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

PLASMODIUM FALCIPARUM RESPONSE TO CHLOROQUINE AND ARTEMISININ BASED COMBINATION THERAPY (ACT) IN GUINEA-BISSAU

Johan Ursing

Karolinska Institutet

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ABSTRACT

Plasmodium falciparum causes the most severe form of malaria and is the most common malaria species in sub-Saharan Africa. Chloroquine used to be the most common drug for the treatment of malaria. Due to development of resistance, chloroquine is no longer efficacious in most of the world. The first line option for treatment of *P. falciparum* is now artemisinin based combination therapy, such as artemether-lumefantrine. In Guinea-Bissau, in contrast to the rest of Africa, chloroquine has been considered to be effective by practitioners in line with clinical trials by our group. Artemether-lumefantrine replaced chloroquine for the treatment of malaria in Guinea-Bissau in 2008.

The main aim of this thesis has been to study if and why chloroquine has remained efficacious in Guinea-Bissau.

We have shown that chloroquine resistance is associated with the CVIET haplotype at amino-acid positions 72-76 of the chloroquine resistance transporter (*pfcrt*). Genotyping and *in vitro* data presented in the thesis indicate that the proportion of resistant *P. falciparum* has been stable and low (~25%) since the early 90's. In line with that, *P. falciparum* with *pfcrt* 76T do not accumulate during the high transmission season, in Guinea-Bissau. Concurrently, approximately 3 times standard dose chloroquine has been routinely prescribed and taken without severe adverse events.

In a randomized clinical trial, we show that the PCR corrected day 28 and day 42 efficacies of double standard dose chloroquine were 95 and 94% respectively. The corresponding artemether-lumefantrine efficacies were 97%. Both antimalarial treatment options were well tolerated. The PCR corrected day 28 efficacies of double standard dose and standard dose chloroquine against *P. falciparum* with *pfcrt* 76T were 78-87% and 38%, respectively. Chloroquine concentrations were lower in children with PCR adjusted treatment failure compared to children with PCR adjusted adequate clinical and parasitological response by day 42. Recrudescent *P. falciparum* following treatment with artemether-lumefantrine or chloroquine appeared to carry opposite alleles at *pfcrt* codon 76 and multidrug resistance gene codon 86.

The dose and concentration dependent efficacy of chloroquine against *P. falciparum* with *pfcrt* 76T indicate that chloroquine resistance can be overcome by higher doses of chloroquine. High total chloroquine doses are well tolerated when split into repeated smaller daily doses. The lack of seasonal accumulation of *pfcrt* 76T indicates that the dosage schedule commonly used in Guinea-Bissau is efficacious and that *pfcrt* 76T is associated with a loss of fitness. Double standard dose chloroquine and artemether-lumefantrine fulfill the WHO efficacy requirements for antimalarials about to be adopted as treatment policy.

RESUMO

O *Plasmodium falciparum* causa a forma mais severa de malária sendo a variante humana mais comum na África subsahariana. O fármaco mais usado para o tratamento da malária costumava ser a cloroquina. Devido ao desenvolvimento de resistência, a cloroquina tornou-se ineficaz em todo mundo. A escolha da primeira linha para o tratamento do *P. falciparum* é actualmente a combinacão com derivativos da artemisinina, por exemplo lumefantrina-artemeter. Na Guiné-Bissau a cloroquina, em contraste com o resto de África, tem sido considerada eficaz por técnicos de saúde juntamente com os ensaios clínicos efectuados pelo nosso grupo. Lumefantrina-artemeter substituiu a cloroquina no tratamento da malária em 2008 na Guiné-Bissau.

O principal objectivo da presente tese é estudar se e porquê, a cloroquina se manteve eficaz na Guiné-Bissau.

Nós demonstramos que a resistência à cloroquina está associada com o haplotipo CVIET dos amino-àcidos nas posicões 72-76 no transportador de resistencia à cloroquina (*pfcrt*). Genotipagem e dados *in vitro* presentes nesta tese indicam que a percentagem de *P. falciparum* resistente à cloroquina mantive-se baixa e estável (~25%) desde os anos 90. Paralelamente, na Guiné-Bissau, o alelo *pfcrt*76T (marcador de resistência) não se acumula durante a época de alta transmissão. Actualmente, a dose receitada de cloroquina é aproximadamente 3 vezes a recomendada e é tolerada sem efeitos secondários.

Através de ensaio clinico aleatório mostramos que a eficâcia, ajustada por PCR para os dias 28 e 42, da dose dupla de cloroquina foram respectivamente, 95 e 94%. A eficácia correspondente de lumefantrina-artemeter foi 97%. Ambas as opcões de tratamento foram bem toleradas. A eficácia ajustada por PCR da dose dupla de cloroquina e a dose recomendada para *P. falciparum* contendo o alelo 76T foram 78-87% e 38%, respectivamente. Para o dia 42, as concentracões de cloroquina foram mais baixas em criancas cujo tratamento falhou quando comparado com as criancas que obtiveram tratamento com resposta parasitologica adequada (ajustado com PCR). As recrudescências em ambos os tratamentos (cloroquina e lumefantrina-artemeter) mostraram a presenca de alelos opostos na posicão 76 do *pfcrt* e na posicao 86 do *pfmdr1* (transportador de resistência múltipla).

Devido à dose e à concentracão, a eficácia da cloroquina contra *P. Falciparum,* contendo o alelo 76T, indicam que a resistência pode ser abolida com o uso de doses aumentadas. As doses elevadas são bem toleradas quando repartidas em pequenas subdoses diárias. A não acumulacao periódica do alelo 76T indica que a dosagem usada na Guiné-Bissau é eficaz e que o alelo 76T no gene *pfcrt* está associado com perda de fitness. Tanto a cloroquina em dose dupla como a Lumefantrina-artemeter preenchem os requerimentos da OMS para serem usadas como planos de tratamento.

LIST OF PUBLICATIONS

I. **Ursing J, Kofoed PE, Rodrigues A, Rombo L, Gil JP.** *Plasmodium falciparum* genotypes associated with chloroquine and amodiaquine resistance in Guinea-Bissau. Am J Trop Med Hyg. 2007 May;76(5):844-8.

II. **Ursing J, Schmidt BA, Lebbad M, Kofoed PE, Dias F, Gil JP, Rombo L.** Chloroquine resistant *P. falciparum* prevalence is low and unchanged between 1990 and 2005 in Guinea-Bissau: an effect of high chloroquine dosage? Infect Genet Evol. 2007 Sep;7(5):555-61. Epub 2007 Mar 31.

III. **Ursing J, Kofoed PE, Rombo L, Gil JP.** No *pfmdr1* amplifications in samples from Guinea-Bissau and Liberia collected between 1981 and 2004. J Infect Dis. 2006 Sep 1;194(5):716-8; author reply 718-9. No abstract available. Erratum in: J Infect Dis. 2006 Oct 15;194(8):1190.

IV. **Ursing J, Kofoed PE, Rodrigues A, Bergqvist Y, Rombo L.** Chloroquine is grossly overdosed and overused but well tolerated in Guinea-Bissau. Antimicrob Agents Chemother. 2009 Jan;53(1):180-5. Epub 2008 Oct 27.

V. **Ursing J, Kofoed PE, Rodrigues A, Rombo L.** No seasonal accumulation of resistant *P. falciparum* when high-dose chloroquine is used. PLoS One. 2009 Aug 31;4(8):e6866.

VI. **Ursing J, Kofoed PE, Rodrigues A, Blessborn D, Thoft-Nielsen R, Björkman A, Rombo L**. Double dose chloroquine fulfils WHO efficacy requirements in Guinea-Bissau despite continued and extensive use of chloroquine: A randomised comparative trial with artemether-lumefantrine Manuscript

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LIST OF ABBREVIATIONS

ABC	Adenosine tri-phosphate binding cassette
ACT	Artemisinin based combination therapy
ACPR	Adequate clinical and parasitological response
AUC	Area under the curve
CI	Confidence interval
Ct	Cycle threshold
EC	Effective concentration
ETF	Early treatment failure
IC	Inhibitory concentration
IQR	Inter quartile range
LCF	Late clinical failure
LNSP	Laboratório Nacional da Saúde Publica
LPF	Late parasitological failure
LTF	Late treatment failure
Mg	Milligram
PfATP 6	*Plasmodium falciparum* adenosine triphosphatase 6 gene
PfATP 6	*Plasmodium falciparum* adenosine triphosphatase 6 protein
PCR	Polymerase chain reaction
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
PfCRT	*Plasmodium falciparum* chloroquine resistance protein
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase gene
Pfdhps	*Plasmodium falciparum* dihydropteroate synthetase gene
Pgh1	P glycoprotein homologue 1
Pfglurp	*Plasmodium falciparum* glutamate rich protein gene
Pfmdr 1	*Plasmodium falciparum* multidrug resistance gene 1
Pfmrp 1	*Plasmodium falciparum* multidrug resistance protein gene 1
PfMRP 1	*Plasmodium falciparum* multidrug resistance protein
Pfmsp 1	*Plasmodium falciparum* merozoite surface protein 1 gene
Pfmsp 2	*Plasmodium falciparum* merozoite surface protein 2 gene
RFLP	Restriction fragment length plolymorphism
SNP	Single nucleotide polymorphism
WHO	World Health Organization

1 BACKGROUND

1.1 HISTORY

Knowledge of malaria dates back thousands of years. The chinese *Nei Ching* (The Canon of Medicine), dated 4700 years ago, refers to epidemically occurring repeated paroxysmal fevers associated with enlarged spleens, suggesting malaria. Hippocrates, described the intermittent fever and spleen enlargement of malaria and related the fever to the time of the year and to where the patients lived. But it was not until 1880 that Dr Laveran discovered the parasite he named *Oscillaria malariae* in fresh blood samples taken from soldiers suffering from malaria in Algeria. Following this discovery Dr. Patrick Manson had the idea that malaria was somehow transmitted by mosquitoes. In 1898, after several years of painstaking work, Dr Ronald Ross identified the parasite in the salivary glands of mosquitoes and proved its infectious nature by demonstrating that birds bitten by mosquitoes developed malaria.

The parasite identified by Laveran has since been classified as a protozoan. The phylum protozoa consists of morphologically similar organisms that are generally unicellular and free living. Plasmodia is the genus of protozoa causing malaria [1]. There are more than 120 species of which *falciparum, vivax, ovale* and *malariae,* are traditionally considered to commonly cause human infection but recently *P. knowlesi* has also been shown to be of regional importance as a human pathogen [2].

1.2 LIFE CYCLE

Plasmodia have an intricate life-cycle involving asexual replication in humans and sexual replication in its definite host - mosquitoes belonging to the genus Anopheles. There are more than 430 species of Anopheles but only 70 transmit malaria and only 40 are of major importance [3]. From a human point of view, the infection starts when a female mosquito injects approximately 20 sporozoites into the bloodstream whilst taking a blood meal. Sporozoites invade liver cells initiating the liver stage. *P. falciparum* and *P. malariae* usually complete their liver stage within 4 weeks whilst *P. vivax* and *P. ovale* can either develop promptly or remain hidden away in the liver cells as hypnozoites and develop into merozoites months or even years later. Each sporozoite develops into 10 000-30 000 merozoites that are released into the bloodstream when the liver cell ruptures. Merozoites invade erythrocytes where they develop from ringforms to trophozoites and finally schizonts that rupture releasing 24-32 new merozoites. This process takes 72 hours for *P. malariae*, 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* and 24 hours for *P. knowlesi* [2]. Each merozoite can invade a new erythrocyte starting a new cycle of development but a minority will eventually develop into sexual forms (gametocytes) that can be ingested by a mosquito taking a blood meal. In the mosquito, gametocytes will finally develop into sporozoites in the salivary glands ready to be injected.

1.3 EPIDEMIOLOGY

Malaria transmission requires a reservoir of infected humans and a suitable Anopheline mosquito. In most tropical regions of Africa, Southeast Asia and Latin America both exist and malaria transmission occurs. *P. falciparum* and *P. malariae* are found worldwide. *P. vivax* is less common in Sub-Saharan Africa but has the widest geographical range. *P. ovale* on the other hand, exists through out Africa but only in foci in Asia and Oceania.

The epidemiology is constantly changing depending on rainfall, movement of people, changes in habitat and measures taken to counter the malaria threat such as the malaria eradication campaign in Africa ca. 1955-1969. A greater than normal rainfall can increase the number of mosquitoes and malaria transmission. People without malaria may be forced to move to an area with malaria and become exposed. Alternatively people with malaria may move to an area without malaria but with a suitable vector thereby introducing malaria.

According to WHO definitions 2.2 billion people live in areas where there is low risk of malaria and 1.1 billion live in areas where there is a high risk of malaria (>1 case per 1000 people per year). In 2006, there were an estimated 247 million malaria cases worldwide. Ninety-one percent or 230 million were due to *P. falciparum* and the vast majority of cases (86%) were in Africa. Of the estimated 881 000 deaths worldwide, 90% occurred in Africa and 88% of the deaths in Africa occurred in children under 5 years of age [4].

1.4 IMMUNITY

Malaria transmission can either be unstable in which case epidemics occur or stable in which case malaria transmission is endemic, occurring more or less continuously. In large parts of Africa, *P. falciparum* is endemic and individuals are repeatedly infected leading to acquisition of immunity. Immunity increases with age, number of malaria episodes and time spent living in an endemic area [5, 6]. Immunity is not sterilising and even in areas with very high rates of transmission, individuals are commonly infected though asymptomatic and usually with fewer parasites as they get older [7]. Maternal antibodies partially protect infants from *P. falciparum* during the first 6 months of life but children then commonly have multiple infections annually. In high transmission areas, children thus gradually acquire immunity and have fewer clinical episodes as they get older [7]. Primigravida are also particularly susceptible to *P. falciparum* infection, probably because *P. falciparum* then express different antigens enabling them to bind to the placenta [8].

1.5 SYMPTOMS

Malaria usually presents as an acute febrile illness with chills, rigors and profuse sweating. Other common symptoms include headache, myalgia, arthralgia, stomach pain, vomiting, pallor and generalised weakness [6]. The acute attack may last several hours occurring irregularly or in a regular pattern coinciding with schizont rupture. Common signs include an enlarged spleen and signs of anaemia. The severity of a clinical attack ranges from a malignant disease causing death in a few days to a mild febrile illness depending upon Plasmodia species, age, immunity, general health and nutritional status.

Because of the variable clinical presentations there are no clinical criteria that can identify malaria with both high sensitivity and high specificity [9]. A study evaluating an algorithm using signs and symptoms found that 16% of children<5 years and 44% of children 6-14 years old who had a history of fever and >5000 parasites per microlitre would have been sent home without treatment [10].

In clinical practice, malaria is considered to be severe or uncomplicated. According to WHO criteria, an individual with *P. falciparum* malaria and one of the following features; prostration, impaired consciousness, multiple convulsions, haemoglobin <50g/L, hyperparasitaemia, hypoglycaemia, respiratory distress, pulmonary oedema, circulatory collapse, abnormal bleeding, jaundice, haemoglobinuria, hyperlactataemia or acidosis is considered to have severe disease [11].

1.6 GUINEA-BISSAU

Guinea-Bissau is a former Portuguese colony in West Africa, sandwiched between Senegal to the north and the republic of Guinea to the south and east. Guinea-Bissau was torn apart by a war of independence between 1963 and 1974 and again in a civil war between 1998 and 1999. There have been several elections but also several coups since independence. The last election in 2008 was deemed calm and organised but even so, the president and chief of the armed forces were assassinated in March 2009. There are more than 30 ethnic-linguistic groups and the common language is Guinean Creole, a mixture of local languages and Portuguese. The estimated population in 2009 is approximately 1.8 million, of which approximately 400 000 live in Bissau [12]. The median age is 16 years and 48% of the population is below 15 years of age.

Guinea-Bissau is one of the poorest countries in the world with a per capita Gross Domestic Product of 237 US$ [12]. There were 188 physicians and 1072 nurses or midwifes in the country in 2005. Total per capita health expenditure is 13 US$ of which 68% is private out of pocket expenditure. The remaining 32% is government financed which mainly comes from international donors. The life expectancy is 46 and 51 years for men and women respectively. The under 5 mortality rate is 200/1000 and the malaria specific under 5 mortality rate is 42/1000 [13].

The national policy for the treatment of uncomplicated malaria was 25 mg/kg of chloroquine until June 2008 when artemether-lumefantrine started replacing chloroquine.

1.7 PROJECTO DE SAÚDE DE BANDIM

The Bandim health project, Projecto de Saúde de Bandim (PSB), performes community based studies within the demographic surveillance site that includes 102 000 inhabitants. PSB includes several neighbourhoods (Bairros) within which all houses are numbered. Housing consists of clay huts, mud brick or sometimes concrete brick houses. Irrespective of type, houses generally do not have internal ceilings and mosquitoes are free to pass in to a house through a gap between the roof and the wall. Houses are generally crowded and without water or sewage systems. As part of PSB's surveillance system, data on births, deaths and migration are routinely registered. Approximately one third of women have been to school for more than 3 years.

Mirroring the situation in Bissau as a whole, many ethnic groups are represented in the study area, the main being Pepel, Balante, Manjaco, Mancanha and Fula. The population residing within PSB demographic surveillance site is very mobile. People often travel to their home village and members of the extended family often travel to Bissau for shorter or longer periods. Of particular note is the period March to June when cashew nuts are harvested and women go to the countryside with their youngest children.

There are 3 health centres, centro de saúde de Bandim, and the smaller centro de saúde de Belém and centro de saúde Cuntum. Bandim and Belém are staffed by both doctors and nurses whilst only nurses work in Cuntum. None of the centres have running water or electricity but all have a small laboratory equipped with a light microscope (Optimus™) enabling examination of blood, sputum and faeces. Hematocrit is also routinely determined. For the purpose of studies, laboratories can be equipped with Haemocue™ (Ängelholm, Sweden) for measurement of haemoglobin concentration. The staff and quality of services of the laboratories are supervised by the national health laboratory Laboratório Nacional da Saúde Publica (LNSP).

The population also has access to the National Hospital, Hospital Nacional Simão Mendes. It is located approximately 5 km from PSB and has a paediatric out patient department and ward. In addition there are numerous pharmacies and also private practitioners.

The Laboratório Nacional da Saúde Publica is located in Bissau and houses several laboratories. It has running water and a generator to supply electricity. One of the laboratories is designated for malaria work and is equipped with an incubator and several microscopes.

1.8 MALARIA ENDEMICITY IN GUINEA-BISSAU

Anopheles Gambiae s.s. and *Anopheles melas* are the most frequent malaria vectors in Guinea-Bissau. Malaria is generally considered to be meso or hyperendemic [14, 15]. In 1990, during the rainy season, 44-79% of children aged 2-9 years in six rural villages in northern Guinea-Bissau had *P. falciparum* in the blood. The mean number of infective bites during the rainy season was then estimated to be 4 per child and 20 per adult [15]. A survey conducted in Prabis, west of Bissau, in 1991 found that *P. falciparum* was meso, hyper or holoendemic amongst 2-9 year olds in 49 out of 50 villages [16]. During the rainy seasons 1995 and 1996, 52/112 (45%) and 42/71 (59%) of asymptomatic individuals screened had *P. falciparum* in the blood in a northern suburb of Bissau [17, 18]. In Bandim, 152/594 (26%) children with a median age of 19 months were parasitaemic in a study conducted in 1993 and 1994 [19]. In 2003, a survey conducted within PSB found that only 28/888 (3%) children <15 years were parasitaemic indicating a considerably decreased endemicity.

1.9 ANTIMALARIAL DRUGS USED IN THIS THESIS

1.9.1 Chloroquine

Pharmacokinetics

Chloroquine is a 4-aminoquinoline that is rapidly and almost completely absorbed. Peak concentrations occur 1-3 hours after oral intake and 50-65% is protein bound in plasma [20]. Following injection of 3 mg/kg over 10 minutes, peak plasma chloroquine concentrations were 9118 (range 2454-20811) nmol/L in one study [21]. The plasma concentrations rapidly decreased (T1/2 ~1.1 minutes) corresponding to a rapid distribution phase [21]. Peak plasma concentrations were approximately 10 times lower following oral intake compared to intravenous injections [22]. The whole blood concentrations are approximately 10 times higher than plasma concentrations after intake of a single oral dose [23].

Following absorption chloroquine is distributed throughout the body accumulating in tissues, especially the liver, lungs, spleen and kidneys [24]. The volume of distribution of chloroquine is very large (>100L/kg) [25]. Chloroquine has complex multi-compartment pharmacokinetics making it difficult to estimate an accurate terminal half-life. Chloroquine is mainly metabolised to desethyl-chloroquine.

Mechanism of action

Chloroquine is a weak base that moves rapidly across cell membranes. It accumulates in the digestive vacuole by binding to haem and by becoming protonated [26, 27]. *P. falciparum* degrades haemoglobin in the digestive vacuole, producing inert haemozoin [28]. Chloroquine interferes with haemozoin production by forming cytotoxic complexes with haem that kill the parasite [29]. Chloroquine concentrations are four to ten times lower in chloroquine resistant parasites compared to chloroquine sensitive parasites. The lower chloroquine concentration in the digestive vacuole decreases the access of chloroquine to haem which probably constitutes the basis of chloroquine resistance [30]. The differences in chloroquine accumulation, and hence chloroquine resistance, are probably energy and proton dependent, as differences disappear in the absence of glucose or when the proton gradient is dissipated [31, 32].

Adverse events

Chloroquine is generally well tolerated, though it is well known to cause pruritus, particularly in dark-skinned people. More unusual adverse events include headache, skin eruptions and gastro-intestinal and visual disturbances [33]. Rapid intravenous injection of chloroquine causes decreased systolic blood pressure that resolve spontaneously and rapidly [22, 34]. Slow infusion of chloroquine is without adverse events [35]. In one study, subjective side effects disappeared when plasma chloroquine concentrations were less than 454-1158 nmol/L [22]. Transient toxic levels may occur following intravenous administration or oral over-dosing as the volume of distribution of the central compartment is relatively small [21, 36, 37]. Chloroquine can then be highly toxic causing death soon after ingestion [38, 39]. The use of multiple smaller doses avoids toxic concentrations in the central compartment and thereby toxicity [36, 40].

Dosing

The standard dose of chloroquine recommended by the WHO for the treatment of uncomplicated malaria is 10 mg/kg on days 0 and 1 and 5 mg/kg on day 2 (total 25 mg/kg). Other dosage regimes have been assessed in small studies including 30 mg/kg given as 10+5+5 mg/kg on day 0 followed by 5 mg/kg twice on day 1. This regime resulted in higher chloroquine concentrations in the blood at 12, 24 and 48 hours and a more rapid decrease of parasite density without an increase of adverse events compared to the WHO dose [41]. In Burundi, the efficacy of 10 mg/kg as a single dose, 25 mg/kg given according to WHO recommendations, 35 mg/kg given as 10 mg/kg on days 0 and 1 followed by 5 mg/kg for 3 days, 40 and 50 mg/kg given as daily doses of 10 mg/kg for 4 or 5 days were assessed in 1985. There was a dose dependent delay before parasites reappeared but the efficacy was not obviously different in this study that was not PCR adjusted and had a small number of participants [42]. In Rwanda 50 mg/kg given as 10 mg/kg per day for 5 days did not improve treatment outcome compared to standard dose in a non PCR adjusted study with a 14 day follow up [43].

1.9.2 Amodiaquine

Pharmacokinetics

Following oral intake, amodiaquine is readily absorbed with peak concentrations reached in 0.5-2.3 hours. The apparent terminal half-life is approximately 5 hours. It is rapidly converted in the liver to the active metabolite desethyl-amodiaquine that reaches peak concentrations in less than 4 hours and has an estimated terminal half life of 6-18 days [25, 44-47]. Due to the longer half-life, it is likely that desethyl-amodiaquine contributes nearly all of the antimalarial effect [48]. Amodiaquine and desethyl-amodiaquine are 90-95% protein bound [49].

Mechanism of action

Amodiaquine competitively inhibits chloroquine accumulation suggesting that amodiaquine has a similar mechanism of action [50].

Adverse events and dosing

The adverse effects of amodiaquine are similar to those of chloroquine [46, 47]. In addition, amodiaquine is associated with a risk of agranulocytosis and, to a lesser degree, of hepatitis when used for prophylaxis (which is no longer recommended) [51, 52]. Even when small doses or treatment doses have been used neutropenia and hepatitis have been reported [47, 53, 54]. Large doses of amodiaquine have been reported to cause syncope, spasticity, convulsions and involuntary movements. Doses from 10-30 mg/kg have been used but the standard dose now employed is 10 mg/kg per day for 3 days.

1.9.3 Lumefantrine

Pharmacokinetics and mechanism of action

Lumefantrine is only used in combination with artemether. Following oral intake, peak concentrations are reached in 10 hours. The elimination half life is 3-5 days [55]. Approximately 8% of lumefantrine is found in erythrocytes and 99.7% of lumefantrine in plasma is protein bound [56]. Lumefantrine is highly lipophilic and the absorption is dependent on concurrent intake of food containing fat. Consequently, the bioavailablity of lumefantrine is 3 times higher by the 3rd and 4th dose compared to the 1st dose as patients appetites improve when they recover [55, 57]. The population mean estimated volume of soya milk required to obtain 90% of maximum effect (in terms of lumefantrine AUC) was 36 ml (corresponding to 1.2 g of fat) [58].

Lumefantrine is structurally similar to quinine, mefloquine and halofantrine and is believed to inhibit haem polymerisation [59].

1.9.4 Artemether

Pharmacokinetics

Artemether is a semisynthetic compound derived from artemisinin, a naturally occurring compound derived from the herb *Artemisia Annua* [60]. It is rapidly absorbed and converted to the active metabolite dihydroartemisinin. Artemether and dihydroartemisinin have similar parasiticidal effect *in vivo* [59]. Peak concentrations of both compounds are reached after 1.8 and 1.2 hours. Terminal elimination half-lives were 0.84 and 0.43 hours [57]. Despite short half-lives both were detected in plasma 8 hours after dosing due to slower absorption than elimination. 11% of artemether is found in erythrocytes and 95.4% of artemether in serum is protein bound [56].

Mechanism of action

Artemisinins contain an endoperoxide bridge believed to be crucial for anti-malarial activity. PfATP6 is a sarcoendoplasmic reticulum Ca^{2+} adenosine triphosphatase, crucial in the control of cellular calcium signalling. Inhibition of the PfATP6 is currently the most probable target of artemisinins [61, 62] though others have been suggested [63, 64]. Supporting this, dihydroartemisinin has been shown to bind to and inhibit PfATP6 [61].

Adverse events

Artemether has been linked to neurotoxicity when high doses were used in experimental animals [65] but when therapeutic doses are used in humans, artemether is very well tolerated [59, 66].

Dosing

Artemether and artesunate are both metabolised to dihydroartemisinin. They have similar efficacy when given for 7 days [66]. The efficacy of artemisinin monotherapy is dependent upon treatment duration. 600 mg of artesunate given as one or two daily doses for 5 days has lower efficacy than a single daily dose for 7 days [67, 68]. When given for 7 days, PCR corrected ACPRs in the central African republic were 95% day 28 and 85% day 42 [69].

1.9.5 Artemether-Lumefantrine

Artemether-lumefantrine is very well tolerated [70-72]. It is co-formulated and each tablet contains 20 mg of artemether and 120 mg of lumefantrine. Each dose consists of 1-4 tablets according to weight and the recommended schedule is two doses per day for 3 days. The lumefantrine area under the curve (AUC) appears to be the principal determinant of cure and a lumefantrine concentration of 280 µg/L, 7 days after the start of treatment, can be used as a cut off value for increased risk of treatment failure [57]. Various dosing schedules have been tried. Six doses given over 3 or 5 days resulted in 60% and 100% greater AUC (area under the curve) respectively compared to 4 doses over 3 days [55]. This in turn results in 204, 252 and 298 hours over the putative lumefantrine minimal inhibitory concentration of 280 µg/L. One daily dose for 3 days resulted in poorer efficacy and an AUC that was 30% lower compared to two daily doses for 3 days [73]. In line with this, a study in Thailand showed that the efficacy of 4 doses over 3 days was 83.3% whilst 6 doses over 3 days and 5 days had efficacies of 96.9% and 99.1% respectively [74].

1.10 DRUG RESISTANCE

P. falciparum evolves resistance to antimalarial drugs in two principal ways. It can alter the target of the antimalarial. For example, sulphadoxine-pyrimethamine is a commonly used antimalarial that disrupts folate synthesis by inhibiting dihydrofolate reductase (DHFR) [75] and dihydropteroate synthase (DHPS) [76, 77]. Single nucleotide polymophisms (SNPs) in the *pfdhfr* and *pfdhps* genes results in enzymes that are less susceptible to the inhibitory effects of sulphadoxine and pyrimethamine, respectively.

Alternatively *P. falciparum* can reduce the amount of drug where the drug exerts its effect. Chloroquine resistance is the most studied mechanism of this type. As mentioned previously, chloroquine accumulates in the digestive vacuole of *P. falciparum* where it disrupts haemoglobin degradation. Chloroquine concentrations are lower in resistant parasites constituting the basis of resistance [30]. The lower concentration is mediated by the chloroquine resistance transporter (PfCRT) that is localized to the parasite's food vacuole and belongs to the drug/metabolite transporter superfamily [78]. PfCRT is most probably a saturable transporter protein [32, 79] that actively transports chloroquine out of the digestive vacuole though others argue that it is a channel [31, 80].

Other putative transporters that have been associated with drug resistance are P-glycoprotein homologue 1 (Pgh1) [81] encoded by *pfmdr1* (multidrug resistance gene 1) and *P. falciparum* multidrug resistance protein (PfMRP1) [82]. They both belong to the adenosine triphosphate binding cassette (ABC) proteins. These are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out biological processes including translocation of various substrates across membranes. Another nine putative ABC transporters, some of which may be involved in drug resistance, are encoded by *P. falciparum* [83].

1.10.1 Resistance associated genotypes

Chloroquine resistance transporter gene (*pfcrt*)

The *pfcrt* K76T amino acid substitution has been shown to be essential for chloroquine resistance, associated with amodiaquine resistance and predictive of treatment failure for both drugs [84-90]. Despite that, high levels of chloroquine resistance have been reported from Madagascar where the *pfcrt* 76T prevalence is very low [91]. *In vitro* tests have also suggested that resistant parasites sometimes lack 76T [92]. The T152A and S163R SNPs have been found in parasites that have lost their chloroquine resistance phenotype despite retaining the 76T allele [93].

Lumefantrine tolerance/resistance and higher IC50 values have been associated with *pfcrt* 76K [94, 95].

Multi drug resistance gene (*pfmdr 1*)

Pfmdr 1 was discovered and associated with drug resistance in 1989 [81, 96, 97]. Polymorphisms occur at five amino acid positions, 86, 184, 1034, 1042, and 1246 [98]. *Pfmdr 1* 86Y has been associated with chloroquine and amodiaquine resistance and shown to increase chloroquine IC50 values in *P. falciparum* with *pfcrt* 76T [99]. In a recent meta analysis it was significantly linked to chloroquine and amodiaquine resistance [100]. Despite that, it has not been linked with chloroquine resistance in several studies [101, 102] and appears to be under less selective pressure by chloroquine compared to *pfcrt* [103].

Pfmdr 1 86N has been associated with lumefantrine tolerance/resistance and higher lumefantrine IC50 [95, 104]. *Pfmdr 1* amplifications have been associated with mefloquine resistance, doubled lumefantrine IC50 and reduced sensitivity to artesunate [105].

Multi drug resistance protein gene (*pfmrp 1*)

In addition to *pfcrt* and *pfmdr1,* other genes have been proposed to modulate chloroquine resistance [106]. *Pfmrp1* Y191H and A437S have been linked *in vivo* [107] and have been associated with chloroquine and quinine resistance *in vitro* [82] but this finding has not been confirmed [108]. The allele 876I has been associated with re-infections following treatment with artemether-lumefantrine [109]. Disrupting PfMRP resulted in *P. falciparum* that were more sensitive to multiple antimalarial drugs, including chloroquine, quinine, and artemisinin [110].

Adenosine triphosphatase gene (*pfATP 6*)

L263E has been shown to decrease the affinity of PfATP 6 for artemisinin [111] and S769N has been associated with decreased artemether IC50 in parasites from French Guiana [112]. However, no *pfATP 6* SNPs have been linked with artemisinin resistance in Southeast Asia [113, 114].

1.10.2 Development and spread of drug resistance

As described above, drug resistance is the result of genetic mutations. One line of reasoning considers resistance to be a single stage process with parasites being either fully sensitive or completely resistant. An example would be resistance to atovaquone that appears to develop following a single mutation [115]. Another line of reasoning considers resistance to be a gradual process resulting from a series of mutations that gradually increase IC50 values until *P. falciparum* can survive drug concentrations achieved during treatment. Intermediate stages are able to survive sub-therapeutic drug concentrations and thus have a selective advantage during re-infections soon after treatment. These *P. falciparum* are considered tolerant and will replace completely sensitive *P. falciparum* under continued drug pressure [116]. An example would be the gradually increasing pyrimethamine IC50 of *P. falciparum* with 1, 2, 3 or 4 SNPs in *pfdhfr* [117, 118]. A drug with a long half-life increases the risk of tolerant parasites developing whilst the very short half-life of artemisinins decreases the risk of tolerant parasites developing [119].

Chloroquine started being used in 1946 but it was not until 1957 that resistance was first detected suggesting that it was a complex probably multigenic process [120]. Supporting this, resistance to standard dose chloroquine (25 mg/kg) has only developed a limited number of times and possibly not at all in Africa [92, 118, 121, 122]. In Africa, chloroquine resistance is primarily associated with the *pfcrt* 72-76 haplotype CVIET although other haplotypes have been described recently [123, 124]. This haplotype arose in Southeast Asia and was probably introduced to East Africa in 1978 [125] from where it spread over the continent during the 1980's [126-128]. Chloroquine resistance was first reported in Guinea-Bissau in 1991 [129] but studies conducted 1995-2004 indicate a stable prevalence of chloroquine resistance [130-132].

1.10.3 Cost of resistance/fitness

The proportion of *pfcrt* 76T genotypes increased during the high transmission seasons in both The Gambia and Sudan [133, 134]. In the absence of a drug selective pressure, such as during the dry season or the situation in Malawi following the ban on use of chloroquine, the *pfcrt* 76T proportion decreased [135, 136]. This suggests that chloroquine resistance is achieved at such a cost to fitness that natural selection acts against the resistant parasite in the absence of drug [137, 138]. *Pfmdr1* amplifications [139] and chloroquine resistance associated mutations in *pfmdr1* have also been associated with loss of fitness [140]. Modelling has suggested that loss of fitness can delay the spread of resistance as has been the case in Sudan [134, 141]. Two main factors govern the proportions of mutations responsible for chloroquine resistance. Their ability to survive in treated patients is the main force driving their expansion whilst natural selection due to loss of fitness acts against them in untreated infections [138].

1.10.4 Pharmacological means of delaying drug resistance

The risk of a resistant mutation arising to a drug is mainly dependent upon the rate at which mutations spontaneously arise and the number of parasites exposed to drug. If two drugs with independent modes of action are used simultaneously, the risk that a parasite will simultaneously develop resistance to both, is the product of the risk of resistance developing to the individual drugs [142]. The WHO therefore recommends artemisinin based combinations (ACT) [11] and artemether-lumefantrine or artesunate-amodiaquine have been adopted by most African countries [143]. The combination of artemether with a short half-life and lumefantrine with a longer half-life ensures that no parasites are exposed to artemether alone reducing the risk of resistance developing [144]. Artemether rapidly reduces the amount of parasites, including those that might have developed tolerance or resistance to lumefantrine thus probably delaying the onset of lumefantrine resistance.

Modelling has suggested that concurrent use of multiple first line treatments will prolong the useful life span of all the drugs used. If several first line treatments are in use concurrently, the amount of each drug used during a certain time period is reduced compared to if only one treatment/drug was being used. This reduces drug pressure of individual drugs and therefore reduces the risk of resistance developing. Furthermore, the selective advantage of resistance to one drug is decreased because of the concurrent use of other drugs [145].

2 AIMS OF THE THESIS

2.1 GENERAL AIMS

The overall aim has been to determine why chloroquine has remained efficacious and to assess the efficacy of AL in Guinea-Bissau

2.2 SPECIFIC AIMS

1. To determine which genotypes and *pfcrt* 72-76 haplotype are associated with chloroquine resistant *P. falciparum* in Guinea-Bissau.

2. To determine the level of chloroquine resistance since the early 90`s when chloroquine resistant *P. falciparum* were first detected.

3. To determine how much chloroquine is prescribed and taken in routine practice in Guinea-Bissau and if there are adverse events associated with intake of high total doses split into multiple smaller doses.

4. To determine if there was seasonal accumulation of *pfcrt* 76T in Guinea-Bissau.

5. To determine the efficacy of higher chloroquine concentrations against *P. falciparum* with *pfcrt* 76T

6. To assess and compare the efficacies and tolerability of artemether-lumefantrine and double standard dose chloroquine in Guinea-Bissau.

7. To determine the prevalence and selection of genotypes associated with artemether-lumefantrine resistance.

3 METHODS

3.1 STUDY LOCATION AND POPULATION

Patients recruited to studies I, III, IV, V and VI resided within the Projecto de Saúde de Bandim demographic surveillance site. Children in study II came from the suburban village of Placc or the rural villages of Nhacra or Pefine. The clinical work, that study I is based on, was conducted at Bandim Health Centre. Study VI was conducted at all 3 health centres (Bandim, Belém and Cuntum) serving the population within PSB. *In vitro* work was conducted at Laboratório Nacional da Saúde Publica. Molecular work was done at Karolinska Institutet, and drug analyses were done at Dalarna University College.

3.2 GENOTYPING

3.2.1 Sampling and DNA extraction

Whenever blood was collected for genotyping, approximately 50-100 μL of capillary blood was put onto a Whatman 3MM filter-paper, dried and then put into a sealable plastic bag for storage. Twenty five to fifty μL of blood was cut from the filter-paper into smaller bits, from which DNA was extracted using ABI Prism 6100 Nucleic Acid Prepstation™ (Applied Biosystems, Fresno, CA). Extraction was performed according to the manufacturer's protocol for isolation of DNA from whole blood with minor modifications. Extracted DNA was frozen in aliquots at –20°C until amplification by PCR.

3.2.2 Polymerase chain reactions

Nested PCR followed by mutation-specific restriction was used to detect *pfcrt* K76T, T152A, S163R, Q271E, N326S, *pfmdr 1* N86Y, F184Y, S1034C, N1042D, D1246Y and *pfmsp 1* Y191H SNPs. The primers and reaction conditions used have been described previously [86, 107, 146, 147].

Pfmsp1, *pfmsp2* and *pf*GLURP were amplified as described previously [148]. In study VI, recrudescent infections were distinguished from reinfections by analysing *pf*GLURP, *pfmsp2* and *pfmsp1* sequentially according to WHO recommendations [149]. In study I only *pfmsp2* was used.

PCR and restriction products were resolved on 2% or 3% agarose gels (Amresco, Solon, OH). All gels were stained with ethidium bromide and visualized under UV transillumination (BioRad GelDoc System, BioRad, Hercules, CA).

3.2.3 Sequencing

The exon containing the *pfcrt* 72–76 haplotype was amplified by nested PCR and resolved as described above. Purification followed by sequencing using the forward nest primer was performed at Macrogen Inc. (Seoul, South Korea).

3.2.4 Real Time PCR

We assessed *pfmdr1* copy number using a real time polymerase chain reaction method [150]. All samples were run in triplicate. Each run contained 3 reference samples of the *P. falciparum* 3D7 clone (1 *pfmdr1* copy) and 1 control sample of Dd2 (2–3 *pfmdr1* copies). We used the same cut off criteria as previous studies [105, 151]; that is, assays were repeated if any of the following results were obtained: ΔΔCt (cycle threshold) spread >1.5, Ct values >35, or copy number equal to 1.3–1.6. Values obtained were then rounded to the closest integer.

3.3 *IN VITRO* ASSESSMENT OF CHLOROQUINE RESISTANCE

In vitro tests were conducted in December or January, following the end of the rainy season. Each year that tests were done, approximately 400 asymptomatic school children aged between 7 and 14 years were screened. Otherwise healthy children with 20 or more *P. falciparum* parasites per 200 leukocytes in the thick film were invited to participate. *In vitro* tests were conducted according to WHO protocols. Development of schizonts in well E, corresponding to a chloroquine concentration of 160 nM was considered as an indication of resistance as defined by the WHO [152]. Effective concentration (EC) values and their 95% confidence intervals were calculated using the probit calculus sheet described in the WHO protocol. In 1992, 1993, 1995, 2004 and 2005 blood was also collected on filter-paper during the screening process.

3.4 *IN VIVO* ASSESSMENT OF CHLOROQUINE AND ARTEMETHER-LUMEFANTRINE EFFICACY

The study protocol adhered to recommendations by the WHO for conducting standardized *in vivo* tests but follow up was extended to 70 days [149, 153]. Inclusion criteria were the same as those used for previous clinical trials conducted within PSB. In essence, children aged 6 months to 15 years with *P. falciparum* mono-infection without signs of severe malaria were recruited.

Treatment was allocated by block randomization. Treatment was not blinded. Medication was given and supervised by an experienced nurse at the health centre. Children were seen at the health centres twice daily on days 0, 1 and 2 and once day 3. They were then visited weekly at home from day 7 to day 70.

Each day a child was seen, including unscheduled visits to the health centres, Giemsa stained thick and thin smears were made to quantify asexual parasitaemia (per 200 white blood cells). A slide was considered negative after examination of 100 high power fields. Blood was put onto filter-paper (Whatman 3MM) for later genotyping whenever a slide was made except for days 2 and 3 and when no parasites were found during unscheduled visits. Blood was taken for drug concentration analyses on day 7. Haemoglobin was measured using a HaemoCue (Ängelholm, Sweden) on days 0, 42 and 70.

PCR adjusted outcome classifications were early treatment failure, late clinical failure, late parasitological failure or adequate clinical and parasitological response [149, 153].

3.5 MONITORING OF CHLOROQUINE PRESCRIPTION AND CONSUMPTION

Between 1994 and 2003 (except 1998, 2001 and 2002) the same laboratory technician attended a health centre in suburban Bissau (not within PSB) for a period of 4–8 weeks. He noted the treatment recommendations given to febrile children but did not in any way interfere with the work or recommendations of the health personnel (Study II).

Study IV was conducted between January and March 2006. As part of the health services provided to the community, PSB provided chloroquine free of charge from a drug dispensary. Children presenting at the drug dispensary with a diagnosis of malaria from a physician and a chloroquine prescription were eligible for participation.

Upon inclusion, prescriptions were copied, and the mother or legal guardian was asked about drug intake and symptoms prior to study entry. Finger prick blood was drawn for thick and thin films and for chloroquine analysis. The child was visited once daily in the afternoon. When the mother reported that she was no longer giving chloroquine, a new blood sample was taken for chloroquine analysis and microscopy. Data and blood were thus collected 1 day after the last dose. The child was then visited two additional times, 4 and 14 days after the last dose. At each visit, the mother or guardian was questioned about symptoms and drug intake and blood was taken for chloroquine concentration analyses.

3.6 DRUG CONCENTRATION ANALYSIS

Exactly 100µL of capillary blood was put onto filter-paper for analysis of drug concentration. Pre-treated filter-papers for analyses of lumefantrine concentrations were stored at -20˚C. Filter-papers were put into separate sealed plastic bags to avoid contamination. Drug concentrations were determined by high performance liquid chromatography [154, 155]. Minimum detectable lumefantrine and chloroquine concentrations were 100 nmol/L and 50 nmol/L, respectively

3.7 ETHICS

The studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent was obtained from all parents/legal guardians orally as most of them were illiterate. The responsible nurse signed a document to indicate that the information had been provided and informed consent had been obtained. All the studies had ethical approval from the Ministério da Saúde Pública in Guinea-Bissau. The ethics committee at Karolinska Institutet approved work done at Karolinska Institutet and did not object to the work done in Guinea-Bissau. Study VI was registered at ClinicalTrials.gov (https://register.clinicaltrials.gov/; study ID: PSB-2006-coartem NCT00426439).

4 RESULTS

4.1 GENETIC POLYMORPHISMS ASSOCIATED WITH CHLOROQUINE AND AMODIAQUINE RESISTANCE

4.1.1 Chloroquine resistance transporter gene (*pfcrt*)

Following treatment with standard (25 mg/kg) and double standard (50 mg/kg) dose chloroquine, the *pfcrt* 76T proportion amongst recrudescent infections were 96% (21/22) and 83% (5/6), respectively (study I). Compared with the Day 0 proportions of 28% (34/121) and 19% (23/122), respectively, this represented a significant selection of the 76T allele ($P<0.001$ and $P=0.002$). The *pfcrt* 76T proportion increased from 45/146 (31%) at day 0 to 8/8 (100%) amongst recrudescent infections at day 42 in study VI. The proportion of *P. falciparum* with *pfcrt* 76T in *in vitro* tests showing schizont maturation gradually increased with increasing chloroquine concentrations in the wells. *Pfcrt* 76T was significantly more common ($P<0.005$) in *P. falciparum* able to develop schizonts at chloroquine concentrations >160 nmol/L compared to the control (study II).

Pfcrt 76T was linked to *pfcrt* 271E OR 7.79 (95% CI 3.01-20.11, $P<0.001$) and 326S OR 4.19 (95% CI 1.87-9.36, $P<0.001$). As with *pfcrt* 76T, the proportion of *pfcrt* 271E and *pfcrt* 326S were significantly higher in *P. falciparum* that developed schizonts at chloroquine concentrations >160 nmol/L.

Pfcrt T152A and S163R alleles were identified in 177 samples collected at day 0 and 44 samples collected from children with recurrent parasitaemia in study I. one successfully treated child had *P. falciparum* with both *pfcrt* 163R and *pfcrt* 76K. *Pfcrt* 152T and 163S were detected in all other samples.

Codons 59–85 of *pfcrt* were successfully sequenced from 45 recrudescent infections, 9 re-infections, and 3 undetermined re-parasitemias. Thirteen successfully treated samples containing *pfcrt* 76T and 15 containing *pfcrt* 76K according to the restriction results were randomly selected and sequenced. The *pfcrt* 72–76 haplotypes identified were CVIET, CVMNK, or a combination of the two.

4.1.2 Multidrug resistance gene (*pfmdr1*)

Neither *pfmdr1* 86Y (study I, II and VI) nor 184F (study I) were associated with chloroquine resistance. Only *pfmdr1* 1034S, 1042N and 1246D were found (study I)

4.1.3 Multidrug resistance protein gene (*pfmrp1*)

Pfmrp1 H191S and S437A were successfully amplified in 172/220 (78%) of samples in study II. Only 191H and 437A were detected.

4.1.4 Linkage disequilibrium

In study V that included data from study I, another clinical trial and data from the first year of study, VI *pfcrt* 76T and *pfmdr1* 86Y were linked (OR, 2.71 [95% CI, 1.99–3.67]; *P*<0.0001; n= 951). Similarly, *pfcrt* 76K and *pfmdr1* 86N were linked (OR, 3.40 [95% CI, 2.45–4.74]; *P*<0.0001; n= 951).

Amodiaquine

Following treatment with 15 mg/kg of amodiaquine, the proportion of *pfcrt* 76T increased from 27/114 (24%) to 7/7 (100%) (*P*<0.001).
The *pfmdr1* 86Y and 184F proportions increased from 56/114 and 92/114, respectively, to 7/7 (100%) in recrudescent infections (*P*=0.01 and *P*=0.05, respectively).

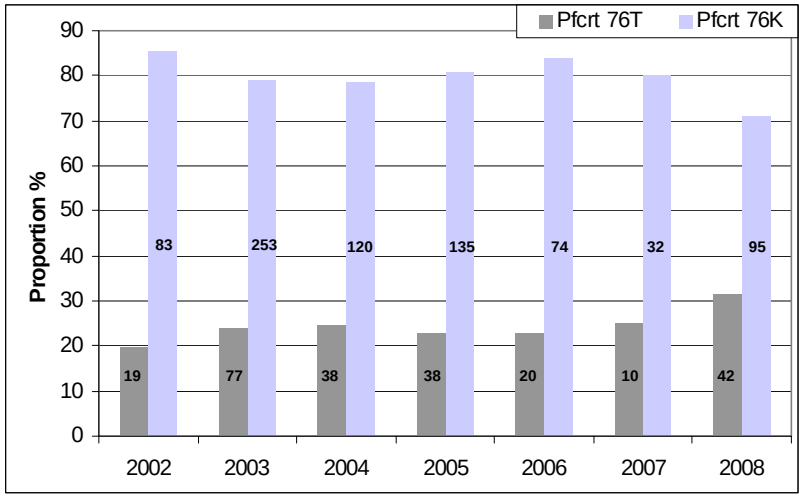
4.2 PROPORTIONS OF CHLOROQUINE RESISTANT *P. FALCIPARUM*

In vitro tests assessing chloroquine resistance in Guinea-Bissau were carried out between 1990 and 2005 except for 1991, 1996, 1999 and 2001 (study II). The mean proportion of tests indicating chloroquine resistance was 33% (range 14–54%). EC 50 values varied between 8 and 117 nM with the exception of the year 2000. In 2000, the chloroquine resistance proportion was 94% and the EC50 value 238 nM. The nonparametric test for trend did not identify a significant trend across the years irrespective of whether the year 2000 was included (*P*=0.22) or excluded (*P*=0.64).

In study II, the proportion of the *pfcrt* 76T allele was 10/31 (32%) in 1992, 4/18 (22%) in 1993, 14/50 (28%) in 1995, 35/93 (38%) in 2004 and 3/23 (13%) in 2005. There was no significant trend of increasing *pfcrt* 76T proportion (*P*=0.89)

The annual proportion of *P. falciparum* with *pfcrt* 76T or *pfcrt* 76K in children (aged 6 months -15 years) with malaria presenting at health centres, was determined in three clinical trials conducted between 2003 and 2008. Data was also available from children presenting at Bandim health centre between October and December 2002. There was no significant trend of increasing *pfcrt* 76T proportion between 2003 and 2008 (*P*=0.21).

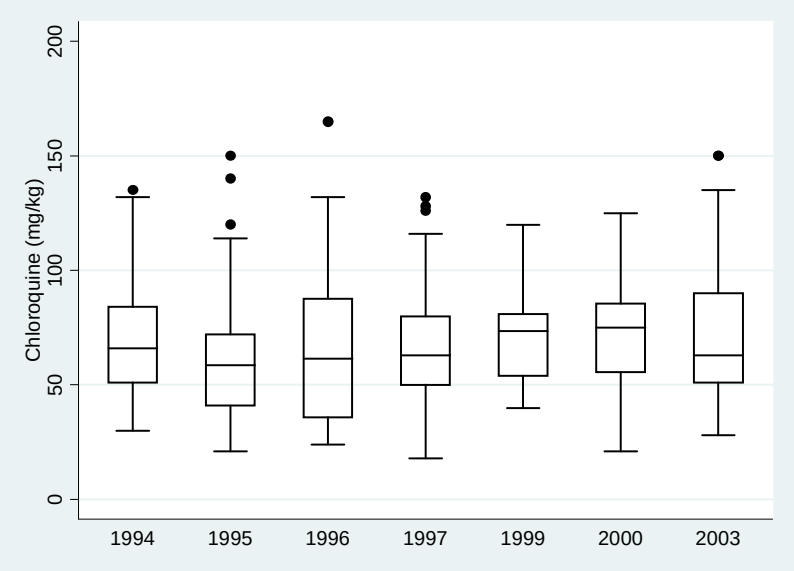
Figure 1. *Pfcrt* 76T and *pfcrt* 76K proportions at Bandim health centre



4.3 CHLOROQUINE PRESCRIPTION AND CONSUMPTION

Between 1994 and 2003 (except 1998, 2001 and 2002) the treatment recommended to 712 febrile children was registered. Four hundred and ninety four children were recommended chloroquine, 116 were recommended quinine, 84 were sent elsewhere for malaria diagnosis and subsequent treatment recommendations were therefore not registered. Only 17 were not recommended anti-malarial treatment. The median number of doses each day was 2 interquartile range (IQR) 2–3. The median dose was 9 mg/kg (IQR 6.6–11) and the median treatment duration was 3 days (IQR 3–3). The median treatment duration did not increase significantly between 1994 and 2003 (non parametric test for trend *P*=0.16). The median total dose was 63 mg (IQR 51–81) chloroquine base/kg over the years with a maximum of 75 mg/kg in 1999 and 2000 and a minimum of 60 mg/kg in 1995. There was a significant increase over the years (non parametric test for trend *P*=0.038).

Figure 2. Total amount of chloroquine prescribed at one health centre in Bissau



In study IV, chloroquine prescriptions by eight physicians and chloroquine intake by 102 children were recorded. The median total chloroquine dose prescribed and reportedly taken were 81 (IQR (72–92) and 77 (IQR 53–102) mg/kg, respectively. The prescribed total dose was usually split into 2 (IQR 2-3) daily doses of 6.5 (IQR 5.7–9.5) mg/kg each for a median 5 (IQR 5-5) days. Four days after the last dose, 34/85 (40%) of the study children had chloroquine concentrations above 820 nM which was the 25th percentile concentration found 4 days after an observed intake of double standard dose (50 mg/kg) over 3 days in a previous study. No severe adverse events were reported. No adverse events were associated with higher chloroquine concentrations.

4.4 CHLOROQUINE CONCENTRATIONS, EFFICACY AND TOLERABILITY

The median chloroquine concentrations measured on day 7 after intake of standard (25 mg/kg) and double standard (50 mg/kg) dose chloroquine and amongst PCR adjusted treatment failures by day 28 are shown in figure 3. Data is pooled from studies I and VI.

Figure 3. Chloroquine concentrations after intake of standard or double standard dose chloroquine and amongst children with treatment failure

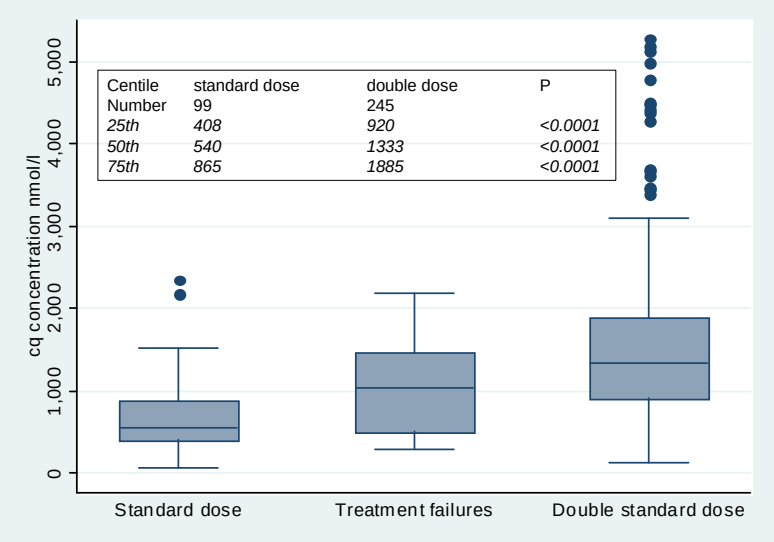
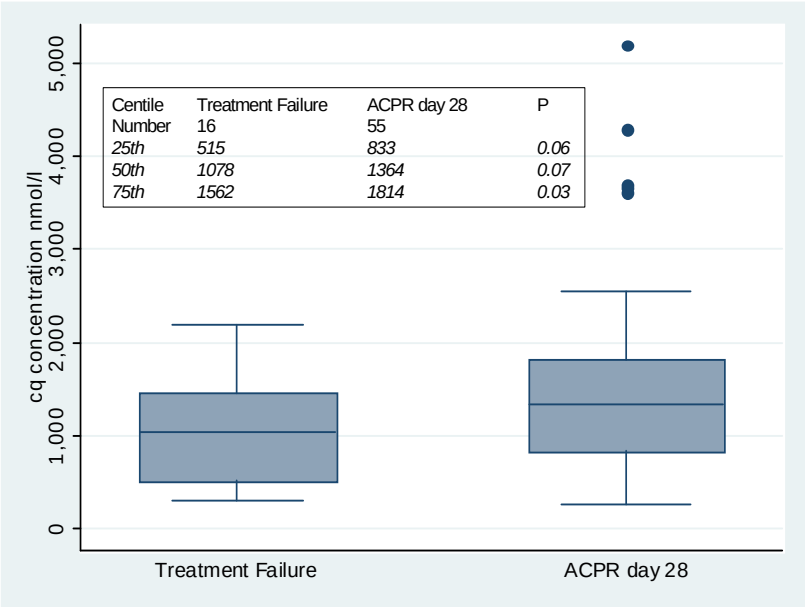


Figure 4 shows the chloroquine concentration in children infected with *P. falciparum* with *pfcrt* 76T. The range of chloroquine concentrations in children with treatment failure was 297-2190 nmol/L. The data includes children given standard or double standard dose chloroquine. The child with a chloroquine concentration of 2190 nmol/L had an initial parasite density of 155700 *P. falciparum*/μL.

As shown in figure 4 the 75th percentile chloroquine concentration found in children with treatment failure was 1562 nmol/L. Treatment with standard dose or double standard dose chloroquine (figure 3) resulted in 2/99 (2%) and 101/245 (41%) of children having concentrations above that.

In study I, the PCR adjusted (*pfmsp* 2 only) day 28 efficacy of standard and double standard dose chloroquine against *P. falciparum* with *pfcrt* 76T was 38% (13/34) and 78% (18/23), respectively (P=0.007). In study VI the PCR adjusted (*pfmsp* 1 and 2 and *pfglurp*) day 28 efficacy of double standard dose chloroquine (50 mg/kg) against *P. falciparum* with *pfcrt* 76T was 87%.

Figure 4. Chloroquine concentrations in children infected with *pfcrt* 76T carrying *P. falciparum*



In study VI, pruritus occurred in 36 children taking chloroquine and 10 taking AL (P<0.001). No other adverse events could be attributed to chloroquine and resolution of symptoms were similar in both treatment arms. Adverse events were not dose related and severe adverse events were not seen in study IV.

4.5 NO SEASONAL ACCUMULATION OF CHLOROQUINE RESISTANCE ASSOCIATED GENOTYPES

There was no continuous monthly trend of changing *pfcrt* 76T or 76K nor *pfmdr1* 86Y or 86N allele proportions during the high (May-December) or the low (January-April) transmission periods (study V). The total *pfcrt* 76T proportions during the high and low transmission periods were 186/777 (24%) and 46/177 (26%), respectively. The total *pfcrt* 76K proportions were 621/777 (80%) and 140/177 (79%), respectively. The total *pfmdr1* 86Y proportions were 338/779 (43%) and 79/179 (44%), respectively. The total *pfmdr1* 86N proportions were 562/779 (72%) and 131/179 (73%), respectively.

P. falciparum with *pfcrt* 76T was associated with lower parasite density compared to *P. falciparum* with *pfcrt* 76K (15254 versus 18664 *P. falciparum*/µl P =0.003; n=755) in children below the age of 10 years. Similarly, *pfmdr1* 86Y was associated with lower median parasite density compared to *pfmdr1* 86N, (16320 versus 18880 *P. falciparum*/µl, P= 0.018; n= 669). If the children over the age of 10 years were included in the analyses, the differences were not significant but the trend the same.

4.6 EFFICACY OF DOUBLE STANDARD DOSE CHLOROQUINE AND ARTEMETHER-LUMEFANTRINE

In study VI, 378 children were enrolled and randomised, 188 to chloroquine and 190 to artemether-lumefantrine. In the chloroquine and artemether-lumefantrine arms, 7 and 4 children were excluded after randomisation. Efficacies were thus assessed in 181 and 186 children. In the chloroquine arm, the number of children with adequate clinical parasitological response or PCR adjusted treatment failure by day 28, 42 and 70 were 158 (87%), 148 (82%) and 125 (69%). In the artemether-lumefantrine arm the numbers were 168 (90%), 161 (87%) and 144 (77%)

The PCR adjusted day 42 ACPRs were 96.6% for artemether-lumefantrine and 93.8% for chloroquine (Hazard ratio 1.76 [95% CI 0.64-4.83], *P*=0.28). The PCR unadjusted day 42 ACPRs were 94.9% for artemether-lumefantrine and 91.3% for chloroquine (Hazard ratio 1.65 [95% CI 0.72-3.82], *P*=0.35).
Age and parasitaemia were not significantly different amongst children with early treatment failure or PCR adjusted late treatment failure compared to children with ACPR day 42.
There were 10 reinfections in both study arms with 3 and 4 occurring before day 42 in the artemether-lumefantrine and chloroquine arms, respectively.

Table 1. Treatment outcomes

	Reparasitaemia classification					PCR unadjusted ACPR		PCR adjusted ACPR	
	ETF	LPF	LCF	Reinfection	ACPR(n)	ACPR(%)	HR (95% CI)	ACPR(%)	HR (95% CI)
Day 28									
CQ	2	2	4	1	150	94.6	1.18 (0.46-3.07)	95.1	1.40 (0.49-4.03)
AL	3	0	3	2	162	95.5		96.6	
Day 42									
CQ	2	2	6	4	138	91.3	1.65 (0.72-3.82)	93.8	1.76 (0.64-4.83)
AL	3	0	3	3	155	94.9		96.6	
Day 70									
CQ	2	3	6	10	114	86.4	1.36 (0.72-2.57)	93.1	1.67 (0.65-4.32)
AL	3	1	3	10	137	89.8		96.0	

HR= Hazard ratio, (95% CI) 95% confidence interval in brackets
ACPR (%) were calculated using a survival analysis and therefore differ from the % calculated by dividing ACPR by ACPR + treatment failures

In the artemether-lumefantrine arm, the day 0, 42 and 70 haemoglobin values were 110, 117 and 120 g/L. Corresponding values in the chloroquine arm were 112, 117 and 118 g/L.

Fever disappeared and the assessed clinical condition improved at the same rate in both treatment arms. Median parasite densities/µL on day 0, 1, 2 and 3 following treatment with chloroquine were 26667, 9080, 280 and 0. Following treatment with artemether-lumefantrine, the parasite densities were 22346, 680, 0 and 0. Parasite densities were significantly lower on day 1 and 2 following treatment with artemether-lumefantrine (*P*<0.0001).

4.7 PREVALENCE AND SELECTION OF GENOTYPES ASSOCIATED WITH ARTEMETHER-LUMEFANTRINE RESISTANCE

Pfmdr1 copy number was successfully determined in 596/641 samples collected between 1992 and 2004 in Guinea-Bissau. No amplifications were found (study III). The 3 recrudescent infections occurring by day 42 following treatment with artemether-lumefantrine had *pfcrt* 76K and *pfmdr1* 86N alleles but the numbers were small and changes not significant. The *pfcrt* 76K (7/10) and *pfmdr1* 86N (8/9) proportions in re-infections did not increase significantly compared to day 0 (study VI).

5 DISCUSSION

5.1 GENETIC BASIS OF RESISTANCE AND PROPORTIONS OF CHLOROQUINE RESISTANT *P. FALCIPARUM*

We have shown that *pfcrt* 76T has existed in Guinea-Bissau since the early 1990's. It is found within the CVIET haplotype that is of Southeast Asian origin and was probably introduced to Africa in the late 70's [120, 127, 128]. We have also demonstrated that the *pfcrt* 76T genotype is associated with chloroquine resistance both *in vivo* and *in vitro*. Thus chloroquine resistant *P. falciparum* most probably have the same genetic basis in Guinea-Bissau as the rest of Africa.

The *pfcrt* 76T proportion and *in vitro* data in this thesis indicate that the prevalence of chloroquine resistant *P. falciparum* has not gradually increased in Guinea-Bissau. This is further supported by *in vivo* data from clinical trials using chloroquine conducted between 1995-2004 and 2007-2008 (study VI) that show a stable level of chloroquine resistance [130-132, 156]. The low proportion of chloroquine resistant *P. falciparum* in Guinea-Bissau contrasts the situation in the rest of Africa where chloroquine resistance has become highly prevalent [157] with an associated increase in morbidity and mortality [158, 159].

The most important factor determining the rate of spread of drug resistance is the proportion of infected people treated [141]. Even if anti-malarial use is strictly controlled, spread of resistance is rapid if the proportion of infected people treated is high [160]. Within our study area, chloroquine has been used for the treatment of fever and the population has easy access to health care. Monitoring prescription patterns in 2003-2004 showed that, 17924/26134 (69%) of children below the age of 5 years consulting a clinician were diagnosed with malaria while only 13% of these presumptively treated children had malaria verified by microscopy [161]. Similar monitoring during 2007 and 2008 revealed that 13310/34884 (38%) of children below 15 years of age attending health centres were treated for malaria, though only 415/13310 (3%) had microscopically verified malaria (study V). In study IV only 3/102 children had *P. falciparum* at day 0. These results show that chloroquine is greatly overused and suggest that the proportion of infected people treated is very high. Consequently the spread of resistant malaria should be fast, contrary to what is seen.

In neighbouring The Gambia and in Sudan, the prevalence of *pfcrt* 76T and consequently chloroquine resistance varies between high and low transmission seasons and the spread of chloroquine resistance is delayed compared to highly endemic regions [133, 134]. A similar situation could exist in Guinea-Bissau but clearly does not as shown in study V. Thus seasonal fluctuation does not account for the low prevalence of resistant *P. falciparum*.

5.2 CHLOROQUINE CONSUMPTION AND EFFICACY

In studies II and IV, we have shown that the median chloroquine dose prescribed and reportedly taken is approximately 3 times the standard dose recommended. This practice has been adopted since at least 1994 in one health centre but all clinicians informally questioned in Guinea-Bissau concur that this has been the generally accepted treatment practice for as long as they recall.

In studies conducted between 1995-1996 and 2001-2004, the day 28 PCR uncorrected efficacies of double standard dose chloroquine where 86 and 90% whilst the efficacies of standard dose chloroquine were 68 and 76% [131, 156]. In studies I and VI, we have shown that double standard dose chloroquine had 78 and 87% PCR corrected day 28 efficacies whilst standard dose only had a 38% efficacy against *P. falciparum* with *pfcrt* 76T. This indicates that *P. falciparum* resistant to standard dose chloroquine can be successfully treated if a higher dose is used. In line with these results, children with treatment failure had lower chloroquine concentrations compared to successfully treated children (study VI).

Furthermore the proportion of children with chloroquine concentrations above the 75th percentile concentration found amongst children with treatment failure was 2/99 (2%) and 101/245 (41%) after intake of standard and double standard dose chloroquine. Thus the probability of achieving a chloroquine concentration in the blood that successfully treats *P. falciparum* with *pfcrt* 76T is considerably greater following intake of double dose chloroquine. It is also interesting to note that recrudescences did not occur in any of the 11/55 children infected with *P. falciparum* with *pfcrt* 76T that had a day 7 chloroquine concentration >2190 nmol/L. The data suggests that there is a chloroquine concentration above which all *P. falciparum* with the current mechanism of resistance will be successfully treated.

PfCRT most likely mediates chloroquine resistance by transporting chloroquine from the digestive vacuole in an energy dependent and saturable manner [32, 79, 162]. It is possible that the higher concentrations achieved with double standard dose chloroquine overcome this export mechanism. Plausible mechanisms include saturation of the efflux mechanism and depletion of energy reserves caused by a prolonged exposure to high drug concentrations [163].

5.3 EFFECT OF ROUTINE USE OF HIGH DOSE CHLOROQUINE IN GUINEA-BISSAU

Our data indicates that *P. falciparum* with *pfcrt* 76T are generally successfully treated with the higher doses of chloroquine that are routinely used in Guinea-Bissau. *P. falciparum* with *pfcrt* 76T thereby loose much of their selective advantage. Even so, they clearly have a selective advantage when chloroquine concentrations are moderate such as soon after treatment or when lower doses are taken. *Pfcrt* 76T is therefore most probably an indicator of tolerance not resistance in Guinea-Bissau [116]. *Pfcrt* 76T has been associated with a loss of fitness [133-136] and results from study V including lower parasitaemias associated with *pfcrt* 76T indicate that this is also the case in Guinea-Bissau. Modelling shows that spread of resistant *P. falciparum* will be retarded

or even curtailed if the fitness cost is too great [141]. This no doubt also applies to tolerance and explains why *pfcrt* 76T has reached equilibrium in Guinea-Bissau at a relatively low proportion.

It took a relatively long period (~12 years) from the massive deployment of chloroquine until resistance developed [164] and resistance has only evolved a limited number of times [92, 121, 122, 127]. This suggests that resistance is a rare, complex multigenic process involving mutations that provide resistance at a cost to fitness, with mutations that compensate for the loss of fitness. The chloroquine resistant parasites that spread throughout Africa have remained less fit than sensitive parasites indicating that mutations that compensate for loss of fitness are rare. We have shown that chloroquine tolerance (*P. falciparum* with CRT 76T) has existed in Guinea-Bissau since the early 90's and that high dose chloroquine has been used since at least 1994. Despite that, double standard dose chloroquine has a day 28 PCR adjusted 95% efficacy. Thus *P. falciparum* able to combine resistance to double standard dose chloroquine with enough fitness to spread have not become established. This is in line with the hypothesis that there is a chloroquine concentration above which resistance can not develop and spread because the energy required to survive will be so great that all fitness is lost [165].

5.4 ABSENCE OF ADVERSE EVENTS

The toxicity of chloroquine is most likely related to transient high plasma concentrations occurring early in the distribution phase [21, 166]. In line with this severe adverse events due to chloroquine are seen soon after intake [167]. Chloroquine is distributed from a central compartment that is approximately one thousand times smaller than the eventual total apparent volume of distribution at steady state [21]. Furthermore the volume of the central compartment decreases with intake of larger doses [23]. Thus a large single dose is acutely toxic whilst repeated smaller doses are not [36, 37]. Study VI and previous studies show that 50 mg/kg in divided doses over 3 days is well tolerated [130, 131, 156], no doubt due to the split dose. A limitation of our studies is that we do not know how close the concentrations achieved are to toxic concentrations.

5.5 HOW SHOULD CHLOROQUINE BE DOSED?

When single daily doses of chloroquine are given, the concentrations fluctuate considerably. Thirty mg/kg given as 10+5+5 mg/kg on day 0 followed by 5 mg/kg twice on day 1 resulted in higher chloroquine concentrations at 12, 24 and 48 hours compared to the standard dose, without an increase of adverse events [41]. Intramuscular chloroquine (3.5 mg/kg) every 6 hours resulted in a 50% reduction of parasite density in approximately 9 hours in children with severe malaria and 30 mg/kg and 25 mg/kg in the schedules above caused a 50% reduction in approximately 14 hours and 37 hours [168]. It is likely that fractionated doses result in a more constant drug pressure and longer time over the minimal paraciticidal concentration. Infections often consist of several unsynchronized strains and mid term trophozoites are the most sensitive to chloroquine. Higher concentrations for longer time will therefore also expose more parasites to paraciticidal concentrations when they are most sensitive. This probably explains the more rapid parasite clearance [23, 41, 168]. The use of

fractionated doses as well as the dosage increase in Guinea-Bissau probably account for the increased efficacy of 50 mg/kg of chloroquine as the same total dose was not superior to standard dose when given once daily [42, 43].

A treatment regimen with multiple doses is complicated and it is likely that doses will be missed with an associated increased risk of treatment failure. However, drugs can be manufactured as sustained release preparations that release drug over 24 hours. A sustained release preparation of chloroquine should theoretically avoid toxicity whilst maintaining a constant drug pressure. It would also reduce the number of daily doses required thereby probably increasing adherence.

5.6 ARTEMETHER-LUMEFANTRINE IN GUINEA-BISSAU

Artemether-lumefantrine started being introduced as first line therapy for uncomplicated malaria in June 2008. As expected and as shown in study VI, the efficacy of artemether-lumefantrine is within the WHO recommendations for an anti-malarial about to be introduced. In the study area and elsewhere in Guinea-Bissau, there is a concept of clinical malaria as opposed to malaria verified by microscopy. This usually applies to a child with fever (but not necessarily) that is deemed to have malaria irrespective of whether microscopy is positive or negative. Consequently microscopy will normally not be done though it has been done as part of screening for the studies in this thesis. Previously, clinical malaria was treated with chloroquine that has therefore been vastly over prescribed (study V) [161]. When informally questioned, clinicians state that they will now use artemether-lumefantrine for clinical malaria. Considering the cost of artemether-lumefantrine and the wish to limit drug pressure in order to extend its useful life, this is a problem that must be tackled.

Pfcrt 76K and *pfmdr1* 86N have been associated with artemether-lumefantrine resistance/tolerance [94, 104] and data from study VI are in line with this. A recent *in vitro* study of field samples found that lumefantrine IC50 was ~5.5 times higher (173 nmol/L) in *P. falciparum* with the *pfcrt* 76K and *pfmdr1* 86N alleles compared to *P. falciparum* that had the *pfcrt* 76T and *pfmdr1* 86Y alleles (31 nmol/L) [95]. In line with this, chloroquine sensitive *P. falciparum* had higher lumefantrine IC50 than chloroquine resistant *P. falciparum* in an *in vitro* study from Senegal [169].

The high prevalence of the 76K and 86N alleles suggest that Guinea-Bissau is one step ahead of other regions as far as developing resistance/tolerance to lumefantrine is concerned. *Pfmdr1* amplifications, that are associated with a 2 fold increase of lumefantrine IC50 [150] can arise rapidly as seen in Gabon [151] and *in vitro* [139]. Furthermore, lower lumefantrine concentrations were seen when intake of artemether-lumefantrine was not observed [170]. The effectiveness of artemether-lumefantrine therefore needs to be assessed in Guinea-Bissau.

6 CONCLUSIONS

1. *P. falciparum* chloroquine resistance associated genotypes are the same in Guinea-Bissau as most of Africa and probably have the same Southeast Asian origin.

2. The level of chloroquine resistant *P. falciparum* as indicated by molecular and *in vitro* tests has been stable in Guinea-Bissau since the early 1990's

3. In Guinea-Bissau approximately 3 times the standard dose of chloroquine is routinely prescribed and taken split into multiple smaller daily doses without commonly occurring adverse events.

4. Seasonal variation of *pfcrt* 76T does not explain its low proportion

5. The efficacy of chloroquine against *P. falciparum* with *pfcrt* 76T is dose and concentration dependent indicating that chloroquine resistance can be overcome by higher doses of chloroquine.

6. Artemether-lumefantrine and double dose chloroquine both achieve the 95% efficacy recommended by the WHO for an antimalarial about to be introduced as first line treatment.

7. Artemether-lumefantrine and chloroquine appear to select for *P. falciparum* with opposite genotypes at codons 76 and 86 of *pfcrt* and *pfmdr1*, respectively.

The use of high total doses of chloroquine split into multiple daily doses, combined with the loss of fitness associated with resistance, is therefore the most likely reason for the low and stable proportion of *P. falciparum* resistant to normal dose chloroquine and the continued efficacy of chloroquine in Guinea-Bissau.

7 FUTURE

We are currently assessing the pharmacodynamics of chloroquine by exposing *P. falciparum* to various concentrations over various times *in vitro*. We also intend to further assess genetic markers of resistance including *pfmdr1* amplifications and *pfATP6* SNPs from samples collected in study (VI). We hope to start an artemether-lumefantrine effectiveness study in Guinea-Bissau.

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