidine-rich protein-2 (*Pf*HRP2) is a parasite derived water-soluble protein and is released in discrete amounts into the plasma predominantly during schizont rupture.\(^17\) Therefore *Pf*HRP2 plasma concentrations reflect the total body parasite burden, including the sequestered parasites. Studies in Asian adults\(^18\),\(^19\) and African children\(^20\),\(^21\) show that in contrast with peripheral blood parasite density, plasma *Pf*HRP2 correlates strongly with disease severity and outcome.

We hypothesized that plasma *Pf*HRP2, as a measure of the total parasite burden determining disease severity, can be used to define malaria-attributable disease in endemic regions where coincidental peripheral blood parasitaemia is common.

In this study, we compared the distribution of peripheral blood parasitaemia versus plasma *Pf*HRP2 concentrations in healthy RDT-negative controls, asymptomatic carriers, uncomplicated and severe malaria patients and used this to estimate the malaria-attributable fraction of severe disease.
Methods

The study was conducted in the rural lowlands of Northeastern Tanzania. Peripheral blood slides and plasma PfHRP2 samples were collected in one community and two hospital studies in neighbouring Handeni and Muheza districts in Tanga region with similar malaria transmission intensity.9,22

Four clinical severity groups were defined: severe malaria, uncomplicated malaria, asymptomatic carriers and healthy RDT-negative controls. Severe malaria cases from the hospital studies were defined by modified clinical WHO criteria confirmed by a positive pLDH (OptiMAL-IT (DiaMed AG, Switzerland) and/or PfHRP2-based (Paracheck Orchid Biomedical, India) rapid diagnostic test (RDT). Severity criteria included decreased consciousness (coma or severe prostration), convulsions, respiratory distress or acidic breathing, shock, severe symptomatic anaemia (haemoglobin concentration <5 g/dL) and hypoglycaemia (glucose concentration <2.5 mmol/L).23 Uncomplicated malaria cases, asymptomatic carriers and healthy controls were identified in the community study by pLDH-based RDT (CareStart, Access Bio, USA). Uncomplicated malaria was defined by fever, absence of severity criteria and a positive pLDH-based RDT.24 Asymptomatic carriers were defined as afebrile children (by history and axillary temp <37.5°C at presentation) with a positive pLDH-based RDT. Controls were afebrile children with a negative pLDH-based RDT. Children aged 6 to 60 months were included.

In the community, asymptomatic children were recruited in the context of the baseline screening (February-August 2008) for a randomised trial that assessed the effect of micronutrient supplementation on the incidence of uncomplicated malaria.25 In four villages in Handeni district, all resident children aged 6-60 months were invited for the screening, and those with height-for-age Z-scores ≤1.5 SD, weight-for-height Z-score >-3 SD and haemoglobin concentrations >7 g/dL were eligible to participate. Those unlikely to comply with interventions, whose parents/guardians refused consent, or with signs of severe or chronic disease upon clinical examination were excluded.

In total, 246 of 612 children had a plasma sample and a positive RDT for Plasmodium falciparum. From these, 177 were afebrile upon examination and without reported fever within the last 48 hours. Slide results were available for 172 asymptomatic individuals, which were included in the present study (“group 2”). All parasitaemic children at baseline were treated with an effective antimalarial (artemether-lumefantrine). We selected the first 60 consecutively enrolled RDT negative children as controls (“group 1”), of whom 11 were subsequently excluded because of the presence or history of fever. Uncomplicated malaria cases were detected during the follow-up period of the trial. Parents were
requested to bring study children to the clinic if their child developed a fever or became unwell. From these, a total of 285 randomly selected febrile children with positive pLDH-based RDT ("group 3") were included in the analysis (Figure 1).

Severely ill parasitaemic patients originated from two consecutive studies conducted at Teule Hospital. The details of these studies have been published elsewhere.\textsuperscript{26,27} The first study assessed the causes of fever in 3639 febrile children ("group 4") admitted from June 2006 to June 2007.\textsuperscript{26} The dataset was selected for all patients with a pathogen isolated by blood culture in the presence of a positive RDT for falciparum malaria, complemented with a random sample of children with RDT-positive severe malaria, but a negative blood culture (n=226). The second severe malaria group ("group 5") was part of a severe malaria treatment trial (AQUAMAT) conducted from February 2007 till July 2010 (n=703).\textsuperscript{27} These subjects were also part of a separate paper describing the prognostic value of plasma \textit{Pf} HRP2 in 3826 children across all AQUAMAT study sites.\textsuperscript{21}

\textbf{Figure 1}

\begin{figure}
\centering
\includegraphics[width=\linewidth]{figure1.png}
\caption{Study population}
\end{figure}
The studies were approved by the Tanzania Medical Research Coordinating Committee. The community study was also approved by the Ethical Review Committee of Wageningen University, The Netherlands. Hospital-based studies were also approved by the London School of Hygiene and Tropical Medicine and Oxford Tropical Research Ethics Committee (UK). In all studies, written individual informed consent was obtained from parents or guardians.

Malaria slide reading was conducted by experienced microscopists at the National Institute of Medical Research (NIMR) Tanga research laboratory in Korogwe, Teule Hospital (Joint Malaria Programme) research laboratory and the Mahidol-Oxford Tropical Medicine Research Unit, which was also responsible for quality control. Parasites/µl was calculated from the thick film per 200 white blood cells (WBC) and the actual WBC or, if missing, assuming 8000 WBC/µl (count/200 WBCx40). In the AQUAMAT study, parasites/µl was calculated from thin film per 1000 red blood cells (RBC; count/1000 RBCx125.6xHct).

Plasma PfHRP2 was assessed from freeze-thawed EDTA plasma samples by a commercial sandwich ELISA kit (Celisa, Cellabs, Sydney, Australia), according to the manufacturer’s instructions with minor modifications. Reference plasma with known PfHRP2 concentration was used to construct standard curves. Concentrations in diluted plasma dilutions were determined in duplicate according to the linear segment of the standard curve. “Positive” cases were defined as those where duplicate derived concentrations were in agreement (ratio 0.5–2) and the OD relative to background was more than 3 SDs of the average background based on all plates.

**Statistical analysis**

Data were analysed with STATA, version 12 (StataCorp, TX, USA). Parasite counts and PfHRP2 concentrations were normalized by log10 transformation. Normally distributed or log10-normalized variables were compared using a Student’s t test, the remainder by Wilcoxon rank-sum test. PfHRP2 concentrations between blood culture positive and blood culture negative patients were compared according to PfHRP2 quintiles in patients with severe malaria (group 4 and 5).

**Modeling PfHRP2 distributions according to diagnostic group**

Analysis of the observed PfHRP2 distributions suggested distinctive distributions according to severity of *P. falciparum* infection (Figure 2, middle column). In addition, the PfHRP2 distributions observed in patients with clinical severe malaria suggested contributions of underlying plasma PfHRP2 distributions as observed in RDT-negative
controls, asymptomatic carriers and patients with uncomplicated malaria (Figure 2, right column), all representing severe illness with alternative causes. It was assumed that each diagnostic group (k) had a distinctive Weibull distribution of plasma PfHRP2 and that the observed plasma PfHRP2 distribution in the different clinical groups (j) is a composite of these Weibull distributions. The diagnostic groups (k) comprised of healthy controls (k=1), asymptomatic carriers (k=2), and patients with uncomplicated (k=3), or severe malaria (k=4). The diagnostic groups of uncomplicated and severe malaria, in contrast with the clinically defined groups, exclude patients with coincidental parasitaemia. A mechanistic model was constructed to infer the most likely Weibull distributions in each diagnostic group (k), described by the coefficients $\alpha_k$ and $\beta_k$. The probability ($P$) that an individual ($i$) has a particular plasma PfHRP2 concentration $P(h_{ij})$ is then determined by the probability, denoted as $m_{jk}$, that this individual belongs to diagnostic group (k).

$$P(h_{ij}) = \begin{cases} m_{ji} & \text{for } h_{ij}=0 \\ \sum_{j=2}^{5} m_{jk} W(\alpha_k,\beta_k) & \text{for } h_{ij}>0 \end{cases}$$

Two different groups with clinical severe malaria were included in the model, of which one was partly selected on the presence concomitant bacteraemia (group 4, see above). The model was used to define the plasma PfHRP2-based malaria-attributable fraction in the unselected group of parasitaemic patients with a clinical diagnosis of severe malaria (group 5). It differentiates severe malaria from both the populations with asymptomatic parasitaemia and uncomplicated malaria. The proportion of malaria-attributable disease ($y$) according to PfHRP2 ($h$) is given by:

$$y(h) = \begin{cases} 100m_{s1} & \text{for } h=0 \\ \frac{100m_{s4} W(\alpha_5,\beta_5)}{\sum_{j=2}^{5} m_{jk} W(\alpha_k,\beta_k)} & \text{for } h>0 \end{cases}$$

That is for each value of plasma PfHRP2 ($h$), the malaria-attributable fraction of severe disease, is $m_{s4} W(\alpha_5,\beta_5)$ divided by the total number of individuals with the same PfHRP2 ($h$) predicted by the model as:

$$\sum_{j=2}^{5} m_{jk} W(\alpha_k,\beta_k)$$

The parameters were estimated by implementing a mixture model within WinBUGS.$^{31}$

Three chains were run for a burn-in of 5000 iterations followed by a further 5000
iterations to obtain posterior distributions. The model parameters were estimated with 95% credible intervals. Sensitivity was calculated using the model derived number of patients with severe malaria as reference.

Results

Subject characteristics
We analyzed data of 49 healthy RDT-negative controls (group 1), 172 children with asymptomatic parasitaemia (group 2), 285 patients with uncomplicated malaria (group 3), and 226 (group 4) and 703 patients (group 5) with clinical severe malaria (Figure 1). Microscopy was negative in all RDT-negative controls, except for one case with parasitaemia of 145 parasites/µL. Baseline clinical and laboratory characteristics according to malaria clinical group are summarized in Table 1. Children with severe malaria were younger than children with uncomplicated malaria (p<0.0001) or asymptomatic parasitaemia (p<0.0001) and also had lower haemoglobin concentrations (p<0.0001). Admission characteristics and outcome of patients with severe malaria (clinical group 4 and 5) are summarized in Table 2.

PfHRP2 concentrations were detectable in 8/49 (16%) healthy controls, 156/172 (91%) asymptomatic cases, 269/285 (94%) uncomplicated and 222/226 (98%) and 698/703 (99%) severe malaria patients and are given in Table 1. The distributions of peripheral blood parasitaemia and PfHRP2 concentrations according to clinical groups are displayed in Figure 2 (left and middle columns). Plasma PfHRP2 concentrations were associated with the severity of P. falciparum infection, whereas peripheral blood parasitaemia was not.

Plasma PfHRP2-based malaria-attributable disease in parasitaemic severe febrile illness
The observed PfHRP2 distributions in the clinical groups were modelled as a composite of the PfHRP2 distributions of the contributing diagnostic groups (Figure 2, right column). The model derived parameter estimates for $m_{jk}$ denoting the probability that an individual from clinical group $j=1$ to 5 belongs to diagnostic groups $k=1$ to 4 are given in the supplement. From these parameter estimates the predicted distributions were fitted to the observed distributions and used to derive malaria-attributable proportions according to plasma log$_{10}$ PfHRP2 in the unselected clinical group of severely ill parasitaemic children (group 5, Figure 3). This shows that PfHRP2 levels above 1000 ng/mL correspond to a malaria-attributable fraction of 99% (95% CI 96–100%), with a sensitivity of 74%
Table 1. Baseline characteristics of the study population according to malaria clinical group

|                         | RDT-negative controls (1) | asymptomatic carriers (2) | uncomplicated malaria (3) | severe malaria (4) | severe malaria (5) |
|-------------------------|---------------------------|---------------------------|---------------------------|-------------------|-------------------|
| n                       | 49                        | 172                       | 285                       | 226               | 703               |
| Female (%)              | 20 (41%)                  | 91 (53%)                  | 141 (49%)                 | 125 (55%)         | 339 (48%)         |
| Age (years)             | 2.3 (1.5–3.6)             | 3.2 (2.3–4.1)             | 2.8 (1.9–4.0)             | 1.7 (1.1–2.6)     | 2.2 (1.2–3.1)     |
| Weight-for-age Z-scoresa | -1.6 (0.7)                | -1.6 (0.7)                | NA                        | -1.5 (1.1)a       | -1.1 (1.2)a       |
| Temperature (°C)        | 36.4 (0.4)                | 36.5 (0.4)                | 37.9 (1.3)                | 38.0 (1.1)        | 38.1 (1.0)        |
| Haemoglobin (g/dL)b     | 11.3 (10.4–11.9)          | 10.3 (9.3–11.2)           | 9.8 (8.9–10.8)            | 4.8 (3.7–6.4)     | 6.5 (4.4–8.2)     |
| Slide Pf positive       | 1 (2.0%)                  | 118 (68.6%)               | 275 (96.5%)               | 208 (92.0%)       | 701 (99.7%)       |
| Parasitaemia (parasites/µL) geometric mean (95% CI), range | 145 (1189–2157) | 1602 (24 390–36 498) | 29 836 (22 312–35 607) | 28 187 (39 476–55 054) | 46 619 (16–1 375 069) |
| PfHRP2 (ng/mL)c, geometric mean (95% CI), range | 4 (1–11) | 19 (15–23) | 163 (137–194) | 1510 (1180–1933) | 1746 (1577–1934) |

Data are No. % of patients, median (IQR), or mean (SD) unless otherwise specified.

Abbreviations: NA, not available; *Pf*HRP2, *Plasmodium falciparum* histidine-rich protein-2

a Missing weight-for-age Z scores in some children; n=219 in group 4 and n=702 in group 5.
b Missing haemoglobin in some children; n=48 in group 1, n=169 on group 2, n=701 in group 5.
c *Pf*HRP2 concentrations shown for individuals with detectable concentrations, n=8, n=156, n=269, n=222 and n=698 in group 1 to 5, respectively.
(95% CI 72–77%). The proportion of malaria-attributable disease declined at lower PfHRP2 concentrations. Below 200 ng/mL, an alternative diagnosis than malaria was suggested in >10% (3–27%) of patients, whereas this proportion increased above 50% (95 CI 31–67%) at concentrations below 50 ng/ml.

**Table 2.** Admission characteristics and outcome of children with severe malaria

| Feature                                      | Severe malaria (4) | Severe malaria (5) |
|----------------------------------------------|--------------------|--------------------|
| Coma (BCS ≤2 or GGS ≤10)                    | 30 (13%)           | 213 (30%)          |
| Prostration (inability to sit)               | 106 (47%)          | 403 (57%)          |
| Convulsions (≥2 within 24 h)                 | 40 (18%)           | 268 (38%)          |
| Severe anaemia (haemoglobin<5g/dL)          | 128 (57%)          | 221 (32%)          |
| Hypoglycaemia (glucose<2.5 mmol/L)          | 27 (12%)           | 145 (21%)          |
| Acidosis (lactate>5 mmol/L or base excess<-8 mmol/L)<sup>a</sup> | 97 (43%) | 314 (49%) |
| Respiratory distress<sup>b</sup>             | 74 (33%)           | 131 (19%)          |
| Shock<sup>c</sup>                            | 21 (9%)            | 111 (16%)          |
| Blood culture positive<sup>d</sup>           | 47 (20.8%)         | 36 (5.1%)          |
| Mortality                                    | 31 (13.7%)         | 99 (14.1%)         |

Data are No. (%) of patients.

Abbreviations: BCS, Blantyre coma scale; GCS, Glasgow coma scale.

<sup>a</sup> Missing lactate or base excess in some children; n=197 in group 4 and n=642 in group 5, respectively.  
<sup>b</sup> Defined as nasal alar flaring, costal indrawing, use of accessory muscles or severe tachypnoea.  
<sup>c</sup> Compensated shock (capillary refill time ≥3 sec or presence of a temperature gradient with systolic blood pressure ≥70 mmHg) and decompensated shock (systolic blood pressure<70 mmHg) combined.  
<sup>d</sup> Missing blood culture in some children; n=700 in group 5.
Frequency distributions of peripheral blood parasitaemia, plasma \( PfHRP2 \) and modelled fitted \( PfHRP2 \) according to malaria clinical group (1=healthy RDT-negative controls, 2=asymptomatic carriers, 3=uncomplicated malaria, 4=severe malaria, 5=severe malaria). The fitted \( PfHRP2 \) distributions (right column) show the modelled \( PfHRP2 \) distributions with the underlying contributing \( PfHRP2 \) distributions of different diagnostic groups (dotted lines), comprised of RDT-negative controls (light green), asymptomatic carriers (green) and patients with uncomplicated (blue turquoise) and severe malaria (bright blue and purple).
Malaria-attributable proportion (black/grey lines, left axis) and sensitivity (median, 95% CI, black/striped lines, right axis) for severe disease according to plasma $\log_{10} \text{PfHRP}_2$ concentration. The malaria-attributable proportion was derived from the predicted $\text{PfHRP}_2$ distributions from the median (95% CI) values of the $m_{ij}$ distributions of individuals in each malaria diagnostic group (see Text S1).

**Blood cultures**

Blood cultures were positive in 83 patients with severe malaria (Table 2), and as expected the proportion was higher in the selected clinical severe malaria group 4. Patients with a positive blood culture were overrepresented in the lowest and highest plasma $\text{PfHRP}_2$ quintiles (Figure 4). In patients with a $\text{PfHRP}_2$ concentrations below the threshold of 200 ng/mL, 16/90 (18%) had positive blood cultures.
Blood culture positivity according to plasma *Pf*HRP2 quintiles in patients with severe malaria.
Gram-positive bacteria included: *Streptococcus pneumoniae, Staphylococcus aureus, beta-hemolytic Streptococcus.*
Other Gram-negative bacteria included: *Haemophilus influenzae* (type b), unspecified Gram-negative rods, *Salmonella Typhi, Acinetobacter baumannii, Burkholderia cepacia, Kingella kingae, Neisseria spp., Pseudomonas oryzihabitans, Pasteurella spp.*
Gram-negative bacteria included *Salmonella spp., Escherichia coli, Enterobacter cloacae, Klebsiella spp.*
Contaminants included *Micrococcus spp., Bacillus spp., coagulase negative Staphylococcus, yeast, Corynebacterium spp. (diphtheroids), unspecified Gram-positive rods, mixed bacterial species, Ralstonia pickettii, alpha-hemolytic Streptococcus viridans, Sphingomonas paucimobilis, Pseudomonas stutzeri, Chryseomonas luteola, Stenotrophomonas maltophilia.*

**Discussion**

This study shows a clear stepwise increase in plasma *Pf*HRP2 concentrations according to disease severity from asymptomatic parasitaemia, to uncomplicated, to severe malaria. There was substantially less overlap in plasma *Pf*HRP2 distributions between groups compared to the distributions of peripheral blood parasitaemia. The distinct distributions between diagnostic groups enabled to model the proportion of malaria-attributable disease based on admission plasma *Pf*HRP2, and distinguish this from patients with coincidental peripheral blood parasitaemia in whom severe disease is caused by an alternative disease. The *Pf*HRP2-based model performed better than a previously described model based on peripheral blood parasitaemia.\(^\text{15}\) The proportion of malaria-attributable disease dropped
below 50% with a sensitivity >99% at plasma PfHRP2 concentrations below 50 ng/mL, in which case additional diagnostic tests are indicated to identify alternative diseases. The current model also accurately identified patients with a very high probability of severe malaria, with still acceptable sensitivity. A threshold of 1000 ng/mL defined a population of patients with severe malaria not diluted by patients with coincidental parasitaemia (<1%), which is mainly useful for defining a study population in a research setting, but also for the treating clinician. Low plasma PfHRP2 in a parasitaemic patient with severity signs should not withhold treatment with antimalarials, but should prompt the treating physician to look for other possible diseases, depending on the clinical presentation and resources (e.g. blood culture, lumbar puncture, chest X-ray, CT scan of the cerebrum). In African settings, were diagnostic facilities are scarce and treatment stock-outs occur, PfHRP2 can also help to prioritize these resources.

A previous study reported the prognostic significance of plasma PfHRP2 for death in a large cohort of African children with severe mala.\textsuperscript{21} PfHRP2 was a better prognostic marker than peripheral blood parasitaemia or in combination with peripheral blood parasitaemia. The current study enabled a more accurate definition of the probability of non-malarial disease at low plasma PfHRP2 concentrations by incorporating individuals with asymptomatic parasitaemia and uncomplicated malaria. It is reassuring that the identified plasma PfHRP2 thresholds denoting a high or low probabilities of alternative disease were highly consistent between these studies, that used different modelling techniques.

Our findings are supported by two recent studies in African children. A small study in Tanzanian children showed a mean PfHRP2 value of 1008 ng/mL in cerebral malaria versus 443 ng/mL in uncomplicated malaria. The diagnostic potential of plasma PfHRP2 in pediatric cerebral malaria was also confirmed in a Malawian study, where presence of malarial retinopathy was used as the reference test.\textsuperscript{32} In contrast, two other studies in moderate to high transmission settings report that PfHRP2 concentration does not reflect severity in children. In Papuan children the median concentrations in uncomplicated and severe malaria were similar 584 versus 456 ng/mL).\textsuperscript{33} However, the case fatality in the severe malaria group was <1% suggesting moderately severe malaria in accordance with the low PfHRP2 concentrations reported. A small study in Kenyan children with severe malaria (n=22) reported low median PfHRP2 concentrations of 63 ng/mL with absence of decay over 48 hours, which could be related to problems in the PfHRP2 assay.\textsuperscript{34} The prognostic utility of plasma PfHRP2 is in line with previous reports in adult populations. A study in Thai adults showed a similar stepwise increase in plasma PfHRP2 according to disease severity.\textsuperscript{18} In Indonesian adults, the mean PfHRP2 value in severe
malaria was 1863 ng/mL versus 314 ng/mL in moderate severe malaria. In both studies plasma PfHRP2 was prognostic for mortality.

This is the first study assessing PfHRP2 concentrations in healthy asymptomatic children in a moderate to high transmission area. Parasite densities that can be tolerated without causing symptoms vary substantially between individuals of different age groups, transmission intensities and seasons. In moderate to high transmission settings, children under-five represent a heterogeneous group regarding levels of immunity. This is reflected by the younger age of children with severe malaria and the older age of asymptomatic children of whom 13/172 (8%) had parasite densities >10 000 parasites/µl. Similar high parasite densities have been reported in cross-sectional surveys in settings with moderate to high malaria transmission. The accuracy of PfHRP2 thresholds for defining malaria-attributable disease will vary with the level of acquired immunity in the population, because this will determine the relative population sizes of individuals with asymptomatic parasitaemia, versus uncomplicated or severe malaria and thus determines the corresponding overlap of plasma PfHRP2 distributions. The model prediction as a function of transmission intensity will be explored in a separate study. In addition, the prevalence of bacteraemia will also affect the size of the population with asymptomatic parasites or uncomplicated malaria but presenting with severe illness. Indeed, in the current study, selection of patients with a positive blood culture (group 4), resulted in a relatively higher proportion of parasitaemic patients with severe illness due to other diseases than malaria.

Detection of malarial retinopathy by fundoscopy is an alternative diagnostic tool evaluated for identification of cerebral malaria versus encephalopathic children with coincidental parasitaemia. In the African setting this has only been evaluated in comatose patients and requires considerable expertise, training and expensive equipment. In comparison, plasma PfHRP2 is positively associated with the entire clinical severity spectrum of *P. falciparum* infection. In this study plasma PfHRP2 was assessed by quantitative ELISA.

Our findings call for the development of a low-cost semi-quantitative rapid test for the detection of plasma PfHRP2 with suitable thresholds.

Positive blood cultures particularly with gram-negative organisms were overrepresented in patients within the lowest and highest PfHRP2 quintiles. Blood cultures are known to have a limited sensitivity (around 40%) in detecting bacteraemia. The actual number of bacteraemic patients could thus be 2.5 fold higher than detected, implying an actual proportion of bacteraemic patients close to 50% in patients with plasma PfHRP2 below 200 ng/mL (2.5 times the observed proportion of 18%). This would be consistent with results from Malawian autopsy series, reporting invasive bacterial infection as the cause
of death in 4/7 (64%) parasitaemic patients with an alternative diagnosis. Positive blood cultures in patients with high PfHRP2 concentrations indicate concomitant bacteraemia in severe malaria. There are several mechanisms that may explain this high rate of concomitant bacteraemia including a reduction in gut barrier function due to intense sequestration, facilitating translocation of gut bacteria, or a general immune suppression due to hemozoin and heme-oxygenase-1 induced macrophagocytic dysfunction. Particularly severe malarial anaemia is associated with invasive disease, mainly non-typhi Salmonella bacteraemia. P. falciparum infection predisposes to gram-negative bacteraemia and can account for more than half of invasive bacterial disease in malaria-endemic areas. Our data show that bacteraemia contributes to severe illness, but also occurs concomitantly in patients with severe malaria, warranting the use of broad spectrum antibiotics in addition to prompt antimalarial treatment, preferably with parenteral artesunate.

The current study has several limitations. This is a retrospective analysis of pooled datasets. Patients with severe malaria in group 5 were also included in a previous publication on the prognostic value of PfHRP2. Patients with severe malaria in group 4 were partly selected on blood culture positivity. However, patients were selected on clinical criteria and RDT results and not on the basis of PfHRP2 concentrations, and the PfHRP2 distributions in both severe malaria groups were similar. In patients with low parasitaemia, the sensitivities of the peripheral blood slide and the RDT are relatively low which could have affected the composition of the clinical groups.

In conclusion, our study shows that plasma PfHRP2 can be used to estimate the proportion of malaria-attributable disease in African children in moderate to high transmission settings and can distinguish severe malaria from severe febrile illness with coincidental peripheral blood parasitaemia. Bacteraemia is prominent in patients with severe illness and low plasma PfHRP2 concentrations, suggesting that malaria may not be their primary diagnosis. Bacteraemia is also more frequent in patients with high plasma PfHRP2, denoting concomitant sepsis with severe malaria, which implies that administration of antibiotics is warranted in all patients with a clinical diagnosis of severe malaria.
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Supporting information

**Text S1. The mechanistic model parameter estimates for \( m_{jk} \) with 95% confidence interval**

The observed *Pf*HRP2 distributions in the clinically defined groups \((j)\) healthy RDT-negative controls \((j=1)\), asymptomatic carriers \((j=2)\), uncomplicated malaria \((j=3)\), or severe malaria \((j=4 \text{ or } j=5)\) were modelled as a composite of the *Pf*HRP2 distributions of the contributing diagnostic groups \((k)\) (see Figure 2, right column). The diagnostic groups of uncomplicated and severe malaria, in contrast with the clinically defined groups, exclude patients with coincidental parasitaemia. The model derived parameter estimate \( m_{jk} \) denotes the probability that an individual from clinical group \( j=1 \text{ to } 5 \) belongs to diagnostic group \( k \): RDT-negative control \((k=1)\), asymptomatic carrier \((k=2)\), uncomplicated malaria \((k=3)\), or severe malaria \((k=4)\).

| Parameter | 2.5% | median | 97.5% |
|-----------|------|--------|-------|
| \( m_{11} \) | 0.7321 | 0.8475 | 0.9285 |
| \( m_{12} \) | 0.07153 | 0.1525 | 0.268 |
| \( m_{21} \) | 0.06734 | 0.1077 | 0.1596 |
| \( m_{22} \) | 0.8404 | 0.8923 | 0.9327 |
| \( m_{31} \) | 0.03531 | 0.05819 | 0.08908 |
| \( m_{32} \) | 0.07897 | 0.1367 | 0.2031 |
| \( m_{33} \) | 0.7341 | 0.804 | 0.8665 |
| \( m_{41} \) | 0.006988 | 0.02048 | 0.04407 |
| \( m_{42} \) | 0.03614 | 0.07437 | 0.1248 |
| \( m_{43} \) | 0.004961 | 0.06056 | 0.1468 |
| \( m_{44} \) | 0.7577 | 0.8406 | 0.9039 |
| \( m_{51} \) | 0.006988 | 0.02048 | 0.04407 |
| \( m_{52} \) | 0.03614 | 0.07437 | 0.1248 |
| \( m_{53} \) | 0.004961 | 0.06056 | 0.1468 |
| \( m_{54} \) | 0.7577 | 0.8406 | 0.9039 |
Chapter 7

Diagnosis, clinical presentation, and in-hospital mortality of severe malaria in HIV-coinfected children and adults in Mozambique

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Summary

Background Severe falciparum malaria with human immunodeficiency virus (HIV) coinfection is common in settings with a high prevalence of both diseases, but there is little information on whether HIV affects the clinical presentation and outcome of severe malaria.

Methods HIV status was assessed prospectively in hospitalized parasitaemic adults and children with severe malaria in Beira, Mozambique, as part of a clinical trial comparing parenteral artesunate versus quinine (ISRCTN 50258054).

Results HIV-1 seroprevalence was 11% (74/655) in children under 15 years and 72% (49/68) in adults with severe malaria. Children with HIV coinfection presented with more severe acidosis, anaemia, and respiratory distress, and higher peripheral blood parasitaemia and plasma Plasmodium falciparum histidine-rich protein-2 (PfHRP2). During hospitalization, deterioration in coma score, convulsions, respiratory distress and pneumonia were more common in HIV coinfected children, and mortality was 26% (19/74) versus 9% (53/581) in uninfected children (p <0.001). In an age- and antimalarial treatment-adjusted logistic regression model, significant, independent predictors for death were renal impairment, acidosis, parasitaemia, and plasma PfHRP2 concentration.

Conclusions Severe malaria in HIV coinfected patients presents with higher parasite burden, more complications and co-morbidity, and carries a higher case fatality rate. Early identification of HIV coinfection is important for the clinical management of severe malaria.
Introduction

Severe malaria and human immunodeficiency virus (HIV) coinfection are common in settings with both high malaria transmission intensity and high HIV-1 seroprevalence.1-3 Various interactions between malaria and HIV have been described, and the diseases negatively affect their reciprocal courses.4,5 HIV transmission and progression may be accelerated by malaria.6-8 Conversely, HIV infection increases the incidence of clinical malaria, severe malaria and malaria-related mortality, particularly in adults with deteriorating immune status.9-13 However, only few studies address the effects of HIV infection on severe malaria morbidity and mortality in African children, who carry the highest burden of disease.2,14-18 We studied the effects of HIV-1 coinfection on diagnosis, clinical presentation and outcome of patients with severe malaria admitted in a tertiary referral hospital in Beira, Mozambique, an area with both a high prevalence of HIV infection as well as malaria.

Methods

This study was part of a large multinational trial comparing artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT, registration number ISRCTN 50258054), the results of which have been published elsewhere.19 This substudy was conducted at Hospital Central da Beira (HCB), Beira, Mozambique, between October 2005 and July 2010. HCB is an 800-bed tertiary referral hospital in the port city of Beira, which is built along the swampy grounds at the mouth of the Pungwe River, where malaria transmission is perennial and malaria is meso-endemic.20 The HIV burden in the city of Beira is reflected in the HIV prevalence in pregnant women reported as approximately 30% in 2004.21 Ethics approval, including confidential HIV and CD4+ testing, was granted by the Comité Nacional de Bioética para a Saúde in Mozambique and the Oxford Tropical Research Ethics Committee.

Children (<15 years) and adults (≥15 years) presenting with suspected severe malaria according to modified World Health Organisation (WHO) clinical criteria were screened using a Plasmodium lactate dehydrogenase (pLDH)-based and Plasmodium falciparum histidine-rich protein-2 (PfHRP2)-based rapid diagnostic test (RDT) and a peripheral blood slide.22,23 Patients with a positive pLDH-based RDT were included in the trial, provided that full written informed consent was given by the patient or carer. Severity criteria included decreased consciousness (coma or severe prostration), convulsions,
respiratory distress or acidotic breathing, shock, severe symptomatic anaemia (haemoglobin <5 g/dL), hypoglycaemia (glucose <3 mmol/L), haemoglobinuria, severe jaundice or a convincing history of anuria or oliguria in adult patients. Patients were excluded if treated with a parenteral antimalarial >24 hours before admission. Patients were randomized to treatment with either parenteral artesunate or quinine.

A venous blood sample was taken for peripheral blood parasite counts, haematocrit (Hct), quantitative assessment of plasma PfHRP2 (a marker of total body parasite burden), biochemistry and acid-base parameters (EC8+ cartridge for i-STAT handheld analyser), as well as HIV testing and CD4+ lymphocyte count. HIV antibody testing was performed according to the sequential test algorithm by the Mozambican HIV-testing guidelines, comprising of a screening test (Determine HIV-1/2, Abbott Laboratories, Abbot Park, IL, USA) followed by a confirmation test (Uni-gold HIV, Trinity Biotech PLC, Bray, Ireland) in case of a positive screening result. Clinical staff and patients did not have access to the HIV-1 test results and laboratory staff did not have access to patient data. Surviving patients or their guardians received voluntary counselling and testing after recovery, and if indicated, were offered further treatment and follow-up according to national guidelines. HIV data were linked to the main database through an anonymized study number.

Quality assessment of slide reading was performed at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok on 1348/2017 (67%) of screening slides and 100% of slides of enrolled patients, and was used as reference. Quantitative parasite counts were calculated from thin film (count per µL=count per 1000 red blood cells x 125.6 x Hct) assuming an MCV of 80 fl or thick film (count per µL=count per 200 white blood cells [WBC] x 40) assuming a WBC of 8000/µL.

**Statistical analysis**

Data were analysed with STATA, version 12 (StataCorp, College Station, TX, USA). Categorical variables were compared between HIV-negative and HIV-positive cases with χ² or Fisher’s exact test. Normally distributed or log₁₀-normalized variables were compared using a Student t test, the remainder using Mann-Whitney U test. Weight-for-age Z scores as a measure of malnutrition were calculated using STATA applications based on the WHO Child Growth Standards. “Strictly” defined severe malaria was based on modified WHO criteria. In HIV-positive patients, CD4+ lymphocyte count or percentage-based immunological staging (not-significant, mild, advanced, severe) was performed according to the WHO classification.

To determine the prognostic significance of HIV coinfection or the WHO HIV immunological stage classification, a logistic regression model was constructed with death
as the dependant variable and HIV infection in addition to established predictors of death as the independent variables, including coma, prostration, convulsions, hypoglycaemia, respiratory distress, shock, haemoglobin (g/dL), base excess (BE; mmol/L), $\log_{10}$ blood urea nitrogen (mg/dL), $\log_{10}$ parasitaemia (parasites/µL), plasma $Pf$HRP2 (as $\log_{10}$ and $[\log_{10}]^2$, ng/mL$^{19,24,25,31-33}$ and weight-for-age $Z$ scores$^{34}$ Since the case fatality rates between children and adults differed significantly ($p<0.0001$) and the number of adult participants was limited, only children were included in the logistic regression model, which was adjusted for age and antimalarial treatment (artesunate or quinine)$^{19,35}$ Using a stepwise approach, only covariates that were significant at $p<0.05$ were retained in the final model. The a priori-specified interaction between HIV infection and $Pf$HRP2 was also assessed.

**Results**

Between October 2005 and July 2010, 896 adults were screened for severe malaria, out of whom 87 (9.7%) had a positive pLDH-based RDT, an inclusion criterion for the treatment trial (Figure 1). Out of these, 68 adults were enrolled in the treatment trial. In 1272 children with suspected severe malaria, 731 or 57.5% (724 positive pLDH and $Pf$HRP2 test, 7 with positive $Pf$HRP2-based test only) had a positive RDT of which 655 were enrolled. In screened adult patients with quality assessment of the peripheral blood slide ($n=513$), the sensitivity of the $Pf$HRP2-based RDT compared to expert microscopy was 44.3% and the specificity was 95.2%. For the pLDH-based RDT, sensitivity was 38.8% and specificity 98.5%. In the children ($n=835$), the sensitivities of these RDTs were 92.6% and 85.3%, and the specificities were 64.5% and 83.3%, respectively. The combination of a negative RDT and a positive malaria slide was associated with low parasitaemia. The parasite density (geometric mean; 95% confidence interval [CI]) with a negative $Pf$HRP2-based test and positive slide with reported parasite density was 232 (141–383) parasites/µl in adults ($n=89$) and 180 (90–359) parasites/µl in children ($n=40$, $p=0.56$). HIV status was assessed in 727/732 (99%) patients with severe malaria. Patients with discordant HIV antibody test results ($n=4$) were excluded from the analysis. Four children aged <18 months were classified as HIV-positive on the basis of their serostatus in absence of viral load testing and were included. HIV-1 seroprevalence was 74/655 (11%) in children and 49/68 (72%) in adults.
Study profile.

Abbreviations: HIV, human immunodeficiency virus; PfHRP2, *Plasmodium falciparum* histidine-rich protein-2; pLDH, *Plasmodium* lactate dehydrogenase; RDT, rapid diagnostic test.

Baseline characteristics are shown in Table 1. HIV-positive status in HIV-coinfected patients was reported on admission in 9/74 (12%) of children and 5/49 (10%) adults, of which 5 and 4 were on antiretroviral treatment, respectively. By history of the carer, 10/74 (14%) of HIV-coinfected children had a prior chronic illness (6 tuberculosis, 2 tuberculosis with chronic otitis media, 2 chronic otitis media), versus 20/579 (3%) of HIV-uninfected children (8 tuberculosis, 1 sequelae of previous cerebral malaria, 4 congenital heart disease, 1 chronic otitis media, 6 bronchial asthma, 2 no information;
p<0.001). In HIV-coinfected adults, 11/49 (22%) had a history of chronic disease (5 tuberculosis, 1 hemiparesis after stroke, 1 epilepsy, 1 hepatitis, 3 herpes zoster) versus 1/16 (6%) (hypertension, 3 no information, p=0.108).

Table 1. Baseline characteristics of children and adults with severe malaria according to HIV status

| Characteristic                              | Children <15 years | Adults ≥15 years |
|--------------------------------------------|--------------------|-----------------|
|                                            | HIV-negative n=581 | HIV-positive n=74 | p value | HIV-negative n=19 | HIV-positive n=49 | p value |
| Female                                     | 279 (48)           | 33 (45)         | 0.58    | 7 (37)            | 22 (45)         | 0.55    |
| Age, years (median, IQR)                   | 3.6 (2.5-5.0)      | 5.0 (3.0-8.0)   | 0.0001  | 18 (16-23)        | 25-38           | 0.0002  |
| Presenting symptoms                        |                    |                 |         |                  |                 |         |
| Coma                                       | 441 (76)           | 54 (73)         | 0.58    | 13 (68)           | 35 (71)         | 0.81    |
| Convulsions                                | 506 (87)           | 56 (76)         | 0.008   | 10 (53)           | 18 (37)         | 0.23    |
| Prostration                                | 132 (23)           | 19 (26)         | 0.57    | 5 (26)            | 12 (24)         | 1.0     |
| Shocka                                     | 21 (4)             | 3 (4)           | 0.74    | 0 (0)             | 3 (6)           | 0.55    |
| Severe respiratory distress                | 37 (6)             | 14 (19)         | <0.001  | 1 (5)             | 6 (12)          | 0.66    |
| Severe acidosis (BE<-8 mmol/L)             | 108 (2)            | 26 (38)         | 0.004   | 1 (8)             | 12 (27)         | 0.26    |
| Hypoglycaemia                              | 32 (6)             | 8 (11)          | 0.07    | 1 (5)             | 2 (4)           | 1.0     |
| Severe anaemia with respiratory distress   | 67 (12)            | 15 (21)         | 0.028   | 0 (0)             | 3 (6)           | 0.55    |
| Blackwater fever                           | 24 (4)             | 5 (7)           | 0.36    | 4 (21)            | 10 (20)         | 1.0     |
| Severe jaundice                            | 15 (3)             | 5 (7)           | 0.06    | 2 (11)            | 4 (8)           | 1.0     |
| Anuria/oliguriab                           | ...                | ...             | ...     | 0 (0)             | 2 (4)           | 1.0     |
| Hyperparasitaemia (>10%)                   | 106 (21)           | 21 (32)         | 0.043   | 3 (21)            | 12 (29)         | 0.74    |
| "Strictly" defined severe malariac         | 533 (94)           | 66 (89)         | 0.08    | 16 (94)           | 41 (89)         | 1.0     |

Data are No. (%) of patients, unless otherwise indicated.
Abbreviations: BE, base excess; HIV, human immunodeficiency virus; IQR, interquartile range.

a In children; compensated and decompensated shock combined. In adults; decompensated shock only.
b In adults only.
c Based on WHO criteria.22

Parasitological markers in paediatric cases

Expert slide readings with quantitative results were available from 575/655 (88%) of enrolled children with known HIV status. Geometric mean (95% CI) parasitaemia was 47 140 (37 988–58 498) in HIV-negative (n=510) and 67 977 (37 143–124 408) in HIV-positive children (n=65; p=0.26). Geometric mean (95% CI) plasma PfHRP2
concentration assessed in 653/655 (>99%) children was 831 (707–975) ng/mL in HIV-negative (n=510) and 1395 (911–2136) ng/mL in coinfected children (n=65; p=0.0321; Figure 2).

Figure 2. Comparison of the circulating peripheral blood parasitaemia (left y-axis) and plasma PfHRP2 concentration as a measure of the total body parasite burden (right y-axis), between human immunodeficiency virus (HIV)-negative (open squares, n=510) and HIV-positive (closed squares, n=65) children with quantified plasma PfHRP2 and peripheral blood parasitaemia. Abbreviations: CI, confidence interval; PfHRP2, Plasmodium falciparum histidine-rich protein-2.

Clinical manifestations of severe malaria according to HIV status
HIV-coinfected children were older and differed in their clinical presentation from uninfected children with an increased frequency of severe acidosis and severe anaemia (both clinically and laboratory assessed) and respiratory distress (Tables 1 and 2). Blood urea nitrogen (BUN) concentrations were also higher in HIV-infected children. On physical examination, HIV-positive children had significantly lower weight-for-age Z scores and more frequent oral candidiasis and lymphadenopathy. In the adult patients, similar nonsignificant trends were recorded.
| Assessment                          | Children <15 years | Adults ≥15 years |
|------------------------------------|--------------------|-----------------|
|                                    | HIV-negative       | HIV-positive    | p value | HIV-negative       | HIV-positive    | p value |
|                                    | n=581              | n=74            |         | n=19               | n=49            |         |
| **Clinical examination**           |                    |                 |         |                    |                 |         |
| Weight-for-age Z score<sup>a</sup> | -1.0 (1.3)         | -1.6 (1.1)      | 0.0001  |                    |                 |         |
| Temperature (°C)                   | 38.2 (0.9)         | 38.3 (1.0)      | 0.76    | 38.1 (1.2)         | 38.2 (1.3)      | 0.76    |
| Blood pressure (mmHg)              |                    |                 |         |                    |                 |         |
| Systolic                           | 103 (16)           | 104 (16)        | 0.48    | 112 (16)           | 113 (22)        | 0.82    |
| Diastolic                          | 64 (13)            | 65 (16)         | 0.65    | 69 (14)            | 69 (13)         | 0.89    |
| Respiratory rate (breaths/min)     | 39 (10)            | 39 (9)          | 0.98    | 26 (5)             | 29 (8)          | 0.14    |
| Coma depth (n, median, range)      |                    |                 |         |                    |                 |         |
| BCS                                | 70, 2 (0–5)        | 7, 2 (2–3)      | 0.29    |                    |                 |         |
| GCS                                | 511, 8 (3–15)      | 67, 9 (3–15)    | 0.29    | 18,10 (3–14)       | 48, 8 (3–15)    | 0.54    |
| Oral candidiasis                   | 1 (<1%)            | 9 (12%)         | <0.001  | 0                  | 4 (9%)          | 0.57    |
| Lymphadenopathy                    | 16 (3%)            | 20 (27%)        | <0.001  | 1 (6%)             | 5 (10%)         | 0.54    |
| **Laboratory assessments**         |                    |                 |         |                    |                 |         |
| BUN (mg/dL)<sup>b</sup>            | 12 (11–12)         | 16 (13–19)      | <0.0001 | 23 (15–35)         | 32 (25–40)      | 0.18    |
| Haemoglobin (g/dL)<sup>c</sup>     | 8.2 (2.7)          | 7.5 (2.7)       | 0.033   | 11.3 (2.6)         | 10.2 (3.2)      | 0.16    |
| pH<sup>d</sup>                     | 7.40 (0.11)        | 7.38 (0.14)     | 0.26    | 7.39 (0.09)        | 7.37 (0.13)     | 0.64    |
| HCO<sub>3</sub> (mmol/L)<sup>e</sup> | 19.9 (4.9)        | 17.7 (6.6)      | 0.0267  | 21.8 (3.1)         | 19.7 (5.7)      | 0.17    |
| Base excess (mmol/L)<sup>f</sup>   | -5 (6)             | -7 (8)          | 0.0461  | -3 (4)             | -6 (8)          | 0.43    |
| Slide Pf positive                  | 547 (99%)          | 72 (99%)        | 1.0     | 18 (100%)          | 45 (96%)        | 1.0     |
| Parasitaemia (parasites/µL)<sup>g</sup> | 47 141          | 68 320          | 0.25    | 133 653            | 61 525          | 0.36    |
| geometric mean (95% CI)            | (38 005–58 474)    | (37 680–123 874) | 0.25    | (59 082–302 343)   | (24 628–153 704) | 0.36    |
| Pf/HRP2 (ng/ml)<sup>h</sup>        | 834                | 1452            | 0.0197  | 457                | 2471            | 0.0072  |
| geometric mean (95% CI)            | (712–977)          | (983–2145)      | 0.0197  | (93–2236)          | (1509–4047)     |         |
### Table 2. Continued

| Assessment                                      | Children <15 years | Adults ≥15 years |
|------------------------------------------------|--------------------|------------------|
|                                                 | HIV-negative       | HIV-positive     |
|                                                 | n=581              | n=74             |
| CD4+ percentage                               | ...                | ...              |
| CD4+ absolute cell count                      | ...                | ...              |
| HIV-associated immunodeficiency               | ...                | ...              |
| Not significant                               | ...                | 6 (14%)          |
| Mild                                          | ...                | 7 (16%)          |
| Advanced                                      | ...                | 8 (19%)          |
| Severe                                        | ...                | 22 (51%)         |

Data are No. (%) of patients, mean (SD) or median (IQR), unless otherwise indicated.

Abbreviations: BCS, Blantyre coma scale; BUN, blood urea nitrogen; CI, confidence interval; GCS, Glasgow coma scale; HIV, human immunodeficiency virus; IQR, interquartile range; Pf HRP2, *Plasmodium falciparum* histidine-rich protein-2.

1. For children ≤10 years (HIV-negative children, n=563; HIV-positive children, n=67)
2. UN was available in n=487 and n=67 children, n=15 and n=45 adults, respectively.
3. Haemoglobin was available in n=551 and n=70 children, n=19 and n=48 adults, respectively.
4. pH was available in n=489 and n=68 children, n=13 and n=45 adults, respectively.
5. HCO₃⁻ was available in n=490 and n=68 children, n=13 and n=44 adults, respectively.
6. BE was available in n=488 and n=68 children, n=13 and n=45 adults, respectively.
7. Parasitaemia reported from all patients with peripheral blood slide count, n=511 and n=66 children, n=14 and n=44 adults, respectively.
8. Pf HRP2 reported for all patients with Pf HRP2 sample n=580 and n=73 children, n=18 and n=48 adults, respectively.
9. CD4+ percentage reported for HIV-infected children <5 yrs (n=34) and adults (n=42).
10. CD4+ absolute cell counts reported for HIV-infected children ≥5 yrs (n=34) and adults (n=43).
11. Based on WHO criteria.²⁰
CD4+ percentages and/or absolute counts were available for 68/74 (92%) of HIV-positive children. Increasing HIV-associated immunodeficiency according to the WHO classification was associated with increasing plasma *Pf*HRP2 concentration (non-parametric test for trend; *p*=0.022) but not with mortality (*p*=0.23).

**Comorbidities, complications and outcome in paediatric cases**

Comorbidities during hospitalization were more common in HIV-coinfected children (15/74 [20%]) compared with HIV-negative children (51/581 [9%]; *p*=.002). Pneumonia was suspected clinically in 19 (3%) of HIV-negative children versus 9 (12%) in HIV-positive children (*p*<0.001). Chest X-rays were sparsely available, and only a minority of cases had a radiologically confirmed diagnosis of pneumonia. Although culture facilities were lacking, clinical sepsis was more common in HIV-coinfected children as well as a variety of other comorbidities (Table 3). After admission, HIV-coinfected children developed more severe malaria-related complications compared to HIV-negative children (Table 3). HIV-coinfected children received more blood transfusions (38/74 [51%] versus 224/581 [39%]; *p*=0.034) and antibiotics (65/74 [88%] versus 447/581 [77%]; *p*=0.033) in HIV-negative children.

In surviving children without neurological sequelae, median hospitalization time (interquartile range [IQR]) was 5 (4–7) days in HIV-coinfected versus 4 days (3–5) in HIV-negative children (*p*=0.0012). The mortality in HIV-coinfected children was 19/74 (26%) versus 53/581 (9%) in HIV-uninfected children (*p*<0.001). This difference remained significant when only patients with “strictly” defined severe malaria (see the “Methods” section) were included in the analysis. Although this substudy was not powered to look at the treatment effect of artesunate versus quinine, the mortality in HIV-positive children treated with artesunate was 22.2% versus 31% with quinine (odds ratio [OR] 95% CI 0.63 (0.22–1.85); *p*=0.40) and in HIV-negative children 8.1% versus 10.1% (OR for artesunate 0.78 (0.44–1.38); *p*=0.39). Stratification according to concomitant antiretroviral treatment (n=5) or antituberculosis treatment (n=5) did not alter any of the results (data not shown).

In a logistic regression model adjusted for age and antimalarial treatment, with death as dependent variable and established predictors of severe malaria as independent variables significant predictors in the final model (n=482) were renal impairment (log₁₀ BUN), acidosis (BE), parasitaemia (log₁₀ parasitaemia; but an inverse correlation), and plasma *Pf*HRP2 concentration (Table 4). HIV infection was correlated with increased mortality (unadjusted OR 3.44, 95% CI 1.88–6.28), but was not an independent prognosticator
Table 3. Comorbidity, complications and outcome according to HIV status

| Characteristic                      | Children <15 years | Adults ≥15 years |
|-----------------------------------|--------------------|------------------|
|                                   | HIV-negative n=581 | HIV-positive n=74 | p value (HIV-negative n=19) | HIV-positive n=49 | p value |
| Co-morbidity                      |                    |                 |                             |                  |
| Suspected pneumonia               | 19 (3)             | 9 (12)          | <0.001                       | 1 (5)            | 2 (4)   | 1.0    |
| Confirmed by CXR                  | 5 (26)             | 4 (44)          | 0.41                         | 1 (100)          | 0 (0)   | 0.3    |
| Clinical sepsisa                   | 9 (2)              | 4 (5)           | 0.049                        | 0 (0)            | 1 (2)   | 1.0    |
| Suspected meningitisa              | 3 (<1)             | 1 (1)           | 0.38                         | 1 (5)            | 0       | 1.0    |
| Gastro-enteritisb                  | 7 (1)              | 2 (3)           | 0.27                         | 0                | 0       | -      |
| Other significant co-morbiditiesc  | 22 (4)             | 7 (9)           | 0.025                        | 0                | 0       | -      |
| Complications (not present on admission) |                |                 |                             |                  |
| Development of coma               | 3 (<1)             | 2 (3)           | 0.101                        | 1 (5)            | 2 (4)   | 1.0    |
| Deterioration coma score          | 22 (4)             | 9 (12)          | 0.001                        | 2 (11)           | 6 (12)  | 1.0    |
| Convulsions developing or persisting >6 h after admission | 61 (11) | 18 (24) | 0.001 | 2 (11) | 3 (6) | 0.61 |
| Respiratory distress              | 6 (1)              | 7 (9)           | <0.001                       | 4 (21)           | 5 (10)  | 0.25   |
| Severe anaemia (Hb<5g/dL)         | 12 (2)             | 2 (3)           | 0.67                         | 0                | 1 (2)   | 1.0    |
| Blackwater fever                  | 13 (2)             | 4 (5)           | 0.11                         | 0                | 2 (4)   | 1.0    |
| Renal failured                    | 3 (<1)             | 2 (3)           | 0.10                         | 3 (16)           | 8 (16)  | 1.0    |
| Outcome                           |                    |                 |                             |                  |
| Mortality                         | 53 (9)             | 19 (26)         | <0.001                       | 4 (21)           | 17 (35) | 0.38   |
| Mortality in “strictly” defined severe malaria | 53/533 (10) | 19/66 (29) | <0.001 | 4/16 (25) | 17/41 (41) | 0.36   |
| Neurological sequelae at 28d      | 6 (1)              | 2 (3)           | 0.23                         | 0                | 0       | -      |

Data are No. (%), unless otherwise indicated.
Abbreviations: CXR, chest X-ray; Hb, haemoglobin; HIV, human immunodeficiency virus.

- **a** No culture facilities available.
- **b** Defined as >6 loose stools/24 h.
- **c** Other significant comorbidities in HIV-negative children included: suspected intoxication with traditional medicine, suspected hepatitis, burn of hand (1%), asthma/bronchitis, undefined skin rash, reactive arthritis, parasitosis (tungiasis, ascaris), Herpes simplex virus labial ulcers, acute otitis media, tonsillitis, conjunctivitis, fever of unknown origin, suspected encephalitis; in HIV-positive children: suspected intoxication with traditional medicine, asthma/bronchitis, impetigo, submandibular abscess, keratitis, suspected encephalitis.
- **d** Defined as urine output <0.5ml/kg/h for >24 h or blood urea nitrogen >60mg/dL.

When plasma PfHRP2 was introduced into the model, which was related to the correlation between HIV status and plasma PfHRP2 (test for trend across ordered groups; p<0.0001). The independent predictors were identical in a model including categorical HIV immunological stages rather than presence of HIV infection as a binary variable.
Also, HIV status did not contribute significantly to the final model when introduced as an interaction term with plasma PfHRP2 concentration.

**Table 4.** Logistic regression analysis for children, adjusted for age, showing the prognostic value of significant risk factors assessed on admission for in-hospital survival of children with severe falciparum malaria

| Variable                                      | Odds ratio (95% CI) | p value |
|-----------------------------------------------|---------------------|---------|
| Plasma base excess                            | 0.89 (0.84–0.94)    | <0.001  |
| [log] Blood urea nitrogen                     | 3.81 (1.90–7.65)    | <0.001  |
| [log] Parasitaemia                            | 0.66 (0.47–0.89)    | 0.007   |
| [log] Plasma PfHRP2 (squared)                 | 1.47 (1.10–1.95)    | 0.008   |
| [log] Plasma PfHRP2                           | 0.12 (0.03–0.48)    | 0.003   |
| Antimalarial drug (artesunate versus quinine) | 0.37 (0.18–0.78)    | 0.009   |

The association between death and [log] plasma PfHRP2 was U-shaped and best described using a quadratic function. The patients with low PfHRP2 concentrations signify children with a low parasite burden where severe illness is likely caused by an alternative diagnosis than severe malaria (Hendriksen et al. *PLoS Med*, in press). Introduction of an interaction term (HIV-1 status x plasma PfHRP2) did not improve the model (p=0.88). $R^2=0.29$ for the final model (n=482).

**Discussion**

This is the first prospective study to report the different clinical presentations of severe malaria, parasite burden, and mortality in HIV-coinfected patients. It was shown that HIV-coinfected children with severe malaria were more undernourished and presented more frequently with severe acidosis, severe anaemia, respiratory distress and elevated BUN concentrations, and similar (albeit nonsignificant) trends were found in HIV-infected adults. Previous studies carried out in areas of high malaria transmission have reported increased prevalence and severity of severe anaemia and a higher 7-, 28-, or 90-day post-admission mortality in HIV-coinfected children without differences in admission parasitaemia. In addition to these findings, we established that a metabolic acidosis is more frequent in HIV-coinfected children. Acidosis in severe malaria has been associated with severe anaemia and respiratory distress or deep breathing and is an established strong predictor of mortality in adult as well as paediatric severe malaria. Total body parasite burden, measured as plasma PfHRP2 concentrations, was higher in HIV-infected children and positively correlated with the severity of immunosuppression
according to WHO immunological classification. This total parasite burden includes the sequestered parasite burden, which causes impaired microcirculatory flow, an important cause of metabolic acidosis in severe malaria. In line with other studies, HIV coinfected children with severe malaria were older and had higher parasite densities. These results suggest that failure of the acquired immunity in HIV infection leads to a decreased ability to control parasitaemia, which increases the risk of developing severe malaria with associated high mortality. The clinical presentation of malaria in HIV-coinfected children depends on malaria-specific immunity, which varies according to age and malaria transmission intensity, as well as HIV-related immunosuppression. Although our study design did not allow such comparison, our findings support the hypothesis that HIV-related immunosuppression increases the risk of severe malaria in adults, since the HIV-1 seroprevalence in severe malaria was more than twice the reported HIV-1 seroprevalence in the adult population. Elevated BUN concentrations have been reported in HIV-coinfected adults with severe malaria, and renal failure due to malaria-induced acute tubular necrosis has been described as a common complication and cause of death in Asian adults. It has been hypothesized that HIV-coinfected adults are more likely to have an HIV-mediated impaired renal function, although normalization of renal function following resolution of the malaria episode has also been described. The significance of elevated BUN concentrations in HIV-positive patients, particularly in children, and its association with mortality as also observed in other studies requires further investigation.

In the adults, sensitivity of the RDTs for diagnosing malaria was remarkably low (<50%). This was explained by low parasite densities on the peripheral blood slide, below the level of detection of the tests. Patients with a negative PfHRP2-based RDT and low peripheral blood parasitaemia include those with very low total parasite burdens, where an alternative diagnosis other than malaria is more likely. Unfortunately, in the setting of the study, possibilities for establishing accurate alternative diagnoses other than malaria were limited. The Mozambican national malaria control program recommends parasitological diagnosis of malaria in adults by rapid diagnostic test, although in referral hospitals, severe malaria is commonly diagnosed by peripheral blood slide. Slide-positive RDT-negative severe illness represents patients with low parasitaemia, warrants diagnosis and treatment of other, possibly HIV-related, alternative illnesses. Further studies to assess the diagnostic work-up and management of this patient group are needed.

Comorbidities were more frequent in HIV-coinfected children than in HIV-uninfected children; notably suspected pneumonia and sepsis. In the AQUAMAT trial site in Muheza,
Tanzania, where blood cultures and confidential HIV testing were performed, 8/38 (21%) of HIV-coinfected children had a positive blood culture versus 45/855 (5%) in HIV-negative children (p<0.001) with an almost 3-fold increased case fatality rate (14/38 (37%) in HIV-coinfected children versus 112/855 (13%) in HIV-uninfected children (p<0.001) [unpublished data; personal communication, Ilse Hendriksen]. Paediatric severe malaria with HIV coinfection has been associated with an increased risk of non-typhi Salmonella (NTS) and/or gram-negative bacteraemia, both leading to an increased risk of death. A study in southern Mozambique (an area with similar high HIV-prevalence) reported an incidence of 5.4% of concomitant bacteraemia in paediatric severe malaria with Streptococcus pneumoniae being the most frequently identified organism, especially in children with respiratory distress and was associated with a higher case fatality. Case fatality with HIV coinfection was 282% higher in children (p<0.001) and 64% higher in adults with severe malaria (p=0.28). However, in a logistic regression model HIV infection was not an independent predictor of death when plasma PfHRP2 was included in the regression model, whereas there was a clear correlation between HIV status and plasma PfHRP2 concentration, which is a measure of the total body parasite burden, including the sequestered parasites. This again suggests that HIV-induced immune incompetence compromises control of the malaria parasite burden and thus severity of the infection. It also suggests that this mechanism is more important than HIV-related comorbidity and underscores the importance of potent antimalarial treatment in these children with parenteral artesunate.

In accordance with data from Asian settings, convulsions, hypoglycaemia, and symptomatic severe anaemia were more frequent in children, whereas renal impairment and severe jaundice were more common in adult patients in the current African study. Acidosis and coma were prominent in both groups, whereas shock was rare.

The main limitations of this study include the lack of diagnostic information to assess the additional pathology responsible for increased severe malaria mortality in HIV-coinfected patients. Chest X-rays were not routinely performed and blood culture facilities were unavailable at the time of this study. In addition, clinical malaria may lower the CD4+ lymphocyte count, which may therefore underestimate the patient’s immunological status.

In summary, severe malaria in HIV-coinfected children presents with more severe acidosis, anaemia and respiratory distress, more complications, and co-morbidity, causing higher mortality and prolonged hospitalization in survivors. HIV coinfection is associated with a higher estimated total parasite burden, which is strongly associated with the observed increased severity. Early recognition of HIV coinfection is important for several reasons.
Higher case fatality and more frequent complications warrant more intense monitoring and a low threshold for additional investigations to diagnose concomitant invasive bacterial infections including chest X-ray, blood culture, and lumbar puncture with CSF examination. Since concomitant pneumonia, sepsis, and severe anaemia are common, prompt parenteral antimalarial and antibiotic treatment, and availability of supportive treatments including oxygen therapy and blood transfusion are of extra importance in this group.

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Chapter 8

The population pharmacokinetic and pharmacodynamic properties of intramuscular quinine in Tanzanian children with severe falciparum malaria

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Submitted
Summary

Although artemisinin is superior, parenteral quinine is still widely used for the treatment of severe malaria. A loading dose regimen has been recommended for 30 years but is often not used.

A population pharmacokinetic study was conducted in 75 Tanzanian children aged 4 months to 8 years with severe malaria receiving intramuscular quinine; 69 patients received a loading dose of 20 mg salt/kg. 21 had plasma quinine concentrations detectable at baseline. A zero-order absorption model with one-compartment disposition pharmacokinetics described the data adequately. Body weight was the only significant covariate and was implemented as an allometric function on clearance and volume parameters. Population pharmacokinetic parameter estimates (%RSE) of elimination clearance, central volume of distribution, and duration of zero-order absorption were 0.977 (6.50%) L/h, 16.7 (6.39%) L and 1.42 (21.5%) h, respectively, for a typical patient weighing 11 kg. Quinine exposure was reduced at lower body weights after a standard weight-based dosing; there was 18% less exposure over 24 hours in patients of 5 kg compared with those of 25 kg. Maximum plasma concentrations after the loading dose were unaffected by body weight. There was no evidence of dose related drug toxicity with the loading dosing regimen.

Intramuscular quinine is rapidly and reliably absorbed in children with severe falciparum malaria. Based on the pharmacokinetic data, a loading of 20 mg salt/kg is recommended, provided no loading dose was administered within 24 hours and no routine dose within 12 hours of admission.
Introduction

Malaria kills over 2000 people each day and young children in Africa account for over 85% of the malaria associated mortality worldwide. The case fatality of paediatric severe malaria usually exceeds 10% with the highest mortality within the first 24 hours. Parenteral quinine has been the mainstay of severe malaria treatment since the global spread of chloroquine resistance. Although parenteral artesunate is now firmly established as the treatment of choice, availability is still limited and so quinine remains very widely used.

Quinine dosing regimens were initially based on studies in Asian adults and have been extrapolated to African children, although there is relatively little detailed pharmacokinetic information in this important group. The currently recommended dosing regimen is a loading dose of 20 mg salt/kg followed by 10 mg/kg every eight hours, given by intravenous rate-controlled infusion or intramuscular (i.m.) injection. Peak quinine concentrations are achieved within 4 hours after i.m. injection, which has been postulated to be less expensive, more practicable and safer in resource limited settings where intravenous (i.v.) infusions cannot be established or reliably monitored.

Quinine is metabolized primarily via the cytochrome P450 enzyme CYP3A4 and the more polar metabolites are eliminated mainly by renal excretion. The major metabolite, 3-hydroxyquinine, contributes approximately 10% of the antimalarial activity of the parent compound. The pharmacokinetic properties of quinine are affected by the severity of infection as well as age. The apparent volume of distribution and elimination clearance are significantly reduced in proportion to increased disease severity, partly because of increased quinine binding to plasma-proteins, mainly alpha 1-acid glycoprotein. Quinine clearance is also altered by drug interactions and to some extent by genetic variability affecting CYP3A function, which will influence the conversion of quinine to its major metabolite. The unbound plasma fraction of quinine determines the therapeutic and toxic actions of the drug. In children under 2 years of age with severe malaria, unbound quinine concentrations were found to be slightly higher than in older children and adults. Although it is generally well tolerated, the therapeutic window of unbound quinine is relatively narrow and side effects include hypoglycaemia, cardiotoxicity, ototoxicity and ocular toxicity.

Rapid achievement of therapeutic quinine concentrations while avoiding toxicity is of vital importance in the treatment of severe malaria. Despite its extensive use in millions of critically ill children at risk of death, there are very few detailed assessments of the pharmacokinetic properties in the target population. The primary aim of this study was...
to characterize the population pharmacokinetic profile of intramuscular m. quinine after a loading dose in children with severe malaria in Tanzania. A limited pharmacodynamic assessment was included as a secondary aim.

Methods

Study design, patients and procedures
This pharmacokinetic assessment of quinine was part of the “AQUAMAT” trial (registration number ISRCTN 50258054), a large multinational trial comparing quinine and artesunate for the treatment of severe malaria, which results have been published elsewhere. This substudy was conducted in Teule Hospital in Muheza, Tanzania from May 2009 to July 2010. Apart from the additional blood sampling, procedures of the current study were part of the AQUAMAT study protocol. The clinical assessment is reported in full elsewhere. Approval for the study including the pharmacokinetic substudy was obtained from Tanzania Medical Research Coordinating Committee and the Oxford Tropical Research Ethic Committee. A total of 21 patients were co-enrolled in the “FEAST” trial evaluating fluid bolus therapy in children with compensated shock. Children ≤14 years with a clinical diagnosis of severe malaria confirmed by a *Plasmodium* lactate dehydrogenase (pLDH)-based rapid diagnostic test (Optimal, Diamed, Cressier, Switzerland) were recruited, provided written informed consent was given by their parent or carer. Severe malaria was defined as at least one of the following: coma (Glasgow coma scale ≤10 or Blantyre coma scale ≤2 in preverbal children), convulsions (of duration >30 min or ≥2 in 24 h before admission), respiratory distress (nasal alar flaring, costal indrawing/recession or use of accessory muscles, severe tachypnoea) or acidotic breathing (“deep” breathing), shock (capillary refill time≥3 sec and/or temperature gradient and/or systolic blood pressure <70 mmHg), severe symptomatic anaemia (<5 g/dL with respiratory distress), hypoglycaemia (<3 mmol/L), haemoglobinuria, severe jaundice or a convincing history of anuria or oliguria in older children. Patients who had received full treatment with parenteral quinine or an artemisinin derivative for more than 24 h before admission were excluded.

Physical examination was done at admission and a venous blood sample was taken for peripheral blood parasitaemia, quantitative assessment of plasma *Plasmodium falciparum* histidine-rich protein-2 (a marker of total body parasite burden), HIV serology (SD Bio-Line HIV 1/2 3.0, Standard Diagnostics Inc, Kyonggi-do, Korea /Determine HIV-1/2, Abbott Laboratories, IL, USA), blood culture, liver function tests (AST, ALT, y-GT, ...
total bilirubin, creatinine and urea, by Reflotron, Roche Diagnostics), Haematocrit (Hct), biochemistry and acid-base parameters (EC8+ cartridge for i-STAT handheld blood analyser). Haematocrit was reported from i-STAT or when not available, derived from haemoglobin (Hb) measured by Haemocue (n=5). At discharge, a neurological examination was performed.

**Antimalarial treatment**
Quinine dihydrochloride (Indus Pharma, Karachi, Pakistan) was given as an i.m. injection. A loading dose (20 mg salt/kg) was given shortly after admission, followed by 10 mg/kg every 8 hours. In case the patient had received pre treatment with a quinine loading dose (20 mg/kg) within 24 hours or a maintenance dose (10 mg/kg) within 12 hours before enrolment, quinine treatment after study enrolment was continued with 10 mg/kg (i.e. no loading dose). Quinine was diluted into normal saline to a concentration of 60 mg salt/ml and injected into the anterior thigh. The dosing was based on body weight and injection volumes over 3 ml were split and divided over both thighs. When the patient was well enough to take oral medication, but after a minimum of 24 hours (3 doses) of i.m. quinine, a full course of oral artemether-lumefantrine (Co-artem, Novartis, Basel, Switzerland) was given to complete the treatment.

**Patient management**
The majority of patients receiving quinine received an intravenous infusion with glucose 5%. Vital signs and glucose were monitored at least 6-hourly and with any deterioration in clinical condition. Hypoglycaemia (defined here as blood glucose <3 mmol/L) was treated with an i.v. 5 ml/kg 10% glucose bolus. A blood transfusion (20 ml/kg) was given to children with haematocrit ≤15% or haemoglobin <5 g/dL. All children were empirically treated with i.v. antibiotics (ampicillin and gentamicin or ceftriaxone if clinically suspected of sepsis or meningitis) until blood culture results were known or changed according to antibiotic sensitivity analysis. Convulsions were treated with diazepam or phenobarbitone if they persisted. A peripheral blood smear was repeated after 24 hours.

**Blood sampling**
Blood samples (1.5 mL) were drawn from an indwelling catheter into lithium-heparin tubes before the first dose (at baseline) and 4 subsequent samples taken at pre-set random times in the following time-windows: 1 to 4 hours, 4 to 8 hours 12 to 16 hours and 20 to 24 hours after the injection of the first dose. Randomization of sample times was done by computer-generated randomization in STATA, version 10 (StataCorp, TX, USA).
Quinine blood samples were centrifuged at 2,000×g for 10 minutes to obtain plasma. Duplicate plasma samples (0.5 mL) were stored at -80°C and sent to AMBRELA/NIMR laboratory in Tanga, Tanzania for plasma quinine quantification. Quinine drug content and quality were checked in ampoules taken randomly from the purchase lots (see supplement of\(^3\)).

**Drug analysis**

Total quinine was quantified in plasma samples using High-Performance Liquid Chromatography (HPLC) with UV-detection.\(^{29}\) Quinine was extracted from plasma samples by liquid-liquid extraction using ethyl acetate/hexane (1:1 v/v). Separation was performed by isocratic elution from a reverse phase Synergi MAX-RP (250 x 4.6 mm; 4 μ) column (Phenomenex, USA) with an acidic (adjusted to pH 2.8 with o-phosphoric acid) mobile phase (25mM KH₂PO₄:methanol; 80:20 %( v/v) + 1 %( v/v) triethylamine) at a flow rate of 1.2 mL/min. Quinidine internal standard (25 μl aliquot of 100 μg/ml quinidine working standard) and quinine were detected at 254 nm and resolved to baseline with retention times of 9.3 min and 11.8 min respectively. The lower limit of quantification was 100 ng/ml. Quality control samples at 1, 10 and 20 mg/L were prepared by spiking drug free plasma. Intra-assay and inter-assay coefficients of variation ranged from 5.4% to 12.7%.

**Population pharmacokinetic-pharmacodynamic analysis**

Quinine concentrations were transformed into their natural logarithms and modelled using NONMEM version 7 (ICON Development Solutions, Hanover MD).\(^{30}\) Automation and model diagnostics were performed using Pearl-speaks-NONMEM (PsN) and Xpose.\(^{31-33}\) The first-order conditional estimation method with interactions was used throughout modelling. The difference in objective function value (ΔOFV) computed by NONMEM as -2×Loglikelihood was used as statistical criteria for hierarchical models (ΔOFV>3.84 was considered statistically significant at p<0.05 with one degree of freedom difference). Goodness-of-fit plots and simulation-based diagnostics were used for model evaluation.

Population pharmacokinetic models were parameterized as a first-order rate constant (ka) or a duration of a zero-order absorption (DUR), elimination clearance (CL/F), inter-compartment clearance (Q/F) and apparent volume of distribution(s) (V/F). The injection sites were considered to be a single depot compartment and bioavailability (F) was assumed to be 100%. Pre-dose concentrations were handled by flagging patients with pre-dose concentrations to allow a baseline value to be estimated for these individuals.
Between-subject variability (BSV) and between-dose occasion variability (BOV) were modelled exponentially. One- and two-compartment disposition models were evaluated. First-order and Michaelis-Menten elimination was evaluated. A Box-Cox transformation was tried on individual population parameters to assess formally the assumption that pharmacokinetic parameters are log-normally distributed. The residual random variability was assumed to be additive since data were transformed into their natural logarithms (i.e. essentially equivalent to an exponential error model on an arithmetic scale).

The implementation of body weight as a covariate on clearance and volume of distribution in the final structural model was assessed using an allometric function (individual clearance value = typical clearance value × [individual body weight/median body weight in the population]^{0.75} and individual volume value = typical volume value × [individual body weight/median body weight in the population]). An age-related enzyme-maturation effect was also investigated on clearance.

Demographic, clinical and laboratory data on admission were considered as potential covariates and investigated using a stepwise forward addition and backward elimination approach. A p value of 0.05 was used in the forward step and a p-value of 0.001 in the backward step to compensate for the relatively small population studied. The following admission covariates were investigated using the stepwise approach: age (y), weight-for-age z scores, temperature (°C), heart rate (beats/min), coma (continuous variable based on GCS/BCS coma score), cerebral malaria (coma and/or convulsions), blood urea nitrogen (mg/dL), haemoglobin (g/dL), base excess (mg/dL), parasitaemia, (parasites/µL), plasma *Plasmodium falciparum* histidine-rich protein-2 (ng/mL) as a marker of total parasite burden, total bilirubin (µmol/L), creatinine (high: age <1 yr ≥44.2 µmol/L, age≥1 year≥62 µmol/L, low: age<1 yr<44.2 µmol/L, age≥1 year>62 µmol/L), HIV coinfection, shock (compensated or decompensated), fluid bolus and/or blood transfusion.

Numerical- and visual predictive checks were used to assess the predictive performance of the final model. The final model with included variability was used to simulate 2000 concentrations at each sampling time-point and the 95% confidence interval around the simulated 5th, 50th and 95th percentiles were overlaid with the same percentiles of observed data to evaluate the predictive power of the model (visual predictive check). The percentages of observations below and above the simulated 5th and 95th percentile were also calculated for a numerical predictive check. Non-parametric bootstrap diagnostics (n=2000), stratified on body weight (above or below 10 kg), were performed for accurate relative standard errors and non-parametric confidence intervals of population parameter
estimates. The final model was also used for Monte-Carlo simulations evaluating the quinine exposure in children at different body weights with or without a loading dose. Survival data were modelled in NONMEM using a time-to-event analysis. Patients were censored at 12 hours after the last intramuscular quinine administration. The survival data were modelled using a constant hazard function, Weibull-distribution hazard function, or an exponential hazard function. OFV and simulation based diagnostics were used to compare models. There were only 13 deaths out of 75 patients in this study which was too few for a formal covariate analysis on the time-to-event. Drug concentration-response relationships were evaluated by a direct effect driven by plasma concentrations or a delayed effect by cumulative quinine exposure.

**Results**

**Clinical details**
Seventy five (75) children were included, of whom 28 (37%) were under 2 years. Demographic, clinical and laboratory characteristics are described in Table 1. Severe prostration, convulsions, severe acidosis, severe anaemia and coma were the most common severity criteria. None of the 18 patients who presented with hypoglycaemia at admission and only 4 out of 7 patients with haemoglobinuria had a history of quinine pre treatment. Seven (9.3%) patients had blood culture confirmed bacteremia (12.5% in shocked patients versus 8.5% in non-shocked patients, p=0.623). The identified organisms were non-typhi Salmonella, Enterobacter cloacae, Klebsiella spp., Escherichia coli, Burkholderia cepacia, Streptococcus Group A. HIV coinfection was found in 5/75 (6.7%) of patients. None of these patients was receiving antiretroviral treatment. Out of 75 patients 13 (17%) died, of whom 10 (77%) within 24 hours. Children who survived had no neurological sequelae at discharge.

Sixty nine (92%) patients received a quinine loading dose at the start of the study, and the remainder started with 10 mg/kg. During the first 24 hours of admission, 37 patients received a blood transfusion and 19 patients received a fluid bolus. Eleven patients (15%) developed hypoglycaemia after admission, including 5/18 (27.7%) of those who presented with hypoglycaemia at admission. Hypoglycaemia occurred in 6/11 children despite intravenous 5% dextrose infusion, five of whom subsequently died.
Table 1. Demographic, clinical and laboratory characteristics of children admitted with severe malaria

| Variable                        | Children with severe malaria, n=75 |
|---------------------------------|-------------------------------------|
| Age (y)                         | 2.4 (0.33-8.1)                      |
| Weight (kg)                     | 11 (5.5-27)                         |
| Weight-for-age Z-score          | -1.0 (-4.1-1.0)                     |
| Coma                            | 27 (36%)                            |
| Prostration                     | 46 (61%)                            |
| Convulsions                     | 34 (45%)                            |
| Shocka                          | 16 (21%)                            |
| Respiratory distress            | 7 (9%)                              |
| Acidosis (base excess < -8 mmol/L) | 32 (46%)                         |
| Hypoglycaemia (glucose < 3mmol/L) | 18 (24%)                           |
| Anaemia                         | 27 (36%)                            |
| Haemoglobinuria                 | 7 (9%)                              |
| Axillary temperature (°C)       | 38.2 (35.4-41.8)                    |
| Heart rate (beats/min)          | 154 (98-202)                        |
| Respiratory rate (breath/min)   | 50 (24-98)                          |

**Laboratory variables**

| Glucose (mg/dL)                  | 95 (15-240)                        |
| Blood urea nitrogen (mg/dL)      | 13 (4-97)                          |
| Haemoglobin (g/dL)               | 7.1 (2.6-13.2)                     |
| pH                              | 7.36 (7.28-7.42)                   |
| HCO₃ (mmol/L)                    | 17.8 (3.5-25.6)                    |
| Base excess (mmol/L)             | -8 (-28-2)                         |
| ASAT (U/L)                      | 85 (7-3180)                        |
| ALAT (U/L)                      | 22 (3-1490)                        |
| Total bilirubin (μmol/L)         | 38 (5-250)                         |
| HIV-positive                    | 5 (6.7%)                           |
| Parasitaemia (parasites/µL) (geometric mean, 95% CI) | 31 900 (17 300-58 900) |
| Plasma P/JHRP2 (ng/mL) (geometric mean, 95% CI) | 2070 (1470 to 2900) |

Data are No. (%) of patients, median (range), unless otherwise stated.

*Compensated and decompensated shock combined

Coma after admission occurred in 7 (9%) patients. Peripheral parasite densities after 24 hours treatment were (geometric mean, 95% CI) 3681 (1422–10 790) parasites/µL with a
median (IQR) fractional reduction of 78% (38%–99%) in 60 patients (1 and 14 patients with missing baseline or 24 hours parasitaemia, respectively, including 10 due to death). A history of oral antimalarial treatment before admission was given for 41 (55%) patients (8 with quinine, 5 with amodiaquine, 17 with artemether-lumefantrine, 10 with sulfadoxine-pyrimethamine and 1 with sulfadoxine-pyrimethamine followed by artemether-lumefantrine). Parenteral quinine pre-treatment within 24 hours prior to admission was reported for 15 patients, with a maximum of 3 doses and a mean (SD) of 10 (1.8) mg/kg. Of these patients, 12 had detectable baseline plasma quinine concentrations ranging from 1.56 to 17.38 mg/L. Three patients treated with oral quinine had baseline drug concentrations of 3.34, 5.10 and 8.26 mg/L. Another 6 patients without history of quinine treatment before admission had detectable plasma quinine concentrations ranging from 0.85 to 14.88 mg/L (of whom 5 had baseline concentrations <4.0 mg/L).

Population pharmacokinetic-pharmacodynamic analysis
Each patient contributed 1 to 5 plasma samples resulting in a total of 341 concentration-time samples distributed randomly over the first 24 hours of the study. All patients were included in the population pharmacokinetic analysis and pre-dose quinine concentrations were accommodated by estimating a baseline concentration in these patients (median [range] concentrations of 6.90 [0.976-14.9] mg/L). All post-dose plasma concentrations were determined to be above the lower limit of quantification, ranging from 0.85 to 33.8 mg/L. Four patients had very high plasma quinine concentrations (>25 mg/L) but no serious adverse events or deaths could be attributed to these high plasma quinine concentrations.

A one-compartment disposition model with zero-order absorption resulted in adequate fit to the observed data. No additional benefit was seen with an additional peripheral disposition compartment ($\Delta$OFV=-4.12, two degrees of freedom difference). There was no substantial between-dose occasion variability in any population parameters ($\Delta$OFV<-0.150) and this was therefore not included in the final model. Michaelis-Menten elimination did not significantly improve the model diagnostics ($\Delta$OFV=-2.75) compared to a first-order elimination model. A Box-Cox transformation of population parameters did not significantly improve the model fit ($\Delta$OFV<-1.88) compared to the usual assumed log-normal distribution. Incorporation of an off-diagonal element in the covariance-matrix of elimination clearance and apparent volume of distribution resulted in a significant correlation (99.9%) and an improvement in model fit. Between-subject variability could not be reliably estimated for the duration of zero-order absorption (RSE=171%) and was therefore not retained in the final model.
Body weight as a fixed allometric function on elimination clearance and apparent volume of distribution resulted in a significant improvement in model fit ($\Delta$OFV=-39.0) and decreased the between-subject variability (%CV) from 47.4% to 35.9% and from 61.2% to 51.3%, respectively. The following covariate relationships were selected in the forward step-wise addition ($p<0.05$): base excess on CL/F, base excess on V/F, haemoglobin on V/F and age on CL/F as a maturation model. However, none of these covariates could be retained in the backward step with a more stringent statistical criterion ($p<0.001$). The final population parameter estimates, variability estimates and post-hoc estimates are summarized in Table 2.

**Table 2.** Parameter estimates of the final model describing quinine population pharmacokinetics in children (n=75) with severe malaria

| Variable                  | Population estimate ($^{\text{a}}$ (% RSE $^{\text{b}}$) | 95% CI $^{\text{b}}$ |
|---------------------------|----------------------------------------------------------|-----------------------|
| **Fixed effects**         |                                                          |                       |
| CL/F (L/h)                | 0.792 (6.50)                                             | 0.692–0.895           |
| V/F (L)                   | 13.7 (6.39)                                              | 12.2–15.5             |
| DUR (h)                   | 1.42 (20.3)                                              | 0.527–1.74            |
| **Random effects**        |                                                          |                       |
| $\eta_{\text{CL/F}}$     | 0.128 (28.7)                                             | 0.062–0.206           |
| $\eta_{\text{V/F}}$      | 0.176 (24.6)                                             | 0.101–0.273           |
| $\eta_{\text{CL/F}} - \eta_{\text{V/F}}$ | 0.15 (23.7)                                             | 0.076–0.206           |
| $\sigma$                 | 0.0942 (8.00)                                            | 0.0652–0.120          |
| **Post-hoc estimates$^{\text{c}}$** |                                                          |                       |
| CL/F (L/h/kg)             | 0.0741                                                   | 0.0455–0.144          |
| V/F (L/kg)                | 1.24                                                     | 0.645–2.89            |
| $t_{1/2}$ (hours)         | 12.1                                                     | 9.63–14.3             |
| $C_{\text{MAX}}$ (mg/L)  | 13.4                                                     | 7.20–24.8             |
| $\text{AUC}_{0-7.5h}$ (h×mg/L) | 78.9                                                  | 42.3–148              |

$^{\text{a}}$ Computed population mean values from NONMEM are calculated for a typical patient with a body weight of 11 kg.

$^{\text{b}}$ Assessed by non-parametric bootstrap method (n=1,413 successful iterations out of 2000) of the final pharmacokinetic model. Relative standard error (% RSE) is calculated as 100×(standard deviation/mean value). 95% confidence interval (95% CI) is displayed as the 2.5 to 97.5 percentile of bootstrap estimates.

$^{\text{c}}$ Post-hoc estimates are displayed as median values with 2.5 to 97.5 percentiles of empirical Bayes estimates.

CL/F=elimination clearance; V/F=central volume of distribution; F=intramuscular bioavailability; DUR=duration of zero-order absorption; $\eta$=inter-individual variability; $\eta_{\text{CL/F}} - \eta_{\text{V/F}}$=correlation of random effects on CL/F and V/F; $\sigma$=additive residual variance error; $t_{1/2}$=terminal elimination half-life; $\text{AUC}_{0-7.5h}$=area under the concentration-time curve from time-point 0 to 7.5 hours; $C_{\text{MAX}}$=predicted peak concentration after first dose.
The final model described the observed data well with adequate goodness-of-fit diagnostics (Figure 1) and calculated shrinkages below 15% (CL/F: 11.3%, V/F: 11.3%, Epsilon-shrinkage: 14.0%). A prediction-corrected visual predictive check of the final model resulted in no model misspecification with good simulation properties (Figure 2). The numerical predictive check resulted in 6.92% (95% CI 2.08–9.00) and 2.08 (95% CI 1.73–9.00) of observations above and below the 90% prediction interval.

**Figure 1**

Goodness-of-fit diagnostics of the final population pharmacokinetic model of quinine in children with severe malaria. Broken line, a locally weighted least-squares regression; solid line, line of identity. The observed concentrations, population predictions, and individual predictions were transformed into their logarithms (base 10).

Body weight was the only significant covariate in the final model with lower body weights being associated with slightly reduced exposure during the first 24 hours after the standard weight-based dose. There was a mean reduction of 7.19% in simulated quinine exposure during the first dose (0 to 8 hours) when comparing patients weighing 5 kg with patients
of 20 kg body weight after a 20 mg salt/kg loading dose (data not shown). This reduction in exposure accumulated with the repeated maintenance dosing of 10 mg/kg over the first 24 hours to a total mean difference of 15.4% (data not shown). Peak concentration after the first dose was unaffected by body weight but accumulated to a mean difference of 15.9% lower peak concentrations for patients weighing 5 kg compared to patients of 20 kg after the third dose (Figure 3A). This difference (<20%) in total exposure and maximum concentration is not likely to have a significant clinical impact since more than 85% of patients irrespectively of body weight reached plasma quinine concentrations over the therapeutic margin of 8 mg/L after a loading dose of 20 mg/kg and more than 95% of patients reached the target during the first 24 hours with the subsequent maintenance dose of 10 mg/kg. In absence of a loading dose, the therapeutic range would only be reached in less than 30% of patients after the first dose and in 89% of patients during the first 24 hours (Figure 3B).

Figure 2

Visual predictive check of the final model describing the population pharmacokinetics of quinine in children with severe malaria. Open circles, observed data points; solid lines, 5th, 50th, and 95th percentiles of the observed data. Shaded area, 95% confidence interval of simulated (n=2000) 5th, 50th, and 95th percentiles. Venous plasma quinine concentrations were transformed into their logarithms (base 10).
Predicted population pharmacokinetic profiles of quinine A) at different body weights (gray solid profiles): 5 kg, (− −), 10 kg (− −) and 20 kg (−) are highlighted in black; and B) after a loading dose of 20 mg/kg (−) versus a routine dose of 10 mg/kg (− −): 10 kg are highlighted in black.

A constant hazard function model with cumulative exposure implemented as an Emax function modulating the hazard described the survival over time well in this study (data not shown). A simulation-based visual predictive check resulted in the observed survival curve to be contained within the 95% confidence interval of the simulated survival. Median time to reach a 50% reduction in hazard was approximately 6 hours. However, no concentration-effect relationship could be established and there was no significant difference in post-hoc estimates of total exposure (p=0.1358) or maximum concentrations (p=0.1786) after the first dose in children who died compared to children who survived.
Intramuscular quinine population pharmacokinetics

(Figure 4). The exposure-effect relationship is likely to describe the delayed antimalarial effect of quinine but this approach is biased since many patients had a pre treatment history of quinine and a larger data set would be necessary for a formal concentration-effect analysis.

**Figure 4**

![Graph showing total quinine exposure and maximum concentration stratified by outcome.](image)

Total quinine exposure ($\text{AUC}_{0-8\text{hrs}}$) and maximum concentration ($\text{C}_{\text{MAX}}$) after the first dose stratified for outcome. Error bars indicate median and interquartile range.

**Discussion**

The therapeutic range for quinine in severe malaria has been estimated between 8 and 15 mg/L based on observations in uncomplicated malaria suggesting reduced therapeutic responses when serum or plasma concentrations fall below 5 mg/L and taking into
account variation in parasite susceptibility and the reduced free fraction observed in severe malaria. Plasma concentrations up to 20 mg/L have not been associated with significant toxicity.\textsuperscript{9,38-41}

In the present study i.m. quinine was rapidly and reliably absorbed and the current loading dose dosing regimen resulted in plasma concentrations that rapidly reached the therapeutic range in African children with severe malaria. A wide range of patient covariates did not significantly affect the pharmacokinetic parameters suggesting that this applied to children of all ages and with all forms of severe malaria. Body weight was the only significant covariate affecting quinine exposure. Monte-Carlo simulations resulted in a modest mean reduction of 7.19\% in total quinine exposure after the loading dose (0 to 8 hours) in children weighing 5 kg compared to that of children weighing 20 kg. This accumulated to a larger difference of 15.4\% over 3 doses (0 to 24 hours). This is unlikely to have a significant clinical impact since therapeutic levels of 8 mg/L were reached rapidly in all weight groups (Figure 2). Dose adjustment is therefore not recommended in younger children based on this pharmacokinetic difference. Simulations resulted in median (95\% CI) maximum concentrations of 12.6 (5.60–28.4) mg/L after a loading dose of 20 mg/kg compared to 6.32 (2.80–14.2) mg/L after a non-loading dose of 10 mg/kg. This supports that a loading dose should be used to achieve target concentrations within the first dose interval.

An i.m. loading dose of quinine was rapidly and reliably absorbed and patients in this study reached estimated peak median (95\% CI) plasma quinine concentrations of 13.4 (7.20–24.8) mg/L within 1.50 hours. This is in accordance with previous studies showing similar peak plasma quinine concentration compared to the intravenous route with similar efficacy.\textsuperscript{17,42,43} Dilution of the quinine solution to 60 mg/mL has been reported to accelerate the absorption from the i.m. injection site.\textsuperscript{8,12,22}

The reported median (95\% CI) estimates of a quinine terminal half-life of 12.1 (9.63–14.3) hours and elimination clearance of 0.0741 (0.0455–0.144) L/hr/kg are in agreement with previously published estimates from small conventional densely sampled pharmacokinetic studies in children with severe falciparum malaria: median half-lives ranging from 8.4 to 23.5 hours and clearance from 0.027 to 0.0816 L/h/kg have been reported (reviewed in\textsuperscript{17}). Minor differences between our findings with that reported in a population pharmacokinetic analysis by Krishna et al (mean (SD) half-life of 19.9 (4.4) hours and elimination clearance of 0.05 L/h/kg) may be explained by using different structural models (a one-compartment model versus a two-compartment model).\textsuperscript{11}

We only sampled patients for 24 hours in this study which could also contribute to the difference in structural models. Thus, a two-compartment model could prove to
be a more appropriate structural model when enough data are collected to support a differentiation between a distribution and a terminal phase. However the terminal elimination half-life estimate reported by Krishna et al is similar to that in adults with severe malaria, whereas the majority of published data point to more rapid elimination in children compared with adults. Body weight has not been described as a covariate for quinine pharmacokinetics before but it was significant in this analysis.\textsuperscript{10,11} Physiological processes do not scale linearly with body weight and consequently children with a lower body weight will have a higher body weight-normalised elimination clearance, which has been reported previously for other antimalarials.\textsuperscript{44,45}

In accordance with the only previous population pharmacokinetic study, we did not find any other covariates explaining the between-subject variability in children with severe malaria despite the different clinical presentations.\textsuperscript{11} Compared with uncomplicated malaria, patients with severe disease have a smaller distribution volume and a slower clearance due in part to a higher fraction of plasma-protein bound quinine.\textsuperscript{5} It is reassuring that intramuscular quinine was reliably absorbed in children with impaired perfusion, shock and severe anaemia, although these were largely corrected for in this study with supportive treatments. In addition, none of the clinical or laboratory parameters with strong prognostic value such as coma, impaired renal function (elevated BUN) or acidosis affected the pharmacokinetics of quinine.\textsuperscript{46} Therefore, the quinine dosing does not need to be adapted according to the presentation of the disease in children with severe malaria.

Intramuscular quinine is painful, but local toxicity is rare when a sterile injection technique is used and the quinine is diluted to 60 mg/ml.\textsuperscript{10} In our study site, all concomitant medications including routinely administered antibiotics were given by i.m. injection in the anterior thigh. However, no mobility problems were noted and all surviving children were well at discharge. The neurological examination at discharge did not disclose any evidence of systemic quinine toxicity such as blindness or hearing problems, even though 4/75 (5\%) of children reached quinine concentrations above 25 mg/L within the first 24 hours of treatment. Peak total plasma concentrations tend to increase during the treatment of severe malaria,\textsuperscript{17} so a higher proportion of patients might have experienced potentially toxic quinine concentrations later in their treatment course. The levels of quinine associated with toxicity in severe malaria are not clear-cut, since toxicity derives from free quinine concentrations, which depend on the levels of plasma-proteins, predominantly AGP that vary substantially.\textsuperscript{18,19} The pharmacokinetic study of Hensbroek et al showed that young children could be more prone to quinine toxicity as evidenced by prolongation of QRS interval on the electrocardiogram (depolarization), although this
was not related to plasma quinine concentrations.\textsuperscript{10} The main adverse effect of quinine in severe malaria is hypoglycaemia resulting from quinine stimulated insulin release. Otherwise given the extensive use of quinine, the widespread and often unreported pre-treatment, its use in severe malaria is otherwise remarkably free from serious toxicity.

In our study, children who died did not have higher or lower plasma quinine concentrations than children who survived (Figure 4) and 12/13 of fatal cases had received a loading dose at admission. The one child who died and did not receive a loading dose at admission had a reported pre treatment with 2 quinine injections; however the baseline quinine concentration was undetectable suggesting that the history was incorrect. Unreliability of the history is commonplace in severe malaria. Administering a loading dose of 20 mg/kg when the history is uncertain may be safest as undertreating severe malaria risks death. The therapeutic benefit of the loading dose has been widely accepted, but unsubstantiated toxicity concerns have long hindered its implementation in the field.\textsuperscript{5,47} Although there is no large randomized controlled trial evidence that the loading dose is life-saving, the faster fever and parasite clearance times and an understanding of the basic pathobiology of severe malaria suggest that it is beneficial in the treatment of severe malaria (reviewed in \textsuperscript{48}). Importantly, the loading dose does not alter the risk of hypoglycaemia due to quinine induced hyperinsulinemia.\textsuperscript{48,49} One fifth of the children in our study had already received routine dosing parenteral quinine prior to admission, none of whom presented hypoglycemia at admission. Hypoglycaemia was also an indicator of severe disease in this series, associated with an increased case fatality.\textsuperscript{49–52} The high incidence of shock and positive blood cultures suggests that concomitant sepsis might also have contributed to the high mortality in our study population.

If artemisinin derivatives are unavailable and quinine is used then a loading dose should be given, unless there is convincing evidence of adequate pre-treatment, since the risk of death of severe malaria is highest and the risk of systemic toxicity is lowest during the first 24 hours. Starting with the routine dose is justified in children that have already received a loading dose within 24 hours prior to admission and those that have received a routine dose within 12 hours of admission, but if in doubt, a loading dose should be given.
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Chapter 9

Population pharmacokinetics of artesunate and dihydroartemisinin following intramuscular administration of artesunate to African children with severe falciparum malaria; implications for a practical dosing regimen

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Summary

Parenteral artesunate is the drug of choice for treatment of severe malaria. The population pharmacokinetic properties of intramuscular artesunate have not been studied in the main treatment group who carry the highest mortality; critically ill children with severe malaria.

A population pharmacokinetic study of artesunate and dihydroartemisinin was conducted from sparse-sampling in 70 Tanzanian children aged 6 months to 11 years admitted with severe falciparum malaria and treated with intramuscular artesunate (2.4 mg/kg at 0, 12, 24 hours). Capillary plasma concentration-time profiles were characterized using nonlinear mixed-effects modeling. A one compartment disposition model described accurately first dose population pharmacokinetics of artesunate and dihydroartemisinin. Body weight significantly affected clearance and apparent volume of distribution (p<0.001), resulting in lower artesunate and dihydroartemisinin exposure in smaller children. An adapted dosing regimen for young children based on the pharmacokinetic model is proposed, including a practical dosing table per weight band.
Introduction

Severe falciparum malaria is one of the main causes of death in African children, who account for >85% of the worldwide malaria mortality.\textsuperscript{1} In children the disease course is more fulminant than in adults, with most deaths occurring within the first 24 hours of admission despite parenteral antimalarial treatment.\textsuperscript{2,3} Parenteral artesunate (ARS) is now the drug of choice for the treatment of severe malaria in all age groups and all malaria-endemic settings.\textsuperscript{4} A large trial (SEAQUAMAT) in mainly adult patients (n=1461) with severe falciparum malaria conducted in Asia showed a 35% reduction in mortality with ARS compared to quinine.\textsuperscript{5} More recently, a larger trial (AQUAMAT) performed in 5425 African children with severe malaria showed a 22.5% lower mortality in children treated with parenteral ARS compared to quinine.\textsuperscript{6} ARS has a broader stage-specificity and more potent parasitocidal effect than quinine.\textsuperscript{7,8} ARS is water-soluble and can be administered as a slow bolus by intravenous (i.v.) route or as an intramuscular (i.m.) injection. The latter route will be more practical in the majority of African hospital and clinic settings with limited facilities. The absorption of i.m. ARS is rapid and reliable with peak concentrations occurring within one hour.\textsuperscript{9,10} After injection, ARS is rapidly and almost completely converted into its active metabolite dihydroartemisinin (DHA).\textsuperscript{11} Elimination of ARS is very rapid, and antimalarial activity is mainly determined by DHA exposure. DHA has a terminal elimination half-life of around 45 min,\textsuperscript{10,12,13} and is approximately 93% plasma protein-bound in patients with malaria infection.\textsuperscript{14} The current dosing recommendation for ARS in the treatment of severe malaria is 2.4 mg/kg, which was mainly empirically derived from adult studies.\textsuperscript{15} In small children with severe disease, the pharmacokinetic properties of antimalarials might differ to that reported in non-pregnant adults.\textsuperscript{16-18} The population pharmacokinetic properties of i.m. ARS have not been studied previously in children with severe malaria, the main target group.\textsuperscript{9,19} The primary aim of this study was to characterize the population pharmacokinetic properties of ARS and its active metabolite DHA in the treatment of severe malaria in African children and to determine a practical dosing regimen.
Methods

Study design
This pharmacokinetic assessment of artesunate was part of the “AQUAMAT” trial (registration number ISRCTN 50258054), a large multinational trial comparing quinine and artesunate for the treatment of severe malaria, which results have been published elsewhere. This substudy was conducted at Teule Hospital in Muheza, Tanzania from May 2009 to July 2010. Apart from the additional blood sampling, procedures for the current study were part of the AQUAMAT study protocol. Ethical approval was obtained from the Tanzania Medical Research Coordinating Committee and the Oxford Tropical Research Ethic Committee. A total of 18 patients were co-enrolled in the “FEAST” trial evaluating fluid bolus therapy in children with compensated shock.

Children ≤14 years with a clinical diagnosis of severe malaria confirmed by pLDH-based RDT (OptiMAL, Diamed, Cressier, Switzerland) were recruited, provided written informed consent was given by their parent or carer. Severe malaria was defined as at least one of the following: coma (Glasgow coma scale [GCS]≤10 or Blantyre coma scale [BCS]≤2 in preverbal children), convulsions (duration >30 min or ≥2 episodes in 24 h prior to admission), respiratory distress (nasal alar flaring, costal indrawing/recession or use of accessory muscles, severe tachypnoea) or acidic breathing (“deep” breathing), shock (capillary refill time ≥3 sec and/or temperature gradient and/or systolic blood pressure <70 mmHg), severe symptomatic anaemia (<5 g/dL with respiratory distress), hypoglycaemia (<3 mmol/L), haemoglobinuria, severe jaundice or a convincing history of anuria or oliguria in older children. Patients who had received full treatment with parenteral quinine or an artemisinin derivative >24 h before admission were excluded.

Physical examination was done at admission and a venous blood sample was taken for peripheral blood parasite count, quantitative assessment of plasma PfHRP2 (a marker of total body parasite burden), HIV serology (SD Bio-Line HIV 1/2 3.0, Standard Diagnostics Inc, Kyonggi-do, Korea /Determine HIV-1/2, Abbott Laboratories, IL, USA), blood culture, liver function tests (ASAT, ALAT, y-GT, total bilirubin, creatinine and urea, by Reflotron, Roche Diagnostics), haematocrit (Hct), biochemistry and acid-base parameters (EC8+ cartridge for i-STAT handheld blood analyser). Haematocrit was reported from i-STAT or when not available, measured by Haemocue (n=5). A neurological examination was conducted at discharge, and repeated at day 28 for children who had not made neurological recovery at discharge.
Antimalarial treatment
Artesunate (Guilin Pharmaceutical Factory, Guangxi, China) was given as an i.m. injection (2.4 mg/kg) shortly after admission, at 12 and 24 hours, then daily thereafter. The content of each artesunate 60 mg vial was dissolved in 1 mL 5% sodium bicarbonate (provided with the drug) and further diluted with 5 ml 5% dextrose (final concentration of 10 mg/ml) before deep i.m. injection into the anterolateral thigh. Dosing was based on measured body weight and injection volumes over 2-3 ml were split and divided over both thighs. When the patient was well enough to take oral medication, but after a minimum of 24 hours (2 doses) of i.m. artesunate, a full 3-day course of oral artemether-lumefantrine (Co-artem, Novartis, Basel, Switzerland) was given to complete the treatment.

Patient management
Vital signs and glucose were monitored at least 6-hourly and with any deterioration in clinical condition. The majority of patients received a drip with dextrose 5%, unless they were able to feed orally. Hypoglycaemia (defined here as blood glucose <3 mmol/L) was treated with an i.v. bolus of 5 ml/kg 10% dextrose. Blood transfusion (20 ml/kg) was given to children with haemoglobin concentration<5 g/dL. Fluid bolus was given to children with signs of shock.20 All children were empirically treated with i.v. antibiotics. Convulsions were treated with i.v. Diazepam or phenobarbitone if persisting. Peripheral blood smears were repeated after 24 hours.

Blood sampling
Blood samples (1.5 mL) were drawn from an indwelling catheter into pre-chilled fluoride oxalate tubes22 for ARS and DHA quantification before the first dose (at baseline). Four subsequent samples were taken at pre-set random times in the following time-windows: 0-1, 1-4, 4-12 and 12-24 hours after the first dose. Randomisation of sampling times was done by computer-generated randomization (STATA version 12; (StataCorp, TX, USA). Immediately after blood collection, ARS/DHA blood samples were centrifuged at 4°C at 2000×g for 7 minutes. Plasma samples (0.5 mL) were stored at -80°C and shipped on dry ice to the Clinical Pharmacology Laboratory of the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, Thailand for drug quantification. Artesunate drug content and quality were checked in vials taken randomly from the purchase lots (see supplement of 6).
**Drug analysis**

ARS and DHA plasma concentrations were measured using liquid chromatography coupled to tandem mass-spectroscopy (LC-MS/MS). Quality control samples at low, middle and high concentrations were analysed in triplicates within each analytical batch to ensure accuracy and precision during the analysis. The total coefficients of variation were below 8% for all quality control samples. The lower limit of quantification was set to 1.2 ng/mL for ARS and 2.0 ng/mL for DHA.

**Pharmacokinetic modelling**

Venous plasma concentrations were transformed into molar units and modelled as natural logarithms using NONMEM v.7 (ICON Development Solutions, MD). Only first dose data (0 to 12 hours) were used during model development since there was a large uncertainty in the collection time of second dose samples. The injection sites were considered to be a single depot compartment. ARS and DHA were modelled simultaneously using a drug-metabolite model with complete in-vivo conversion of ARS into DHA.

The first-order conditional estimation method with interactions was used throughout the modelling. Model selection was based on the objective function value (OFV) computed by NONMEM, goodness-of-fit graphical analysis and physiological plausibility.

One- and two-compartment disposition models for ARS and DHA were considered. First-order and zero-order absorption models were evaluated for the distribution of artesunate from the injection sites to the central compartment. A Box-Cox transformation was tried on individual population parameters to assess formally the assumption that pharmacokinetic parameters are log-normally distributed. Separate additive error models for artesunate and dihydroartemisinin were assumed since logarithmic data were modelled.

Potential covariates were investigated using a stepwise forward addition and backward elimination approach. A p-value of 0.05 was used in the forward step and a p-value of 0.001 in the backward step to compensate for the relative small population studied. Body-weight was evaluated as a simultaneous allometric function on all clearance (power of 0.75) and volume (power of 1) parameters. An age-related enzyme-maturation effect was also investigated on clearance.

The following admission covariates were investigated: age (y), weight-for-age Z scores, body mass index (kg/m²), coma (continuous variable based on GCS/BCS score), cerebral malaria (coma and/or convulsion), shock (compensated or decompensated), systolic blood pressure (mmHg), heart rate (beats/min), respiratory rate (breaths/min),
haematocrit (%), haemoglobin (g/dL), base excess (mmol/L), blood urea nitrogen (mg/dL), total bilirubin (µmol/L), plasma creatinine (µmol/L), ALAT (U/L), ASAT (U/L), parasitaemia (parasites/µL), plasma \( P_f \text{HRP2} \). Simulation-based diagnostics (prediction-corrected visual predictive check and numerical predictive check) were used to evaluate the predictive performance of the final model. Monte-Carlo simulations by use of the final model with the observed variability were performed for different body weights to obtain representative population estimates of the exposure during the first day of dosing (AUC\( _{0-12\text{hrs}} \)) after prospective dose regimens. For definition of a practical parenteral dosing regimen, different body weight “bins” were evaluated to ensure a similar target exposure in all weight bands with reference to the exposure for children of 25 kg. The same simulations were used to evaluate the effect on drug exposure for other significant covariates.

Pharmacodynamics
Peripheral blood smears were performed at admission and after 24 hours. Parasite reduction over 24 hours, survival and severe neurological sequelae were evaluated in the pharmacodynamic analysis. The effects of artesunate and dihydroartemisinin exposure on outcome were investigated using a time-to-event analysis in NONMEM where the predicted drug concentrations were used to modulate the hazard function in a traditional E\( _{\text{MAX}} \) relationship. Group comparisons were performed with the non-parametric Mann-Whitney U-test in STATA.

Results
Clinical details
Seventy patients aged 7 months up to 11 years were included, of whom 59 (84%) were <5 years. Nine children died (case fatality 13%). One patient died within 15 minutes of admission and two patients died within the first 24 hours of admission. One patient aged 2.5 years had severe neurological sequelae 28 days after discharge comprising of spastic hemiparesis, blindness and hearing impairment. At admission this child had a parasite count of 880 205 parasites/µL and plasma \( P_f \text{Plasmodium falciparum histidine-rich protein-2 (PfHRP2)} \) concentration of 1875 ng/mL, both indicating a high parasite burden. At the 3-monthly follow-up these sequelae had completely resolved.
Table 1. Demographic, clinical and laboratory characteristics of children admitted with severe malaria

| Variable                           | Children with severe malaria |
|------------------------------------|-----------------------------|
| Total number of patients           | 70                          |
| Age (y) (median, range)            | 2.5 (0.6–11)                |
| Weight (kg)                        | 10.8 (9–13.5)               |
| Weight-for-age Z-score<sup>a</sup> | -1.2 (1.0)                  |
| Coma (based on GCS/BCS)            | 19 (27%)                    |
| Prostration                        | 46 (66%)                    |
| Convulsions                        | 26 (37%)                    |
| Shock<sup>b</sup>                  | 11 (16%)                    |
| Respiratory distress               | 1 (1%)                      |
| Acidosis (base excess < -8 mmol/L) | 28 (43%)                    |
| Hypoglycaemia (glucose < 3 mmol/L) | 11 (16%)                    |
| Anaemia (haemoglobin < 5 g/dL)     | 21 (30%)                    |
| Haemoglobinuria                    | 1 (1%)                      |
| Axillary temperature (°C)          | 38.3 (1.0)                  |
| Heart rate (beats/min)             | 158 (141–176)               |
| Respiratory rate (breaths/min)     | 49 (40–58)                  |
| Laboratory variables               |                             |
| Glucose (mg/dL)                    | 102 (88–127)                |
| BUN (mg/dL)<sup>c</sup>            | 12 (8–16)                   |
| Haemoglobin (g/dL)                 | 7.1 (5.1–9.2)               |
| pH<sup>c</sup>                     | 7.39 (7.33–7.43)            |
| HCO₃ (mmol/L)<sup>c</sup>          | 17.8 (13.2–21.5)            |
| Base excess (mmol/L)<sup>c</sup>   | -7 (-13–3)                  |
| ASAT (U/L)<sup>d</sup>             | 71 (49–116)                 |
| ALAT (U/L)<sup>d</sup>             | 25 (14–42)                  |
| Total bilirubin (μmol/L)<sup>d</sup>| 31 (24–49)                  |
| HIV-positive                       | 3/70 (4.3%)                 |
| Parasitaemia (parasites/μL), (geometric mean, 95% CI) | 88 391 (53 547–145 909) |
| Plasma PfHRP2 (ng/mL), (geometric mean, 95% CI)<sup>e</sup> | 1893 (1387–2584) |

Data are median (IQR), mean (SD) or n (%), unless otherwise stated.

<sup>a</sup> Weight-for-age Z scores for children ≤10 years; 27 data missing for n=1 aged >10 years.

<sup>b</sup> Compensated (n=4) and decompensated shock (n=7) combined

<sup>c</sup> Missing data for n=5 due to missing i-STAT measurement

<sup>d</sup> Missing data for n=5, n=6, n=4 for ASAT, ALAT and total bilirubin, respectively

<sup>e</sup> Missing data due to missing sample in n=2 and n=1 with undetectable PfHRP2 concentration.
Demographic, clinical and laboratory characteristics are described in Table 1. Severe prostration, severe acidosis, convulsions and severe anaemia were the most common severity criteria. Eleven patients (16%) presented with decompensated or compensated shock. Three patients (4.3%) had blood culture-proven septicaemia (unspecified gram-negative rods, *Klebsiella pneumoniae*, *Staphylococcus aureus*), none of whom presented with shock. HIV coinfection was detected in 3/70 (4.3%) of patients. None of these patients were receiving antiretroviral treatment.

Pre treatment with an oral antimalarial was reported for 31 patients (6 with quinine, 3 with amodiaquine, 13 with sulfadoxine-pyrimethamine, 8 with artemether-lumefantrine and 1 with amodiaquine followed by artemether-lumefantrine). Twelve patients had received pretreatment with i.m. quinine within 24 hours before admission with a median (range) total dose of 16.1 mg/kg (10.1–53.7 mg/kg).

All patients received an i.m. ARS injection of 2.4 mg/kg shortly after admission. Supportive treatments included blood transfusions and fluid resuscitation. Hypoglycaemia was corrected with a dextrose 10% bolus at admission and in 8 patients who developed hypoglycaemia after admission. Peripheral blood asexual parasite counts after 24 hours were negative in 12/66 (18%) patients (4 patients with missing 24 hour slide; 3 due to death). The geometric mean (95% CI) parasite count was 1,128 (537-2,368) parasites/µL in the remainder (n=54). The geometric mean (95% CI) fractional reduction was 96% (94% to 98%) in 66 patients.

**Population pharmacokinetic-pharmacodynamic analysis**

A total of 274 ARS and DHA post-dose samples randomly distributed over the first 12 hours of the study were analysed and included in the model. A total of 136 (49.6%) ARS and 106 (38.7%) DHA concentrations were reported below the quantification limit (BQL). Coding BQL samples as missing data resulted in no model-misspecification and there was no additional benefit of implementing a Laplacian estimation method for categorical BQL data. Modelling all data over 24 hours resulted in similar population parameter estimates but with substantially larger residual errors (2- and 3-fold increased variance) that confirmed the suspicion that sampling times were unreliable after the second dose (data not shown).

A one-compartment disposition model for both ARS and DHA was adequate to describe the observed plasma concentration-time data. All combinations of two-compartment disposition models displayed significant model misspecification of BQL data despite significantly lower OFV. This has been reported previously for DHA. Zero-order distribution/absorption from the injection site to the central compartment
provided the best description of the data but too few samples were collected during the absorption phase for an accurate estimation. The absorption rate was estimated to be very rapid with a high degree of uncertainty and therefore fixed to 1 minute for a robust pharmacokinetic model. Box-Cox transformation of ARS clearance and ARS and DHA volume of distribution resulted in a significantly better model ($\Delta$OFV=-22.3, 3 degrees of freedom). However, the additional transformation parameters could not be estimated reliably (RSE: 242% to 656%) and the confidence intervals of the estimates all spanned from large negative to large positive values. A traditional log-normal distribution of pharmacokinetic parameters was therefore used in the final model. Inter-individual variability could be reliably estimated for ARS and DHA clearance and ARS volume of distribution with correlation between ARS clearance and volume.

Body weight implemented as a fixed allometric function on all elimination clearance and apparent volume of distribution parameters resulted in a significantly (p<0.001) better model fit ($\Delta$OFV=-14.6). An age-dependent maturation effect on clearance parameters resulted in no additional benefit ($\Delta$OFV=-8.24, four degrees of difference). Bilirubin and haemoglobin as a linear covariate on DHA clearance were selected in the forward covariate stepwise search (p<0.05) and could also be retained in the backward elimination step (p<0.001). However, the effect of bilirubin was very small with a 0.323% decrease with every bilirubin unit increase. This effect could not be estimated with adequate precision (RSE: 94.2%) and the bootstrap confidence intervals contained zero effect (-0.500% to 0.5%). This covariate was therefore removed from the final model. DHA clearance increased with 10.2% with each unit (g/dL) of haemoglobin decrease. Inter-individual variability in clearance decreased from 63.6% CV to 55.3% CV after the inclusion of this covariate suggesting that it accounts for a limited but significant part of the observed variability. The final model described the observed data well with adequate goodness-of-fit diagnostics (Figure 1) with calculated shrinkages below 34% (CL/$F_{ARS}$: 10.6%, V/$F_{ARS}$: 12.2%, CL/$F_{DHA}$: 6.81, Epsilon-shrinkage: 33.3%).
Table 2. Parameter estimates of the final model describing artesunate and dihydroartemisinin population pharmacokinetics in children (n=70) with severe malaria

| Variable | Population estimate<sup>a</sup> (% RSE<sup>b</sup>) | 95% CI<sup>b</sup> |
|----------|-----------------------------------------------|-----------------|
| **Fixed effects** | | |
| CL/F<sub>ARS</sub> (L/h) | 45.8 (8.10) | 38.8–53.7 |
| V/F<sub>ARS</sub> (L) | 28.2 (11.4) | 22.7–35.2 |
| CL/F<sub>DHA</sub> (L/h) | 22.4 (8.40) | 19.2–26.5 |
| V/F<sub>DHA</sub> (L) | 13.5 (9.69) | 11.2–16.3 |
| DUR (min) | 1.00 (fixed) | – |
| **Covariate effects** | | |
| Negative effect of haemoglobin on CL/F<sub>DHA</sub> (%) | 10.2 (14.9) | 6.84–12.8 |
| **Random effects** | | |
| η<sub>CL/F<sub>ARS</sub></sub> | 0.415 (45.3) | 0.0890–0.755 |
| η<sub>V/F<sub>ARS</sub></sub> | 0.680 (54.6) | 0.111–1.373 |
| η<sub>CL/F<sub>ARS</sub> – η<sub>V/F<sub>ARS</sub></sub> | 0.497 (52.3) | 0.0732–0.969 |
| η<sub>CL/F<sub>DHA</sub></sub> | 0.306 (37.9) | 0.136–0.546 |
| σ<sub>ARS</sub> | 0.0942 (29.2) | 0.0266–0.249 |
| σ<sub>DHA</sub> | 0.211 (12.5) | 0.122–0.320 |
| **Post-hoc estimates**<sup>c</sup> | | |
| CL/F<sub>ARS</sub> (L/h/kg) | 4.27 | 1.18–11.0 |
| V/F<sub>ARS</sub> (L/kg) | 2.58 | 0.479–8.06 |
| t<sub>1/2</sub><sub>ARS</sub> (h) | 0.425 | 0.238–0.727 |
| C<sub>MAX</sub><sub>ARS</sub> (ng/mL) | 943 | 329–5090 |
| AUC<sub>0–12h</sub><sub>ARS</sub> (h×ng/mL) | 570 | 281–2170 |
| CL/F<sub>DHA</sub> (L/h/kg) | 2.01 | 0.736–5.95 |
| V/F<sub>DHA</sub> (L/kg) | 1.24 | – |
| t<sub>1/2</sub><sub>DHA</sub> (h) | 0.427 | 0.145–1.18 |
| T<sub>MAX</sub><sub>DHA</sub> (h) | 0.608 | 0.321–1.04 |
| C<sub>MAX</sub><sub>DHA</sub> (ng/mL) | 547 | 284–890 |
| AUC<sub>0–12h</sub><sub>DHA</sub> (h×ng/mL) | 890 | 297–2510 |

Abbreviations: ARS, artesunate; DHA, dihydroartemisinin; CL/F, elimination clearance; V/F, central volume of distribution; F, intramuscular bioavailability; DUR, duration of zero-order absorption; η = inter-individual variability; η<sub>CL/F – ηV/F</sub>, correlation of random effects on CL/F and V/F; σ, additive residual variance; t<sub>1/2</sub>, terminal elimination half-life; T<sub>MAX</sub>, time to maximum concentration; AUC<sub>0–12h</sub>, area under the concentration-time curve from time-point 0 to 12 hours; C<sub>MAX</sub>, predicted maximum concentration.

<sup>a</sup> Computed population mean values from NONMEM are calculated for a typical patient with a body weight of 10.9 kg and a haemoglobin value of 7.1 g/dL.

<sup>b</sup> Assessed by non-parametric bootstrap method (n=974 successful iterations out of 1000) of the final pharmacokinetic model. Relative standard error (% RSE) is calculated as 100×(standard deviation/mean value). 95% confidence interval (95% CI) is displayed as the 2.5 to 97.5 percentile of bootstrap estimates.

<sup>c</sup> Post-hoc estimates are displayed as median values with 2.5 to 97.5 percentiles of empirical Bayes estimates.
Goodness-of-fit diagnostics of the final population pharmacokinetic model of artesunate (A, B, C) and dihydroartemisinin (D, E, F) in children with severe malaria. Broken line, a locally weighted least-squares regression; solid line, line of identity. The observed concentrations, population predictions, and individual predictions were transformed into their logarithms (base 10).

A prediction-corrected visual predictive check of the final model resulted in no model misspecification with good simulation properties (Figure 2). The numerical predictive check (n=2000) resulted in 4.35% (95% CI 1.45–9.42) and 5.07 (95% CI 1.45–9.42) of observations above and below the 90% prediction interval for ARS, respectively, and 2.97% (95% CI 1.79–9.52) and 5.95 (95% CI 1.79–8.92) of the observations above and below the 90% prediction interval for DHA, respectively.

ARS and DHA exposure were simulated after the standard 2.4 mg/kg dose at each body weight (i.e. 1000 simulations per kg body-weight at 6 to 25 kg) using a uniform distribution of haemoglobin within the observed range (2.72 to 13.6 g/dL) to account for the observed covariate-relationship (Figure 3 A/B). Children between 6 and 10 kg body weight had a 20.4% mean reduction (p<0.0001) in DHA exposure compared to that in children between 21 to 25 kg body weight (median [25th to 75th] percentile exposure: 3380 [2130–570] ng×hr/mL for 6-10 kg patients, 3780 [2430–6060] ng×hr/mL for 11-15 kg patients, 4100 [2570–6590] ng×hr/mL for 16-20 kg patients, 4240 [2700–6840] ng×hr/mL for 21-25 kg patients).
Simulation-based diagnostics of the final model describing the population pharmacokinetics of artesunate (A, C) and dihydroartemisinin (B, D) in children with severe malaria. Graph A and B display a prediction-corrected visual predictive check with venous plasma concentrations transformed into their logarithms (base 10); open circles, observed data points; solid lines, 5th, 50th, and 95th percentiles of the observed data; shaded area, 95% confidence interval of simulated (n=2000) 5th, 50th, and 95th percentiles. Graph C and D display the observed fraction of data points below the limit of quantification as solid lines and 95% confidence interval of the simulated (n=2000) fraction below the limit of quantification as shaded area.

This suggests that smaller children need a higher dose to achieve the same exposure compared to children with a higher body-weight. We evaluated various body weight-bands based dosing regimens and the proposed regimen (Table 3) resulted in similar exposure in all weight bands after the first dose of intramuscular artesunate (Figure 3 C/D). The same simulations were performed for a uniform distribution of body weights at different levels of haemoglobin and resulted in lower DHA exposure with decreasing haemoglobin levels (Figure 4).
Simulated total first-dose exposure ($\text{AUC}_{0-12\text{ hr}}$) of ARS (A) and DHA (B) after the standard 2.4 mg/kg dosing in children at different body weights, and simulated total first-dose exposure ($\text{AUC}_{0-12\text{ hr}}$) of ARS (C) and DHA (D) after the suggested adjusted dose regimen (Table 3). Open circles represent median values and bars indicate the 25th to 75th percentile of simulations (1000 simulations at each body weight). The broken line represents the median exposure for the largest weight group (i.e. 700 h×ng/mL and 1230 h×ng/mL for ARS and DHA, respectively).

**Table 3. Proposed body weight adjusted i.m. artesunate dosing regimen**

| Weight (kg) | Dose i.m. (mg) | Dose i.m. (mg/kg) |
|------------|----------------|-------------------|
| 6–7        | 20             | 2.86–3.33         |
| 8–9        | 25             | 2.78–3.13         |
| 10–11      | 30             | 2.73–3.00         |
| 12–13      | 35             | 2.69–2.92         |
| 14–16      | 40             | 2.50–2.86         |
| 17–20      | 50             | 2.50–2.94         |
| 21–25      | 60             | 2.40–2.86         |
Simulated total first-dose exposure ($\text{AUC}_{0-12\text{hr}}$) of DHA (A) after the standard 2.4 mg/kg dosing in children at different haemoglobin levels. Open circles represent median values and bars indicate the 25th to 75th percentile of simulations (1000 simulations at each haemoglobin level). Simulated total first-dose exposure ($\text{AUC}_{0-12\text{hr}}$) of DHA (B) in children at different body weights at low (open circles: 3 g/dL), medium (open squares: 8 g/dL) and normal (open triangles: 13 g/dL) haemoglobin levels. The broken line represents the median exposure for the largest weight group (i.e. 1230 $\text{h} \times \text{ng/mL}$).

There was no statistical difference in total exposure (ARS $p=0.8060$, DHA $p=0.4828$) or maximum concentration (ARS $p=0.7655$, DHA $p=0.6865$) after the first dose between survivors versus children who died. Similarly, an exposure-response relationship could not be established using nonlinear mixed-effects modelling in a time-to-event approach. However, this might be a consequence of a relatively low number of deaths and high percentage of pre treatment with different antimalarial drugs, doses, and administration routes.
Chapter 9

Discussion

Parenteral artesunate is now the drug of choice for the treatment of severe malaria. Optimal treatment strategies depend upon detailed knowledge of the pharmacokinetic (PK) properties of drugs in the target population where the drug is used. Age, disease status and severity may all affect drug absorption, distribution, metabolism and elimination. The importance of pharmacokinetics in determining the therapeutic response is illustrated by artemether, the first parenteral artemisinin derivative compared with quinine for the treatment of severe malaria in large clinical trials. In a meta-analysis of randomized trials in severe malaria, artemether significantly reduced mortality in Southeast Asian adults but did not in African children. Subsequent pharmacokinetic studies showed that the oil-based artemether was released slowly and erratically from the i.m. injection site, which counterbalanced its pharmacodynamic advantages over quinine in African children.

Dosing regimens for children are often derived from adult studies, which have led to important underdosing. A pharmacokinetic study on sulfadoxine-pyrimethamine (SP) in African children with uncomplicated falciparum malaria showed that with the usual dose of 25/1.25 mg/kg, the area under the concentration-time curves (AUCs) in children 2 to 5 years old was half those in adults. This may have caused antimalarial treatment failures in small children, and thereby contributed to the spread of resistance. This information came decades after introduction of SP. Piperaquine exposure has also been shown to be lower in small children after a standard body weight-based dose regimen.

This is the first population pharmacokinetic study of i.m. ARS in African children with severe malaria. A one-compartment model described accurately the distribution of ARS and DHA. ARS was converted rapidly into DHA with an approximate ARS elimination half-life of 26 minutes. This is in agreement with reported ARS half-life in other pharmacokinetic studies of i.m. ARS, but is considerably longer than after i.v. injection. This is because the ARS elimination rate is limited by the rate of absorption from the i.m. injection site (i.e. “flip-flop” pharmacokinetics). The volume of distribution, maximum concentration and AUCs of ARS and DHA are also comparable with the findings from previous small dense-sampling PK studies of i.m. artesunate in adults and children.

Despite a lower Cmax after i.m. injection compared to i.v. administration, this is still far above the in-vitro defined DHA IC99 value of 2.28 ng/mL. The excellent bioavailability after i.m. injection (approximately 90%), fast absorption and comparable estimates for ARS and DHA exposure, support the use of i.m. ARS as a suitable alternative to i.v. ARS.
The most significant covariate identified in the present study was body weight. This has also been reported in other population pharmacokinetic studies of oral and rectal artesunate in paediatric,37,38 or mixed adult-paediatric populations. In general, physiological processes do not scale linearly with body weight and consequently children with a lower body weight will have a higher body weight-normalised elimination clearance. This has also been reported previously for other antimalarials.37,38 Dosing simulations with the exact dosing of 2.4 mg/kg resulted in lower ARS and DHA exposure in small children compared to those with a body weight of 25 kg, suggesting the need for higher dosing in small children. In addition, haemoglobin concentration was also a significant covariate resulting in lower DHA exposure in more anaemic children. In severe malarial anaemia, a degree of intravascular haemolysis occurs and the released heme might cause iron-mediated degradation of the artemisinin peroxy bond, as has been found to be a cause of artesunate degradation in hemolysed plasma samples ex-vivo.39 Since haemoglobin concentration was associated with reduced exposure independent of body weight, this further supports the need for an adjusted higher dose in young children who are generally more anaemic than older children.40,41

Underdosing in young children with severe malaria may have immediate adverse consequences for outcome. Parenteral and oral artesunate are extremely well tolerated. The only dose dependent toxicity identified to date is neutropenia. A study from Cambodia has recently shown that oral artesunate in a dose of 6 mg/kg/day for 7 days resulted in a reduction in neutrophil counts and short lived neutropenia <1.0x10^3/μL in 19% of patients.42 With an oral bioavailability of approximately 80%,43,44 this corresponds to a total parenteral dose of 33.6 mg/kg, which would only be reached if parenteral treatment were continued for 14 doses. In the AQUAMAT trial, the median (IQR) duration of parenteral treatment in surviving children was 3 (2-4) doses.6 Artesunate is now the first choice treatment of severe malaria and a GMP qualified formulation is available. To facilitate implementation of an optimized dosing regimen in the treatment of severe malaria in African children, we defined a simplified weight-bands based dosing regimen based on the current population pharmacokinetic model, which incorporated accuracy and practicality issues. We considered the minimum volume of prepared artesunate solution that can be measured accurately and administered with commonly available types of syringes as 0.5 ml. Since the weight bands in children below 14 kg were smaller, incrementing the dose of artesunate with 5 instead of 10 mg, we propose an artesunate dilution of 10 mg/ml for i.m. administration in this group, as was used in the current study. Larger injection volumes in children of 14 kg and above can be avoided by diluting the artesunate to 20 mg/ml. Doses should be split over both thighs at...
injection volumes over 2–3 ml. Binning of weight bands was also chosen by the current available vial size of 60 mg, demarcating upper limits of weight bands at 25 kg.

The dosing recommendations do not extend beyond the weight ranges of the children included in this study. No children below 6.5 kg were included, so additional artesunate population pharmacokinetic studies are urgently needed to evaluate dosing in these very young children and to support the current dosing recommendations. Sample decay could have contributed to the high percentage of samples below the quantification level. However, negative samples were not more frequent than expected based on earlier studies and will not have affected the differences between subgroups.15 More extensive sampling in the first 15 minutes and 12 hours post-dose could give more information than our current study provided.

In conclusion, ARS and DHA exposure after i.m. administration of ARS in severe malaria were lower in small children, warranting dose adaptation in this group. Independently, anaemia may aggravate lower exposure in young children. We propose a bodyweight adjusted and convenient dosing regimen for i.m. ARS in children from 6 to 25 kg.

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Chapter 10

General discussion and conclusions
General discussion

This thesis describes studies performed between 2005 and 2011 on a number of important issues concerning the diagnosis, prognosis and treatment of severe falciparum malaria in African patients with a focus on children. The AQUAMAT study was the largest clinical trial to date in hospitalized patients with severe malaria, and provides the definite answer that artesunate is the best antimalarial treatment of severe malaria. The studies on plasma PfHRP2 in African children provide a method to define malaria-attributable disease in children with severe febrile illness in high transmission settings. The study on rapid diagnostic tests shows that these are a reliable and easy to use alternative to microscopy for the diagnosis of severe malaria in African children and highly suitable for this setting. The pharmacokinetic studies define a practical dosing regimen for artesunate and underline the need for a quinine loading dose, both particularly especially important for small children. The descriptive clinical studies provide a novel predictive severity score for children with severe malaria and show the different clinical presentation of severe malaria in case of HIV coinfection, which are of importance to the treating clinician. This chapter discusses in some detail the key findings of this thesis and aims to put these in perspective, including possible future directions for research.

The WHO estimated 890 000 malaria deaths in 2004, the majority of whom are African children.1 A recently published systematic analysis, using a wide range of key predictors of malaria mortality into a variety of predictive models, including verbal autopsies, estimated that the total number of malaria deaths in 2004 was almost double as high.2 Differences in research methodology aside, these heterogeneous estimates of the worldwide malaria mortality also reflect the challenges in diagnosing severe malaria. Improvements in the diagnosis of severe malaria are needed to quantify the burden of malaria, to define study populations in clinical studies, and to assist the clinician in the management of severe febrile illness including severe malaria.

Diagnosis of severe malaria

The WHO definition of severe malaria is based on the presence of one or more clinical severity criteria and limited laboratory markers in the presence of asexual Plasmodium falciparum parasites on the peripheral blood film.3 This definition was derived from the associated risks of death corresponding to clinical severity signs3,4 and was aimed towards optimal sensitivity, since untreated severe malaria approaches a case fatality of
100%. Missing the diagnosis and not treating the patient with a parenteral antimalarial may result in death and therefore sensitive parasitological tests are of utmost importance. Reliable microscopy of the peripheral blood film, the gold standard, is often not available in African health care settings.\textsuperscript{5,6} We hypothesized that malaria rapid diagnostic tests detecting malaria specific antigens would be suitable alternatives to routine microscopy in the diagnosis of severe malaria. Theoretically these tests may well perform better than microscopy, since the plasmodial antigens are freely circulating in the plasma and do not sequester like the malaria parasites.

Various types of RDT have been available, but none have been evaluated for the diagnosis of severe malaria in African children.\textsuperscript{7,8} For the AQUAMAT research project, a rapid and reliable diagnosis of severe malaria, that would be applicable across various levels of health care and transmission settings, was essential. In a substudy, we compared the performance of a \textit{Pf}HRP2- and a pLDH-based RDT versus expert microscopy for the diagnosis of severe malaria in two settings with different transmission intensities (Chapter 4). We found that the \textit{Pf}HRP2-based test was a reliable alternative to routine microscopy with a sensitivity of 96.9\% for parasite densities >100 parasites/\textmu L. The \textit{Pf}HRP2 based test was more sensitive than the pLDH-based test. The sensitivity of both tests was positively correlated with the parasite density, with a significantly better performance of the \textit{Pf}HRP2 based test at lower parasite densities. The specificity of the \textit{Pf}HRP2 based test was lower which is related to the longer half-life of \textit{Pf}HRP2 versus the parasite enzyme pLDH.\textsuperscript{9,10} Of practical importance, the used \textit{Pf}HRP2 based test was easier to perform, more heat-stable and cheaper than the used pLDH based test.\textsuperscript{11-14}

The prior probability of having severe malaria depends on malaria transmission intensity and the prevalence of alternative diseases causing severe febrile illness. The malaria transmission intensity in the study site of Beira was lower than in Muheza, reflected by the different age and clinical pattern of severe malaria (Chapter 4). The reported HIV-prevalence was much higher; 30\% in sentinel surveys in pregnant women in the city of Beira.\textsuperscript{15} Mozambique was in the top-3 of countries with the highest estimates of mortality in children >5 years and adults in 2000 and 2010.\textsuperscript{2} This was one of the reasons for including adult patients in the AQUAMAT trial in Mozambique (Chapter 7). However, during the almost 5 year study period we recruited only 68 adults with severe malaria, despite extensive screening of clinically suspected severe malaria patients. Although our study was not designed as an epidemiological survey, our observations suggest that the absolute numbers of adults dying from severe malaria are not that high, even though HIV might increase the risk of severe disease and increase the case fatality of severe malaria (Chapter 7). The malaria rapid diagnostic tests performed well in children in this setting.
(Chapter 4, 7), but overdiagnosis of severe malaria in adults was more common. Not restricted to adults, our findings are in line with other studies reporting a low predictive value of clinical signs and symptoms for peripheral blood parasitaemia. Pneumonia, meningitis, bacteraemia and other HIV-associated severe illnesses may present with similar signs and symptoms to severe malaria.

In malaria-endemic areas, an additional challenge in the diagnosis of severe malaria is the aspecificity of presence and malaria parasites in the peripheral blood film due to the development of host partial immunity early in life. Overdiagnosis of severe malaria is also an important problem in young children in Africa who are at highest risk of death of malaria or other severe febrile illness.

*Plasmodium falciparum* specific sequestration in the second half of the erythrocytic cycle renders the more pathogenic mature parasites invisible to the microscopist assessing the peripheral blood slide. Peripheral blood parasite density is therefore not a reliable measure of total parasite burden and severity of disease. *Pf*HRP2 is produced by *Plasmodium falciparum* and approximately 90% is released into the circulation at the moment of schizont rupture. Since this is distributed through the total plasma volume, quantitative plasma *Pf*HRP2 can be considered as a measure of the total parasite burden of the preceding asexual cycle. We compared the distributions of peripheral blood parasitaemia versus plasma *Pf*HRP2 concentrations in control, asymptomatic, uncomplicated and severe malaria cases from a community and a nearby hospital in a high transmission setting in Tanzania (Chapter 6). We showed a stepwise increase in plasma *Pf*HRP2 concentrations according to the severity of infection, ranging from asymptomatic parasitaemia, uncomplicated malaria to severe malaria. There was substantially less overlap in plasma *Pf*HRP2 distributions between these groups compared to the distributions of peripheral blood parasitaemia. Notably, peripheral blood parasitaemia distributions were entirely overlapping between patients with uncomplicated and severe malaria. The distinct plasma *Pf*HRP2 distributions were used to model the malaria-attributable proportion of severe disease, in order to distinguish patients with “true” severe malaria from those with coincidental peripheral blood parasitaemia in whom severe febrile illness is caused by an alternative disease. *Pf*HRP2 levels above 1000 ng/ml corresponded to a malaria-attributable fraction of 99% with a sensitivity of 74%. The proportion of malaria-attributable disease declined at lower *Pf*HRP2 concentrations. Below 200 ng/ml an alternative diagnosis was suggested in >10% of patients and below 50 ng/ml in >50% of the patients (Chapter 6).

These results are in strong agreement with the *Pf*HRP2 thresholds identifying high or low probability of alternative disease derived from a mechanistic model based on the malaria-
attributable mortality in the AQUAMAT population (Chapter 5). In the latter study, we found that a PfHRP2 concentration >1000 ng/ml corresponded to a malaria-attributable proportion >95%, and that a PfHRP2 concentration <174 ng/mL suggested an alternative illness in >10% and was responsible for more than 50% of the mortality. Since >60% of patients with severe malaria in the AQUAMAT study had PfHRP2 concentrations >1000 ng/mL, we conducted the former study incorporating the asymptomatic carriers and uncomplicated malaria patients for a more accurate definition of the probability of non-malarial disease with low plasma PfHRP2 concentrations (Chapter 6). It is reassuring that the identified PfHRP2 thresholds denoting a high or low probability of alternative disease respectively were consistent between these two studies using different study populations and modelling techniques.

This work contributes to a growing body of evidence about the diagnostic utility of PfHRP2 in African children. A recently published study from Malawi reported that a PfHRP2 concentration >1700 ng/ml had sensitivity of 98% and a specificity of 94% for cerebral malaria with histological evidence of sequestration.29 Compared to malarial retinopathy, the sensitivity and specificity of this plasma PfHRP2 threshold were 90% and 87%, respectively. Their chosen PfHRP2 thresholds seem to be within the plausible ranges of our findings, although our studies included also non-cerebral malaria. We estimated that with plasma PfHRP2 concentrations between 1000 to 3162 ng/mL, the probability that death was caused by severe malaria varied between 83% and 93% (Chapter 5). In this large study we described a U-shaped relation between PfHRP2 and the probability of in-hospital death with a nadir in case fatality at a plasma PfHRP2 concentration of 174 ng/mL. Below this threshold, we found an increasing risk of death with decreasing plasma PfHRP2 concentrations, presumably representing deaths caused by non-malarial disease. This assumption is supported by increased frequency of positive blood cultures in patients with low PfHRP2 concentrations (Chapter 6). On the other hand, positive blood cultures were also overrepresented in patients with very high PfHRP2 concentrations (>5000 ng/mL) indicating concomitant sepsis in patients with severe malaria.

**Prognosis of severe malaria**

In line with studies in Asian adults,28,30 we reported that plasma PfHRP2 has strong prognostic significance in African children (Chapter 5). We showed a 20% increase in risk of death per unit increase in log10 PfHRP2 above 174 ng/mL. These studies and the Malawian study taken together, confirm the central pathophysiological role of the total
parasite burden including the sequestered parasite burden.\textsuperscript{28-30} This severe malaria in Asian adults as well as African children, whereas potential differences in pathophysiology of severe malaria between these groups have been much debated, related to differences in clinical presentation and findings of autopsy studies.\textsuperscript{3,23,31-33} Autopsy studies have demonstrated the sequestration of infected red blood cells in the vital organs and particularly the brain.\textsuperscript{34} However, assessment of the sequestered parasite burden in the alive patient was only possible by detection of malarial retinopathy in patients with cerebral malaria.\textsuperscript{23,35,36} Ophthalmoscopy requires equipment and considerable expertise and will therefore not be practicable in most clinical settings in malaria-endemic areas.

Plasma $Pf$HRP2 reflects the total parasite burden including the sequestered parasites in the entire clinical spectrum of severe malaria and is closely correlated with outcome. This can be a useful tool for the case definition of severe malaria in studies addressing the pathophysiology in severe malaria, as well as for epidemiological and intervention studies, and also assist the clinician in diagnosing and treating individual patients.

Our data call for the development of a semi-quantitative $Pf$HRP2 rapid diagnostic test, with suitable plasma $Pf$HRP2 thresholds for the case definition of severe malaria. We would propose plasma $Pf$HRP2 thresholds of 200 and 1000 ng/ml, indicating high (>10%) and low probability (1%) of alternative disease, respectively. Particularly the lower threshold may depend on the level of malaria-specific immunity and the prevalence of alternative disease in the population. Extending to low transmission areas, these effects may be balanced out by the strong association of malaria and bacteraemia, where the burden of bacterial disease has reported to be lower.\textsuperscript{37} In addition, the prognostic value of $Pf$HRP2 was similar in low versus high transmission settings (Chapter 5). It will be impractical to define different thresholds for every transmission, clinical care or research setting; therefore our proposed thresholds will be a compromise depending on the planned use of the test.

In addition to plasma $Pf$HRP2, the large dataset of the AQUAMAT study was used to identify other clinical and laboratory indicators that could predict the outcome of severe malaria (Chapter 3). We identified 5 highly significant independent predictors of mortality, including acidosis indicated by a large base deficit, cerebral manifestations, elevated blood urea nitrogen and signs of chronic illness at admission. The overall mortality of severe malaria was 9.8% which increased up to 43% in presence of the 3 most frequent predictors cerebral malaria (coma and/or convulsions), acidosis ($BE < -8$ mmol/L) and elevated blood urea concentrations ($BUN \geq 20$ mg/dL). Numerous other studies have identified cerebral manifestations and acidosis as important prognostic factors, which have been associated with sequestration of infected red blood cells leading to impairment
of microcirculatory flow, causing tissue hypoxia and anaerobic metabolism.\textsuperscript{4,35,38,39} The association of an elevated BUN concentration with poor outcome has scarcely been reported in children.\textsuperscript{26,40} We also confirmed the prognostic value of elevated BUN concentrations in the substudy in Mozambique, wherein the logistic regression model was adjusted for age and a high proportion of patients was HIV-coinfected (Chapter 7). The origin and significance of the elevated BUN levels remains unclear. Renal failure is a common complication in adults with severe malaria,\textsuperscript{41,42} but this is uncommon in African children.\textsuperscript{3} Protein break down might contribute to elevated BUN concentrations and hence be more prominent in children with malnutrition and HIV. For many years, hypovolaemia has been postulated to contribute to the metabolic acidosis and be of relevance in the pathophysiology of severe malaria.\textsuperscript{43-45} Hypovolaemia could also attribute to elevated BUN concentrations. However, a recently completed large randomized trial on the treatment of children with impaired perfusion and shock showed that a fluid bolus with either normal saline or human albumin increased mortality in children with severe malaria or other severe infection compared to no fluid bolus.\textsuperscript{46} Prognostic indicators can be of practical relevance to the clinician or clinical researcher and plea for the use of point-of-care test, but may be aspecific clues to the underlying pathophysiology of severe malaria.\textsuperscript{47} More research into the pathophysiology of malaria is urgently needed in order to understand the specific pathways leading to death and identify new targets for treatment. To date, apart from supportive care that includes glucose for hypoglycaemia and blood transfusion for anaemia, there are no adjunct therapies that reduce mortality from malaria.\textsuperscript{48} Treatment with an effective antimalarial remains the pillar in the management of the severe malaria.

**Treatment of severe malaria**

The AQUAMAT study was the first large randomized controlled trial demonstrating a life-saving treatment for severe malaria in African children (Chapter 2). This trial included 5425 patients in 11 sites in 9 African countries across various transmission settings and was the largest trial in the treatment of severe malaria ever. In African children, we found that parenteral artesunate reduced the mortality by 22.5\% compared to parenteral quinine. In addition to the SEAQUAMAT trial,\textsuperscript{49} and various earlier small comparative studies comparing artesunate versus quinine for the treatment of severe malaria,\textsuperscript{50-53} the evidence is conclusive that artesunate is a better treatment than quinine.\textsuperscript{54}
Artesunate has a greater parasitocidal effect than quinine, which is related to the broader stage specificity of action. The artemisinins kill the young stage parasites, before they mature, and hereby prevent the sequestration of the infected red cells in the vital organs leading to potentially fatal microvascular obstruction. This is supported by the AQUAMAT subgroup analysis, showing that the benefit of artesunate was greater in patients with hyperparasitaemia on the peripheral blood film. Notably, children with the highest total body parasite burden (PfHRP2 values >2300 ng/ml) showed the greatest benefit of artesunate. In this subgroup, the treatment effect was similar to that observed in Asian adults, whereas the overall treatment benefit in the AQUAMAT trial was lower than that observed in the SEAQUAMAT trial due to the dilution of the study population with non-malarial disease. The life-saving benefit of artesunate was not at the expense of more children with neurological sequelae as assessed at 28 days post-discharge, although a recent study challenges the appropriate length of follow up for the detection of malaria-related neurological sequelae.

Parenteral artesunate has several other advantages compared to parenteral quinine. It is safe and easy to administer and can be given by i.v. or i.m. route. Artesunate is also a safe drug, since no serious adverse effects were identified in this study or previous studies with similar dosing. The only known side-effect is neutropenia when artesunate is given at high dose (6 mg/kg) during 7 days, which dose will not be reached at the current recommended dosing regimen. The current dosing regimen has been derived from adult studies and our population pharmacokinetic (POPPK) assessment of intramuscular artesunate was the first to examine the covariates on the pharmacokinetic (PK) profiles of artesunate and its active metabolite dihydroartemisinin in African children. Following an intramuscular injection of artesunate, artesunate and dihydroartemisinin exposure were lower in small children. Increased clearance of antimalarial drugs resulting in lower exposure has been observed in other antimalarials, and may contribute to the development of antimalarial drug resistance. Independently, the presence of anaemia may aggravate lower exposure in young children. This has not yet been observed in other studies and may be a drug-specific phenomenon due to iron-mediated degradation of the artemisinin peroxide bridge.

We proposed a body weight-adjusted and practical dosing regimen for intramuscular artesunate in children from 6 to 25 kg. Based on our experience from the AQUAMAT trial, we considered various practical dosing issues like the vial size and the minimum volume of prepared drug solution that can accurately be administered. This dosing scheme may assist in the implementation of artesunate in malaria-endemic countries.
Until parenteral artesunate becomes wider available, quinine remains commonly used and is a suitable alternative for the treatment of severe malaria. Despite longstanding and wide-spread use of quinine, there is no evidence of the development of high-grade resistance. Since the available body of evidence is small for treatment of one of the most life-threatening diseases in sub-Saharan Africa, we re-addressed the PK co variates and dosing regimens of quinine with POPPK modelling methods assessing a wide range of co variates. A loading dose of 20 mg/kg has been recommended for over a decade, yet has not been implemented everywhere due to toxicity concerns. More than 85% of patients reached therapeutic quinine blood concentrations (above 8 mg/L) after an initial loading dose, whereas only 30% did so if a routine dose (10 mg/kg) of quinine was given. Intramuscular quinine was rapidly and reliably absorbed and none of the studied covariates affected the PK parameters. Also for quinine, body weight was identified as a co variate; however this did not affect therapeutic drug levels. Quinine has reliable pharmacokinetics at the current recommended dosing regimen. If artesunate is unavailable and quinine is used, then a loading dose should be given unless a loading dose was already given within 24 hours before admission or a routine dose was given within 12 hours prior to admission.

Conclusions and recommendations for clinical practice and research

PfHRP2-based RDTs are an acceptable alternative to routine microscopy for the diagnosis of severe malaria in African children. In settings where reliable microscopy is available, negative RDT results should be confirmed by microscopy and effectuate the search for an alternative diagnosis.

Plasma PfHRP2 concentrations have prognostic significance and can distinguish severe malaria from alternative diseases with coincidental peripheral blood parasitaemia in moderate to high malaria-endemic areas. In parasitaemic children with clinically suspected severe malaria and a plasma PfHRP2 concentration <200 ng/mL, the probability of non-malarial illness is greater than 10% and warrants additional diagnostic tests and treatment with effective antibiotics. A plasma PfHRP2 concentration >1000 ng/mL indicates a very high probability of severe malaria (>99%), although concomitant sepsis may occur. In malaria-endemic areas with a high HIV prevalence, HIV testing is recommended in the diagnostic work-up of clinically suspected malaria. It will assist in the contemplation of alternative HIV-related diagnoses. In addition, severe malaria in HIV-coinfected
patients presents with a higher parasite burden, more severe disease and carries a higher case fatality rate.

In African children with severe malaria, cerebral malaria (coma and/or convulsions), acidosis (base excess < -8 mmol/L), elevated BUN concentrations (≥20 mg/dL) and signs of chronic disease (wasting or oedematous malnutrition, generalized lymphadenopathy, oral candidiasis) are independent predictors of a poor outcome.

Artesunate reduces the mortality of severe malaria compared to quinine with 22.5%. Parenteral artesunate is now the antimalarial treatment of choice for children as well as adults with severe malaria. It is safe and easy to administer and has no serious side effects. The recommended dose is 2.4 mg/kg at admission, after 12 hours and then once daily until the patient is able to take oral antimalarial treatment. Body weight and Hb affected the clearance of dihydroartemisinin (the active metabolite of artesunate), resulting in a lower exposure in young children (<15 kg). We propose a body weight-adjusted practical dosing regimen for children. Where parenteral artesunate is not available, parenteral quinine including a loading dose is a suitable alternative with reliable pharmacokinetics. In all children with severe malaria, routine administration of broad spectrum antibiotics is recommended. Prompt and appropriate treatment of sepsis could further reduce the mortality of severely ill children with malaria.

**Future perspectives and implications for health policies and research**

Over the past few years, an increasing number of reports have shown declining malaria transmission in several parts of Africa. There are concerns that international efforts to fight malaria will lose interest now major improvements have been made to control malaria and economic recession affects donor funds. With declining malaria transmission, the acquisition of malaria-specific immunity is delayed and severe malaria with high case fatality will continue to be a problem in all age groups, with an extended risk to older children and adults. To further reduce malaria mortality, ongoing research is needed to improve our understanding of the pathophysiology and ameliorate the diagnosis and treatment of severe malaria. Since most patients suffering of severe malaria live in sub-Saharan Africa, strategies should be tailored to these settings.

Plasma *Pf*HRP2 is an ideal biomarker for the development of a (semi-) quantitative RDT for severe malaria, because of its predictive value for outcome and its ability to distinguish severe malaria from non-malarial disease in parasitaemic children in moderate to high transmission areas. Currently existing whole blood *Pf*HRP2-based
RDTs show substantial differences in performance, which might be related to the test-specific PfHRP2 antibodies or the presence of PfHRP2 deletions or polymorphisms in the parasite population, amongst other factors. We have assessed the possibility of PfHRP2 (and PfHRP3) deletions and the relationship of PfHRP2 polymorphisms with plasma PfHRP2 concentrations in a subset of AQUAMAT patients with low PfHRP2 concentrations and high peripheral blood slide counts. In agreement with findings from Baker et al, we did not find evidence for PfHRP2 deletions in field isolates causing severe malaria in African children. In addition, sequence polymorphisms were not a significant cause of variation in plasma PfHRP2 concentration. This is promising for the utility of (semi-) quantitative plasma PfHRP2-based RDTs. An evaluation of various PfHRP2-based rapid diagnostic tests for the semi-quantitative measurement of plasma PfHRP2 is currently underway. In line with WHO methods for RDT testing, we recommend the use of patient-derived plasma PfHRP2 standard curves over test calibration with recombinant PfHRP2 standards.

With declining malaria transmission and an increasing number of patients living with HIV/AIDS in sub-Saharan Africa, distinguishing malaria from other causes of severe febrile illness is increasingly important. A PfHRP2-based (semi-) quantitative rapid diagnostic test would therefore be a first useful test. Since bacteraemia has been associated with a higher case fatality rate than severe malaria alone, studies investigating alternative causes of severe febrile disease, including in HIV-positive patients, are urgently needed. Such studies are likely to be challenged by the low sensitivity of blood cultures, which is the gold standard for the detection of bacterial disease. Studies identifying and including biomarkers of bacterial disease are warranted, of which procalcitonin might be one of the most promising.

Despite the challenges to diagnose bacterial disease, our findings suggest that bacteraemia and malaria parasitaemia commonly co-exist in severely ill African children. Positive blood cultures in severely ill children with a low parasite burden likely represent alternative disease in children with asymptomatic parasitaemia, whereas bacteraemia in children with a high parasite burden represents concomitant illness. Routine administration of broad spectrum antibiotics is therefore recommended in the treatment of severe malaria in African children. Prompt and appropriate treatment of sepsis could further reduce the mortality of severely ill children with malaria parasitaemia, which was still 8.5% in children treated with parenteral artesunate. Further trials are needed to assess which antibiotic and regime would be most suitable for that.

Our studies support the central role of red blood cell sequestration in the pathophysiology of severe malaria. Improvement of the microcirculation may be an important target
for adjunctive malaria treatment. A potential adjunct treatment under study is a depolymerized heparin with strongly reduced anticoagulant properties that has been shown to inhibit merozoite invasion of the erythrocytes and the microvascular cytoadherence of \textit{P. falciparum} infected erythrocytes.\textsuperscript{80}

The recently confirmed artemisinin resistance in Western Cambodia poses a threat to the treatment of severe malaria, and efforts to reduce malaria mortality and the burden of malaria worldwide.\textsuperscript{81} Hyperparasitaemia and reduced ARS/DHA exposure in young children are potential population risk factors that may contribute to development of artemisinin drug resistance.\textsuperscript{61} The findings of our POPPK study suggest that the current dose of artesunate should definitely not be reduced.\textsuperscript{82,83} Additional POPPK studies are urgently needed to confirm our findings, identify other potentially relevant covariates and optimize the artesunate dosing regimen for African children.

Lastly, the translation of research findings demonstrating life-saving interventions in the treatment of severe malaria has been very slow. Although usually a body of evidence consisting of many studies and expert opinion changes medical practice, rather than a single large randomized controlled trial like the AQUAMAT study. Not only drug efficacy, but also costs will convince policy makers to adopt parenteral artesunate for the treatment of severe malaria. An economic analysis conducted alongside the AQUAMAT showed that the cost of averting malaria-related deaths in sub-Saharan Africa by switching from quinine to artesunate was extremely low, with a mean value of US$ 123 per death averted.\textsuperscript{84} This is very cost-effective compared to other interventions aimed to reduce malaria mortality, and in view of the high malaria burden in sub-Saharan Africa, also affordable.\textsuperscript{84}

Another hurdle in the implementation of artesunate has been overcome since the Chinese factory that developed artesunate (Guilin Pharma) has improved the manufacturing process and the consequent quality of the formulation which has led to WHO GMP prequalification. The factory claims that it can produce enough artesunate to fulfil the demand and has promised to keep costs at or below current prices. Meanwhile the US Army project has also developed a GMP formulation. Medicines for Malaria Venture (MMV) has liaised with WHO, Guilin Pharma and Mahidol-Oxford Tropical Medicine Research Unit in order to facilitate the implementation of artesunate in the field, including the drug registration issues and training materials for local health care staff. It is expected that within the next few years, artesunate will replace quinine for the treatment of severe falciparum malaria everywhere in the world.
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Chapter 11

Summary
Acknowledgements
Curriculum Vitae
Publications
Samenvatting (voor niet ingewijden)

Malaria tropica wordt veroorzaakt door de parasiet *Plasmodium falciparum* en is een van de belangrijkste oorzaken van ziekte en sterfte in de wereld. De meeste patiënten zijn jonge kinderen in Afrika. Naar schatting serven jaarlijks bijna 800 000 kinderen jonger dan 5 jaar.
In de jaren ’80 en ’90 werd een toename gezien van de met malaria samenhangende sterfte. Dit was met name het gevolg van de ontwikkeling van resistentie van de malariaparasiet tegen veelgebruikte antimalariamiddelen zoals chloroquine en sulfadoxine-pyrimethamine, en de opkomende HIV-AIDS pandemie in sub-Sahara Afrika. Wereldwijd introductie van de bijzonder effectieve artemisinine combinatie therapieën en een sterke toename in het gebruik van geïmpregneerde muskietennetten, heeft ook weer een daling van de malaria transmissie en sterfte tot gevolg gehad in het afgelopen decennium.
Malaria tropica presenteert zich gewoonlijk als een koortsende ziekte met griepachtige verschijnselen, braken en diarree, die goed te behandelen is met orale antimalaria middelen. In een kleine minderheid van de gevallen ontwikkelt de ziekte zich tot een levensbedreigend ziektebeeld met ernstige ziekteverschijnselen zoals coma of een verminderd bewustzijn (cerebrale malaria), stuipen (convulsies), ernstige bloedarmoede (anemie), kortademigheid of een toegenomen ademarbeid tengevolge van een overmaat aan zuren in het bloed (acidose). Onbehandelde ernstige malaria kan binnen enkele uren lijden tot de dood, dus een snelle diagnose en effectieve behandeling zijn essentieel om de sterftekans te verminderen. Bij gebrek aan basisgezondheidszorg op het Afrikaanse platteland, overlijden vele kinderen voordat een kliniek bereikt kan worden. In ziekenhuizen in malaria-endemische gebieden is de sterfte van behandelde ernstige malaria onacceptabel hoog: 15-20%.
Kinine wordt al meer dan 300 jaar gebruikt voor de behandeling van ernstige malaria. Het is een goedkoop en effectief middel, waartegen tot op heden geen belangrijke resistentie is aangetoond. Nadelen van kinine zijn dat het een smalle therapeutische breedte heeft (met gevaar voor onderdosing zonder therapeutisch effect of overdosering met toxiciteit, met name voor het hart) en dat het niet gemakkelijk toe te dienen is. Het moet driemaal daags worden toegevoegd als een langzaam lopend intraveneus infuus of als een intramusculaire injectie. Een belangrijke bijwerking van kinine is verlaging van het bloedsuikergehalte, wat levensbedreigend kan zijn indien dit niet tijdig wordt onderkend en behandeld. De potente antiparasitaire eigenschappen van de artesimisine plantenextracten werden ontdekt in de jaren ’70, nadat deze middelen al meer dan 2 millennia in gebruik waren
voor de behandeling van koortsende ziekten in China. Deze antimalaria middelen doden de malaria parasieten snel en effectief.

De eerste klinische studies naar artesimisine behandeling werden verricht met artemether, een vetoplosbaar artemisinine derivaat dat uitsluitend intramusculair toe te dienen is. Vanwege deze eenvoudige toedieningsvorm verdiende artemether de voorkeur van de Wereldgezondheidsorganisatie om verder ontwikkeld te worden. Grote vergelijkende studies tussen artemether en kinine voor de behandeling van ernstige malaria lieten echter geen significant verbeterde overleving zien bij patiënten behandeld met artemether. Achteraf bleek de initiële voorkeur voor artemether een ongelukkige keuze, toen werd aangetoond dat artemether niet altijd goed werd opgenomen in het bloed vanuit de intramusculaire injectieplaats, met name bij de meest ernstig zieke patiënten.

Artesunaat is een wateroplosbaar artesimisine en kan zowel intraveneus als intramusculair toegediend worden en heeft sterkere antimalaria eigenschappen dan artemether. Dit veelbelovende middel werd eerst getest in Azië, in een grote vergelijkende studie tussen artesunaat en kinine voor de behandeling van ernstige malaria bij voornamelijk volwassen patiënten. Deze zeer succesvolle studie (SEAQUAMAT) liet zien dat mortaliteit in de groep patiënten behandeld met artesunaat 35% lager was dan in de groep patiënten behandeld met kinine. Malaria experts waren echter van mening dat deze studie resultaten niet zondermeer naar Afrikaanse kinderen vertaald konden worden, omdat bij kinderen het ziektebeloop fulminanter is, waarbij het merendeel van de sterfte binnen de eerste 24 uur na ziekenhuisopname optreedt. Hierdoor zou zelfs een effectief middel onvoldoende werkingstijd zou kunnen hebben. Daarom werd besloten een vergelijkbare studie als de SEAQUAMAT studie op te zetten met het acroniem AQUAMAT (African Quinine Artesunate Malaria Trial); een grote multicenter vergelijkende studie tussen artesunaat en kinine voor de behandeling van ernstige malaria bij Afrikaanse kinderen.

AQUAMAT vormde de basis voor dit proefschrift. De studie werd uitgevoerd op 11 locaties in 9 Afrikaanse landen en includeerde 5425 patiënten. De deelnemende centra vertoonden verschillende malariatransmissie intensiteit, mate van co-morbiditeit (inclusief HIV/AIDS) en kwaliteit van zorg (variërend van academische ziekenhuizen tot rurale eenvoudige ziekenhuizen). De studie was de grootste klinische interventie studie op het gebied van ernstige malaria en bood daarmee een unieke gelegenheid om naast de behandeling ook onderzoek te doen naar de diagnose en prognose van ernstige malaria. Het belangrijkste resultaat van de AQUAMAT studie was dat artesunaat de sterfte in Afrikaanse kinderen met ernstige malaria tropica verminderde met 22,5%; de sterfte in de groep kinderen behandeld, met artesunaat was 8,5% en in de groep behandeld
met kinine was 10,9% (*Hoofdstuk 2*). Samen met de SEAQUAMAT studie en een aantal kleine eerdere studies, levert dit het definitieve bewijs dat artesunaat het beste antimalariamiddel is voor de behandeling van ernstige malaria, zowel bij kinderen als bij volwassenen. Artesunaat kan in belangrijke mate de ziekenhuissterfte van ernstige malaria reduceren. Het levensreddende effect van artesunaat bleek niet ten koste te gaan van een groter percentage overlevende kinderen met neurologische restschade. Bovendien was artesunaat veilig en gemakkelijk toe te dienen en deden zich geen ernstige bijwerkingen voor.

De sterfte ten gevolge van ernstige malaria tussen de verschillende studie locaties varieerde van 4 tot 15%. Deze variatie is mede afhankelijk van verschillen in klinische presentatie, wat mede bepaald wordt door de intensiteit van de malaria transmissie per gebied. Dit heeft te maken met de ontwikkeling van malaria-specifieke immuniteit, die geleidelijk opgebouwd kan worden naarmate een kind vaker geïnfecteerd wordt met *P. falciparum*. In gebieden met een hoge malaria transmissie intensiteit zullen kinderen sneller deze immuniteit opbouwen. In dergelijke gebieden komt ernstige malaria vooral voor bij hele jonge kinderen en manifesteert zich hoofdzakelijk met ernstige bloedarmoede. In gebieden met een lagere malaria transmissie of bij niet-immune individuen zal ernstige malaria zich hoofdzakelijk presenteren met cerebrale verschijnselen. Voor de definitie van ernstige malaria is het van belang om universele risicofactoren voor sterfte te identificeren. Voor de arts is het herkennen van deze risicofactoren van belang voor de behandeling van de individuele patiënt. Ook is dit relevant voor de inclusie van patiënten in klinische studies en voor studies naar de onderliggende mechanismen van een fataal beloop.

In onze multivariatie secundaire analyse van de AQUAMAT resultaten bleken cerebrale malaria, acidose, verhoogde ureum concentratie en tekenen van chronische ziekte onafhankelijke voorspellende factoren van sterfte aan ernstige malaria bij Afrikaanse kinderen (*Hoofdstuk 3*). De sterfte nam toe tot 23% in aanwezigheid van cerebrale malaria en acidose. Deze factoren werden reeds in eerdere studies geïdentificeerd als belangrijke risicofactoren en houden sterk verband met de onderliggende ziektemechanismen. De betekenis van een verhoogde ureumconcentratie bij kinderen is nog onbekend. Meestal is het een teken van een verminderde nierfunctie, maar dit werd bij kinderen met malaria zelden vermeld. HIV/AIDS is een van de belangrijkste oorzaken van chronische ziekte in Afrika. In Mozambique, een gebied met een hoge prevalentie van HIV/AIDS werd een substudie verricht naar de effecten van HIV coinfectie op de klinische presentatie en het beloop van ernstige malaria. We vonden dat ernstige malaria bij HIV-positive patiënten gepaard gaat met ernstiger ziekteverschijnselen, meer complicaties en een aanzienlijk
hogere sterfte; 26% in HIV-positieve kinderen versus 9% in HIV-negatieve kinderen (Hoofdstuk 7). In Muheza, Tanzania werd HIV coinfectie in verband gebracht met een verhoogd risico op een bacteriële infectie en sterfte. Het vaststellen van HIV-infectie is dus van belang bij de behandeling van ernstige malaria, waarbij aanvullend onderzoek naar bijkomende ziekten en behandeling, onder andere met antibiotica, geïndiceerd is.

Startpunt voor de behandeling van ernstige malaria is een snelle en accurate diagnose. De klinische diagnose dient bevestigd te worden met het aantonen van malaria parasieten in het bloed. De gouden standaard hiervoor is microscopisch onderzoek van de dikke druppel of bloeduitstrijk. Betrouwbaar microscopisch onderzoek vergt tijd en materialen en vereist expertise van de laborant. In de afgelopen jaren zijn verschillende soorten malaria-sneltesten op de markt gebracht die berusten op de detectie van malaria-antigenen. Deze sneltesten zijn voornamelijk ontwikkeld en geëvalueerd voor de diagnose van ongecompliceerde malaria. In Mozambique en Tanzania vergeleken we twee soorten sneltesten voor de diagnose van ernstige malaria. De sneltest berustend op detectie van het Plasmodium falciparum histidine-rich protein-2 (PfHRP2) had een hogere gevoeligheid voor het aantonen van malariaparasieten in het bloed dan de sneltest berustend op detectie van het malaria enzym Plasmodium lactaat dehydrogenase (pLDH). PfHRP2 sneltesten zijn gebruiksvriendelijk en een goed en betrouwbaar alternatief voor microscopisch onderzoek voor de diagnose van ernstige malaria (Hoofdstuk 4).

De diagnostiek van ernstige malaria wordt bemoeilijkt door een tweetal problemen. Het eerste probleem houdt verband met de neiging van geïnfecteerde rode bloedcellen om zich vast te hechten aan de wand van bloedvaten in de hersenen en andere organen. Deze vastgehechte rode bloedcellen verdwijnen daardoor uit het circulerende bloed en zijn niet langer detecteerbaar in het malaria preparaat voor microscopisch onderzoek. Beoordeling van de parasitemie middels microscopisch onderzoek kan dus leiden tot een onderschatting van het aantal malariaparasieten in het lichaam van de patiënt. Echter, naarmate er meer parasieten in het lichaam aanwezig zijn, zal de malaria infectie ernstiger verlopen. Een tweede probleem is de ontwikkeling van tolerantie voor de malariaparasiet bij mensen in malaria-endemische gebieden. Hierdoor kan een hoeveelheid malariaparasieten verdragen worden, zonder dat daarbij ziekteverschijnselen optreden (asymptomatisch dragerschap). Andersom, worden (zelfs ernstige) ziekteverschijnselen dus niet altijd verklaard door de aanwezigheid van malariaparasieten en kan er een andere ziekte in het spel zijn. Bovengenoemde problemen maken dat parasitemie, zoals gezien onder de microscoop, geen betrouwbare voorspeller is van de ernst van de malaria.
Het antigeen PfHRP2 wordt uitgescheiden door de malariaparasieten en blijft enige tijd vrij circuleren in het bloed. De concentratie van dit antigeen geeft een betere afspiegeling van het totaal aantal malariaparasieten in het lichaam, dan het aantal parasieten in een bloedpreparaat. Bij kinderen met verschijnselen van ernstige malaria en malaria parasieten in het bloed, bleek de PfHRP2 concentratie een betere voorspellende waarde te hebben voor sterfte dan parasitemie in het perifere bloed aangetoond middels microscopisch onderzoek (Hoofdstuk 5). Hierbij was een belangrijke observatie dat de sterftekans ook verhoogd was bij patiënten met hele lage PfHRP2 concentraties. We toonden aan dat dit meest waarschijnlijk patiënten zijn met een andere ziekte dan malaria, bij wie als bijkomende bevinding ook malariaparasieten in het perifere bloed aanwezig zijn, maar bij wie het totale aantal parasieten in het lichaam heel laag is. Om dit nader te onderzoeken werd een vervolgstudie gedaan, waarbij de PfHRP2 concentratie werd gemeten bij verschillende groepen; niet-zieke kinderen met aangetoonde malariaparasieten in het bloed (“asymptomatische dragers”), bij kinderen met koorts en ongecompliceerde malaria en bij kinderen met verschijnselen van ernstige malaria. De verdelingen van de PfHRP2 concentraties tussen deze groepen was duidelijk verschillend, terwijl de perifere bloed parasitemie veel meer overlap vertoonde (Hoofdstuk 6). Met behulp van mathematische modellen werd ingeschat dat bij een PfHRP2 concentratie onder de 200 ng/ml, meer dan 10% van de ernstige zieke kinderen geen malaria heeft. Bij PfHRP2 concentraties boven de 1000 ng/mL heeft slechts minder dan 1% geen malaria. Bepaling van de PfHRP2 concentratie in het plasma is dus een belangrijk hulpmiddel om onderscheid te maken tussen malaria en een ernstige koortsende ziekte met een alternatieve oorzaak.

Voor de behandeling van ernstige malaria is niet alleen de effectiviteit van het anti-malaria middel van belang, maar ook de optimale dosering, die kan verschillen tussen verschillende patiëntenpopulaties. Studies naar de geneesmiddel farmacokinetiek bestuderen wat er in de verschillende compartimenten van het lichaam met het medicijn gebeurt. Een voorbeeld hiervan waren de eerdergenoemde studies die uitwezen dat artemether niet goed werd opgenomen na injectie in de spieren. Leeftijd en ernst van de ziekte kunnen de farmacokinetiek in belangrijke mate beïnvloeden. Onderdosering kan fatale gevolgen hebben voor de patiënt, alsmede bijdragen aan de ontwikkeling van resistentie. In dit proefschrift werden in een substudie in Tanzania de populatie-farmacokinetische eigenschappen van intramusculair toegediende kinine en artesunaat bestudeerd. Voor kinine wordt al meer dan 10 jaar een oplaaddosering aanbevolen, echter deze wordt veelal niet toegepast vanwege ongegronde angst voor toxiciteit. We beschrijven snelle adequate, doch niet toxische, concentraties in het bloed na
toediening van een oplaaddosering kinine *(Hoofdstuk 8)*. Indien geen oplaaddosering, maar een normale dosering gegeven zou worden, dan zou het ongeveer 3 keer zo lang duren voordat therapeutische concentraties in het bloed bereikt worden. De huidige artesunaatdosering voor kinderen werd afgeleid van studies bij volwassenen, want er zijn geen eerdere populatie farmacokinetische studies verricht naar artesunaatbehandeling bij kinderen met ernstige malaria. We beschrijven dat het gewicht van de kind in belangrijke mate bepalend was voor de artesunaatconcentratie in het bloed *(Hoofdstuk 9)*. Bij kleine kinderen was de uitscheiding verhoogd, wat resulteerde in lagere artesunaat concentraties in het bloed. Aan de hand van ervaringen opgedaan bij de AQUAMAT studie, werd een praktische doseringstabel opgesteld, met licht verhoogde doseringen voor kleine kinderen.

**Tot slot**

De resultaten van dit onderzoek hebben in belangrijke mate bijgedragen aan de behandeling van ernstige malaria wereldwijd, waarbij artesunaat nu door de Wereldgezondheidsorganisatie wordt aanbevolen als eerste keus bij zowel kinderen als volwassenen, onafhankelijk van de endemische setting. Daarnaast heeft het onderzoek ook bijgedragen aan de diagnostiek van ernstige malaria in malaria-endemische gebieden, waarbij *Pf*HRP2 een belangrijk hulpmiddel bleek om onderscheid te kunnen maken tussen ernstige ziekteverschijnselen ten gevolge van een andere ziekte met parasitemie als toevalsbevinding, danwel ernstige malaria. Malaria-sneltesten vormen een betrouwbaar alternatief voor microscopisch onderzoek voor de diagnose van ernstige malaria. De ontwikkeling van semi-kwantitatieve sneltesten met geschikte grenswaardes voor *Pf*HRP2 zal de diagnostiek van malaria kunnen verbeteren. Ondanks artesunaatbehandeling blijft de sterfte hoog; 8,5%. Bijkomende bacteriële infectie zal mogelijk hieraan bijdragen, waarvoor antibiotische behandeling geïndiceerd is bij alle kinderen met ernstige malaria. Klinische studies naar het beste antibioticabeleid bij de behandeling van ernstige malaria verdienen prioriteit. Nader onderzoek naar de pathofysiologie van malaria is relevant in de zoektocht naar nieuwe aanknopingspunten voor de behandeling met adjuvante middelen met als doel de sterfte verder te verlagen. Met het oog op resistentieontwikkeling zijn aanvullende farmacologische studies van belang om de dosing van artesunaat te optimalizeren en dient de zoektocht naar nieuwe antimalaria middelen voorgezet te worden.
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Chapter 11

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Curriculum Vitae

Ilse Hendriksen was born in 1979 in Zevenaar, The Netherlands. During her study medicine at the Radboud University Nijmegen, she conducted various electives abroad and gained interest in paediatrics, infectious diseases and research and clinical work in resource-limited settings.

After completing her medical degree in 2004, she assisted in a study on cryptococcal meningitis in Thailand, which unfortunately did not lead to implementation. During this period, she met Prof. Arjen Dondorp and Prof. Nick White from the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, who inspired her to become trial physician on the AQUAMAT trial. Working on the largest severe malaria trial in Africa was an excellent opportunity to learn about clinical tropical medicine, paediatrics, international research and studying the diagnosis, pathophysiology and treatment of severe malaria for a PhD project.

In 2005, she initiated the AQUAMAT trial in “Hospital Central da Beira”, in the city of Beira, Mozambique, in collaboration with Dr. L. von Seidlein. She was responsible for clinical data collection, supervision and local trial management tasks and clinical care. She assisted in the set-up of various new study sites and was closely involved in the overall trial management and coordination. She moved to Tanzania in 2007, where she worked in “Teule Hospital” in Muheza, until the completion of the trial in 2010. This was a more established research site, including collaborations with various other research groups (Joint Malaria Program and The London School of Tropical Medicine and Hygiene, Dr R. Reyburn and Dr L. von Seidlein). The study site in Tanzania was located in an area with high malaria transmission and high patient burden, which facilitated data collection and additional studies for the thesis.

From October 2010 to April 2012, Ilse worked at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, where she conducted the PfHRP2 laboratory work, analysed the data and prepared the papers presented in this thesis. She intends to pursue a further career in paediatrics and has started her residency at the University Medical Centre in Groningen in May 2012.
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Diagnosis, prognosis and treatment of severe falciparum malaria in African children

Ilse C.E. Hendriksen