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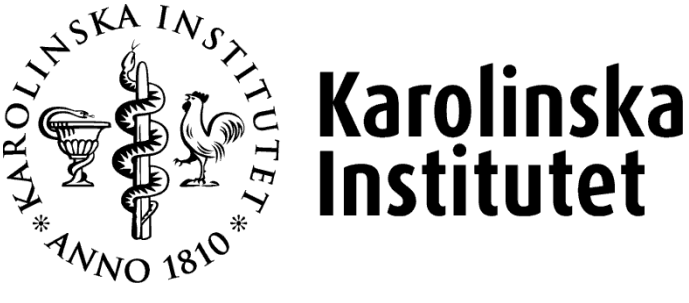
SEARCH FOR GENETIC DETERMINANTS OF *PLASMODIUM FALCIPARUM* MALARIA DRUG RESISTANCE IN VITRO AND IN VIVO

Aminatou Kone



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Search for genetic determinants of *Plasmodium falciparum* malaria drug resistance in vitro and in vivo.

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ABSTRACT

Malaria remains the most deadly disease in the world with nearly 627 000 deaths and more than 200 million new clinical cases every year, the large majority occurring in sub Saharan Africa children aged < 5 years. This represents anyway a significant decrease as compared with the situation in the start of the Millennium. This is due in part to the worldwide adoption of artemisinin-based combination therapy (ACT). However these gains are being threatened. A pattern of progressive decreased susceptibility of the parasite to the ACT key drugs, the artemisinin derivatives is emerging. Another central drug is quinine, still the mainstay for the treatment of severe malaria in Africa.

The aim of this thesis was to contribute to the understanding of the genetic determinants of *Plasmodium falciparum* resistance to two key short half-life antimalarials, quinine and artesunate and to assess the parasite susceptibility to these drugs in Mali.

In a clinical study on the efficacy of quinine 100% of severe *Plasmodium falciparum* infected patients were cured. For the first time, the *pfnhe1* microsatellite allele ms4760-1, previously proposed to be involved with parasite in vitro resistance to this drug was selected post treatment pointing for this marker as also involved in the in vivo sensitivity of the parasite to quinine. Conversely, the ms4760 status of the initial infections was not predictive of the clinical outcome, leading to the conclusion that the ms4760-1 is likely a secondary factor of quinine resistance. The *pfcrt* K76T SNP was not shown to be under selection. In conclusion, albeit *pfnhe1* has an undeniable contribution to the parasite response in vivo, other factors must be involved, supporting the view of quinine resistance as a complex multigenic trait.

P. falciparum decreased sensitivity to artemisinin and its derivatives have been recently reported in SE Asia, including Thailand. We therefore performed an explorative study based on the determination of the in vitro sensitivity (IC_{50}) of 47 culture adapted parasites from Mae Sot (Thai-Myanmar boarder) to a number of ACT drugs. These included artemisinin and dihydroartemisinin (DHA), the key metabolite of both artemether and artesunate. The open reading frames of the drug transporter genes *pfcrt, pfmdr1, pfmrp1* and *pfmrp2* were further studied. Correlation analyses revealed two novel candidate markers of multidrug resistance: the *pfmdr1* F1226Y and *pfmrp1* F1390I SNPs, which were associated with 2-3 fold, increases in the IC_{50}s of artemisinin and also the ACT partner drugs lumefantrine and mefloquine.

An artesunate monotherapy (7 days) efficacy trial was performed at Malian malaria setting with the objective of detecting possible delayed *P. falciparum* clearance phenotypes, an early sign of decreased drug susceptibility. The microscopic based assessment of the infections did not reveal any extended parasitaemia clearance times with a median clearance time of 32 hours. Nevertheless there were clear inter-individual differences in the clearance dynamics.

Recently, SNPs in the *P.falciparum* K13 propeller gene has been proposed to be markers of artemisinin resistance, i.e. of significantly increased clearance time in SE Asia. We therefore studied the polymorphisms in this gene in Mali and any possible association with the range of clearance times observed above. In addition, a set of samples from a previous cross section survey study, conducted prior to ACTs implementation, were analyzed in order to try to detect temporal changes in the sequence of the K13 propeller gene. The SE Asian mutations associated with artemisinin resistance were not found in Mali in any of the periods. Nevertheless, the K13 gene was found to be polymorphic in Mali even before the wide use of ACTs. No association was however found between polymorphism and parasite clearance rate. Interestingly, the SNPs found in the early cross-sectional study were different from those found in the later study. Further, the later study revealed mutations present near one of the key a.a. positions linked with resistance in Asia. These patterns merit further investigations.

Finally, a new qPCR based approach was used to revisit the artesunate monotherapy study. This had the aim of increasing the sensitivity of parasite detection, in order to obtain an improved phenotype of parasite clearance, and hence improved conditions to search for a correlation between the presence of K13 mutation and the trend of prolonged parasite clearance.

No clear association could be found even though the qPCR approach was able to find evidence of parasites 72 hours after artesunate treatment in more than 46% of infections previously considered as cleared by microscopy. Intriguingly no mutations in the K13 propeller gene were found among the parasites classified as fast clearers by this method (parasites cleared at 24 hours after treatment).

The result of the clinical trials showed high in vivo efficacies for both artesunate and quinine. However, this situation can rapidly change, as demonstrated by the recent emergence of artemisinin resistance in Asia. Molecular monitoring of any possible evolution and selection of antimalarial drug tolerance/resistance associated polymorphisms of

genes such as Pfnhe-1 or K13 propeller are critical for optimal drug policies and sustained efficacy

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I. **Kone A**, Mu J, Maiga H, Beavogui AH, Yattara O, Sagara I, Tekete MM, Traore OB, Dara A, Dama S, Diallo N, Kodio A, Traoré A, Björkman A, Gil JP, Doumbo OK, Wellems TE, Djimde AA. Quinine treatment selects the pfnhe-1 ms4760-1 polymorphism in Malian patients with Falciparum malaria. J Infect Dis. 2013 Feb 1;207(3):520-7. doi: 10.1093/infdis/jis691. Epub 2012 Nov 16.

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Content

LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ACT	Artemisinin based Combination Therapy
ACPR	Adequate Clinical and Parasitological Response
AL	Artemether-Lumefantrine
ART	Artemisinin
ASAQ	Artesunate-Amodiaquine
ATS	Artesunate
AUC	Area Under the Curve
AQ	Amodiaquine
BHQ1	Black Hole Quencher1
BTP/POZ	BR-C, ttk and bab/Pox virus and Zinc finger
BLAST	Basic Local Alignment Search Tool
CNV	Copy Number Variation
Ct	Cycle threshold
CQ	Chloroquine
CYP	Cytochrome P
DEAQ	Desethylamodiaquine
DELI	Double sites Enzyme Linked Immunoassay
DHA	Dihydoartemisinine
DNA	Deoxyribonucleic Acid
dNTP	Dinucleotide Tri Phosphate
ETF	Early Treatment Failure
G6PD	Glucose-6-Phosphate Dehydrogenase
HumTuBB	Human Tubulin
IC	Inhibitory Concentration
IRS	Indoor Residual Spray
ITN	Insecticide Treated Net
KDa	Kilo Dalton

KEAP1	kelch-like ECH-associated protein 1
LUM	Lumefantrine
LCF	Late Clinical Failure
LPF	Late Parasitological Failure
MQ	Mefloquine
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NIH	National Institute of Health
NIAID	National Institute of Allergies and Infectious Diseases
ORF	Open Reading Frame
PC	Parasite Clearance
PCE	Parasite Clearance Estimator
PCR	Polymerase Chain Reaction
Pgh	P glucoprotein homologue
PgMET	*Plasmodium falciparum* tRNA Methionine
P*f*ATP6	*Plasmodium falciparum* Adenosine Tri Phosphate 6
P*f*EXP	*Plasmodium falciparum* Exported Protein 1
P*f*CRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
pfcrt	*Plasmodium falciparum* chloroquine transporter gene
P*f*HRP	*Plasmodium falciparum* Histidine Rich Protein
pfmdr	*Plasmodium falciparum* multi drug resistance gene
P*f*MRP	*Plasmodium falciparum* Multi drug Resistance associated protein
pfmrp	*Plasmodium falciparum* multi drug resistance associated gene
pfmsp	*Plasmodium falciparum* merozoite surface protein
PfNHE	*Plasmodium falciparum* Sodium/Hydrogen Exchanger
Pfnhe	*Plasmodium falciparum* sodium/hydrogen exchanger gene
PPQ	Piperaquine
PRR	Parasite Reduction Rate
QN	Quinine
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
RBM	Roll Back Malaria

RFLP	Restriction Fragment Length Polymorphism
RSA	Ring stage Survival Assay
RT-PCR	Real Time Polymerase Chain Reaction
tRNA	Transfer Ribonucleic Acid
SERCA	Sarco/Endoplasmic Reticulum Ca2+-ATPase
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-pyrimetamine
WHO	World Health Organization
WWARN	World Wide Antimalarial Resistance Network

1 INTRODUCTION

1.1 MALARIA

Malaria is globally the largest vector borne disease with over 200 million new clinical cases happening in low and middle-income countries every year. Presently it has an associated annual death toll of above 600 000 lives, the large majority among children under the age of 5 years and pregnant woman (130, 216). In parallel with this mortality rate, the disease imposes a significant morbidity in society, with economic costs in the poorest part of the world representing up to USD 30 billion lost in Gross Domestic Product per year (168) Management of malaria accounts for 40% of all public health spending in those countries where the disease is a cause, but also a consequence of poverty.

1.2 THE *PLASMODIUM* PARASITE

Malaria is caused by a single cell protozoan parasite of the *genus Plasmodium* comprising more than hundred species (105), Only 5 species infect Humans: *Plasmodium ovale, Plasmodium malariae, plasmodium knowlesi, plasmodium vivax* and *Plasmodium falciparum.* This latest represents the most virulent species, responsible for the severe form of malaria and its highest mortality. Due to its importance in the African Continent, as well as notorious capacity to develop resistance against antimalarial-based therapies, the present thesis is focused in *P. falciparum* malaria.

1.3 *PLASMODIUM* LIFE CYCLE

The full life cycle of the malaria parasite, allowing the disease transmission and its spreading, is based on the alternately infection of human and mosquito hosts illustrated in Fig. 1. It starts with the need of an infected fertilized female anopheles to obtain blood, necessary for its eggs maturation. Upon a blood meal it injects *plasmodium* sporozoites at the same time as anticoagulants from her salivary glands, into the derma. In order to avoid as much as possible the host immune system, the sporozoites rapidly reach the liver, invading hepatocytes. There it starts its first asexual reproduction, developing schizonte forms in which thousands of merozoites develop. This hepatic phase of malaria (sometimes referred as the “silent phase”) is asymptomatic and develops for 5 to 16 days, depending of the species, being 5-10 days for *P. falciparum* (133). In the case of *P. vivax* and *P. ovale* infections, a fraction of the parasites

enrolled in the hepatic stage develop a dormancy state, the hypnozoite. These forms are able to stay dormant for large periods of time. Their reactivation is associated with disease bursts ("relapsing fever"), months or even years after the first infection (126). Mature hepatic merozoites, are released after rupture of infected hepatocytes, reaching the blood stream where they rapidly invade red blood cells (RBCs), initiating a cyclic asexual reproduction of 24 to 72 hours depending on species (*ca.* 48 hours for *P. falciparum*). With each schizont producing up to 30 merozoites, every cycle releases millions of merozoites, causing the clinical effects of the infection. The merozoites swiftly invade new RBCs, reinitiating the intra-erythrocytic cycle, and the destruction of an increasing number of erythrocytes.

In parallel, and responding to still not clearly identified stimuli, a fraction of merozoites exits the asexual multiplication cycle through a process of sexual differentiation. This leads to the development of male and female gametocytes, ready to be sucked during a female anopheles blood meal. Once in the mosquito gut, male and female gametocytes mature becoming fully functional gametes, fuse, and form a diploid zygote. This develops towards an ookinete, which migrates into the mosquito midgut wall. There it undergoes intense asexual division giving rise to an oocyst full of several thousands of haploid unicellular infecting sporozoites. After one to two weeks the oocyst bursts, releasing a large number of mature sporozoites in the mosquito abdominal cavity. This mobile stage then migrates upstream and invades the mosquito salivary gland. The cycle of human infection closes (and re-starts) upon the mosquito next blood meal, by injecting the sporozoites present in its salivary glands into the human host bloodstream.

1.4 THE MOSQUITO HOST

From over 400 species in the *Anopheles* genus, only 30 to 40 of them transmit malaria in natural condition. The highest reproduction rate of anopheles is observed in tropical regions where the humidity and heat are optimal for the mosquito to live long enough to allow the parasite to complete its life cycle into the anopheles host (female anopheles have a life expectancy of approximately one month). The time required for the mosquito to be infective to a human is 10 to 21 days following its infecting blood meal. This timeline depends on the parasite species and the temperature. In Africa, *An. Gambiae* and *An. Funestus* are both strongly anthropophilic, making of them the most efficient malaria vectors worldwide.

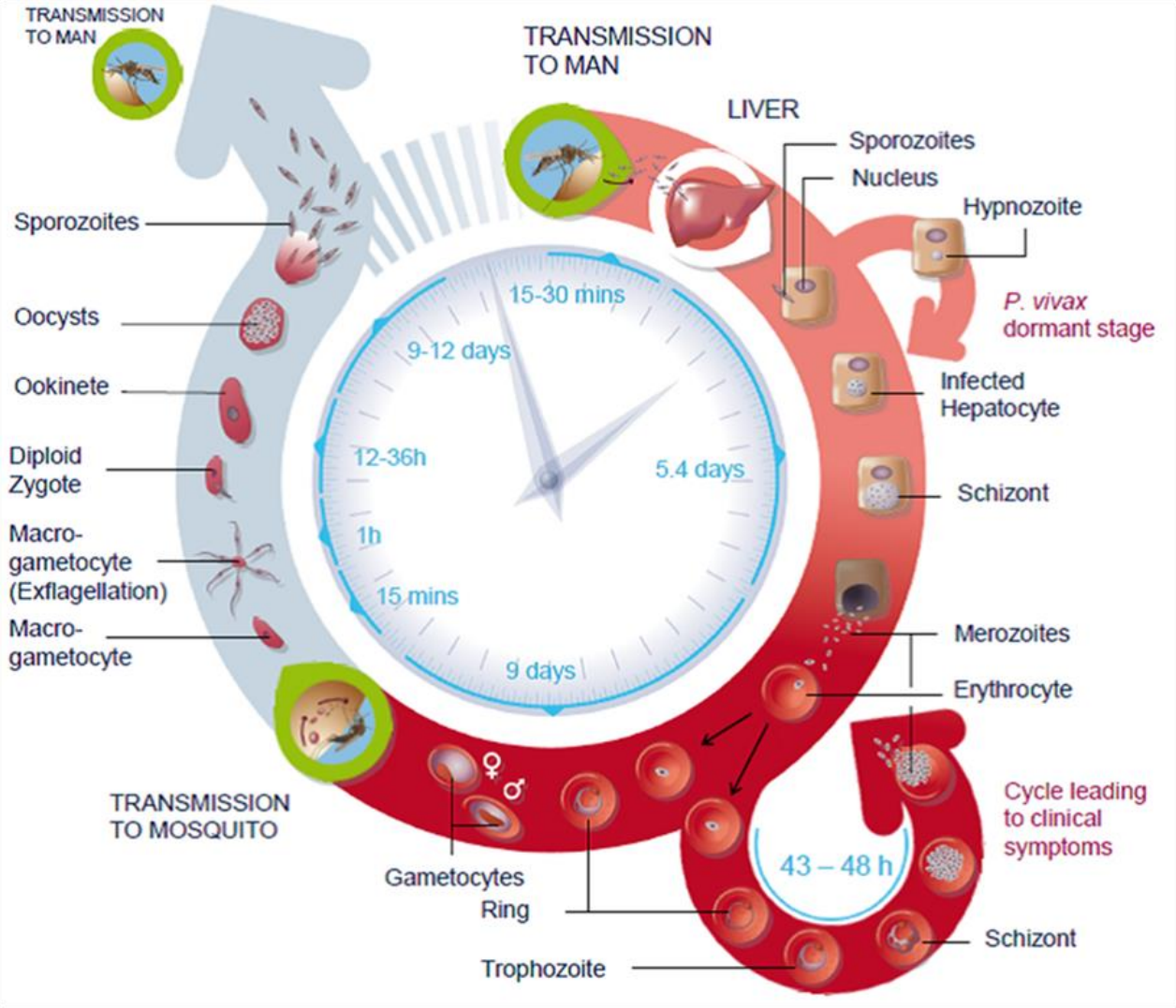


Figure 1 - The malaria life cycle (http://www.mmv.org/malaria-medicines/parasite-lifecycle)

1.5 THE HUMAN HOST – ASPECTS OF SUSCEPTIBILITY

There is strong evidence that humans and the malaria parasites have been evolving together for a large period of time. This co-evolution is visible by the geographical distribution of specific population genetic characters.

It is generally accepted that malaria has its origins in Africa, where some genetic disorders have been shown to be protective against the severe forms of malaria. This is mostly visible in specific hematologic characteristics. Hemoglobinopathies like the presence of hemoglobin C or S are known to limit the parasite development in malaria infected RBCs and have been associated to a protective potential during an acute malaria infection(3, 30, 102), Similarly, deficiencies in the pentose phosphate pathway enzyme Glucose-6-Phosphate Dehydrogenase

(G6PD), as well as Alpha Thalassemia are considered to provide a certain degree of protection against severe malaria (65, 113, 198). The significant presence of those anomalies in Africa, particularly in heterozygote forms, is likely to be the result of the selection pressure of the lethal *P. falciparum* malaria on the human populations, thus modeling some characteristics of their genomes throughout a long co-evolution in the African continent. Similarly, the almost absence of Duffy antibody on melanoderma RBCs make the African populations resistant to *P. vivax* infection, common in other regions, namely in Asia and Latin America. (125)

1.6 MALARIA PREMUNITION

Premunition is the immunity developed to the infection after repeated exposure. It protects the host against hyperparasitemia without clearing the infection (167). It is rapidly and progressively acquired, short-lived, and partially effective (127). Thus, if an individual departs from an endemic area for an extended period of time, he or she is likely to lose the built up premunition, become again susceptible to malaria (127). Antibody action contributes to premunition, although it is generally accepted that this phenomenon is probably much more complex than simple antibody and antigen response (4, 221).

1.7 CLINICAL MALARIA

By the clinical manifestation, malaria can be classified into the most common uncomplicated malaria and its severe, life-threatening, forms.

Uncomplicated malaria

Uncomplicated malaria is characterized by recurrent attacks showing stages of chilling, followed by fever and a sweating phase. Those phases are combined to a number of diffuse symptoms including tiredness, vomiting, abdominal and muscle pains and headache, in absence of clinical or laboratory indicator of severity, including vital organ dysfunction. Anemia states are also frequent. Uncomplicated malaria is more seen among adults in high transmission areas (220), an observation probably related with the aforementioned premonition status which is acquired with several successive malaria infections. Left untreated, the condition poses the risk of developing towards its complicated forms, associated to a severe picture of the disease and a potentially fatal prognosis for the patient.

Severe malaria

Complicated cases of malaria also called severe malaria are typically observed in individuals with a low level of premunition, essentially children under year of 5, pregnant woman and non-immune adults travelling to malaria transmission areas (220). Major clinical manifestations related to severe malaria include prostration, coma, severe anemia, breathing difficulties and low blood sugar. The mentioned prostration and coma symptoms are strongly associated with the specific condition referred as cerebral malaria. This represents one of most deadly manifestation of severe malaria, being a particular characteristic of *P. falciparum* infections; this particular severity of *P. falciparum* malaria is due to the ability of this parasite to express adhesive variant protein on the surface of their host RBC (27, 28, 162). The resulting knobs-like structures mediate the interaction of infected RBC with receptors located at the venal and capillary endothelium, leading to its attachment and cytoadherence. *P. falciparum* infected RBCs are also able to adhere to uninfected ones forming conglomerates designated as "rosettes". Cytoadherence and rosetting are both central to the pathogenesis of *P. falciparum* malaria, resulting in the clogging in vascular structures of vital organs, namely the brain (28, 190).

1.8 MAJOR ANTIMALARIAL DRUGS

We give in here a brief description of the presently used major antimalarials, with a special emphasis on the ones under focus in this thesis, artemisinin derivatives and quinine.

1.8.1 Aminoquinoline structure drugs - Amodiaquine and piperaquine

Amodiaquine (AQ) is an old drug, having been developed in the late 1940s. Its use was limited for decades by the acceptance and implementation of chloroquine (CQ) as the global standard of anti-malaria treatment, as well as by a number of reports of rare - but serious - neutropenia and liver toxicity related side effects observed with prophylactic regimens (68, 78, 132). The use of AQ as a prophylaxis agent is associated with its metabolism, as this drug is extensively bio-transformed towards a main active metabolite desethylamodiaquine (DEAQ), characterized by a very long half-life up to more than 20 days. AQ has been proposed to act in a similar fashion as CQ, i.e. by inhibiting the bio-polymerization or the glutathione dependent destruction of the heme group resulting from the parasite haemoglobin degradation process (72, 225). By dint of its safety, AQ was recovered in the XXI Century as an ACT partner for the treatment of uncomplicated malaria, nowadays available as a

Artesunate-AQ (1:2.7) fixed combination tablet (Cuarsucam/Winthrop, Sanofi-Aventis, Paris), in a three day regimen (141).

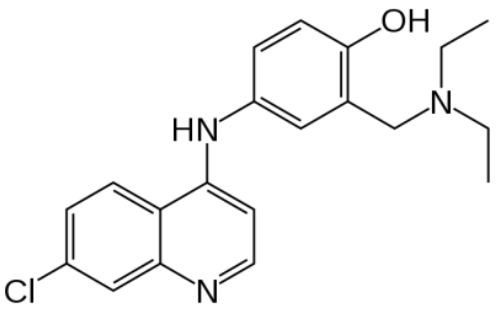


Figure 2: Chemical structure of amodiaquine.

Piperaquine (PPQ) represents a bisquinoline structure related to chloroquine (CQ) synthesized in France for the first time in the 1960s (179). The drug was re-discovered in China during the 1970s, having been developed towards a fully operational antimalarial. It was subsequently launched as a monotherapy regimen in the South of China during the 1980s (84) as a response to the rise of chloroquine resistance. Unfortunately, resistance to PPQ readily emerged, forcing its abandonment in 1992. In 2000, PPQ use was resurrected in combination with dihydroartemisinin (DHA) for the Vietnamese national malaria control program. Its success in SE Asia has prompted the initiative to introduce the DHA-piperaquine ACT as a fixed combination in the African continent. PPQ is a very long half-life drug, making DHA-PPQ particularly interesting as an ACT, due to an expected long post-treatment protection against new infections (39) On the other hand, this long pharmacokinetic tail makes it prone for the progressive selection of resistance, as it has been proposed for lumefantrine (77). Its mode of action is still not known.

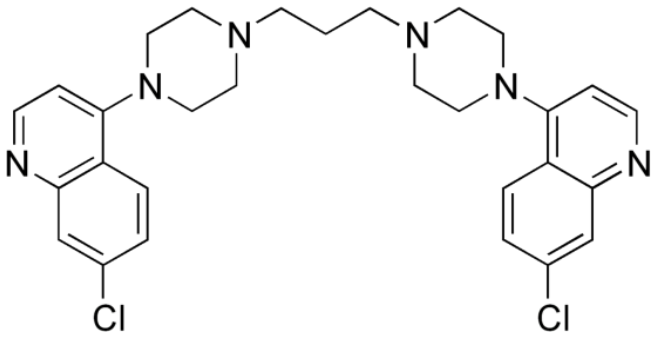


Figure 3: chemical structure of piperaquine.

1.8.2 The aminoalcoholquinolines – mefloquine and lumefantrine

Mefloquine (MQ), a drug resulting from the Vietnam War driven research at the Walter Read Institute, is a very long half-life antimalarial (12-25 days) (42, 43, 214). Massively launched in Thailand during the 1980s as a response to increased CQ and sulfadoxine-pyrimethamine (SP) clinical failure, resistance to this drug readily developed. MQ was partially rescued during the 1990s through its combination with artesunate, constituting the first formal ACT (140). Its long half-life has made this antimalarial attractive for use as a prophylaxis agent for non-immune travellers (66). The MQ mode of action is not completely understood, with some authors proposing to act as an heme biopolymerisation blocker (as CQ), while others providing evidence for an action outside the food vacuole (59, 164).

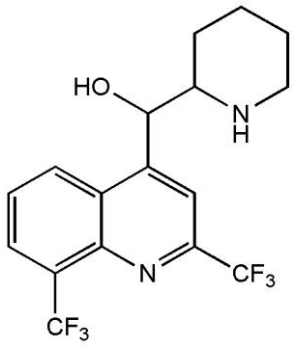


Figure 4: Chemical structure of mefloquine.

Lumefantrine (also called benflumetol), another arylaminoalcohol quinoline (fig. 5) was developed by the National Military Academy of Sciences in China during the 1980s (31). Lumefantrine is at present exclusively used in combination with artemether (Coartem®, Novartis, Basel, Switzerland). Artemether-lumefantrine is globally the most implemented ACT, being present in the malaria control programs of the majority of the African countries (189). Its mode of action is still unknown, although the positive correlations in vitro with MQ IC_{50}'s and potential common aspects of the resistance mechanisms (e.g. both involving the *pfmdr1* gene), suggests a possible overlap of pharmacological targets (158).

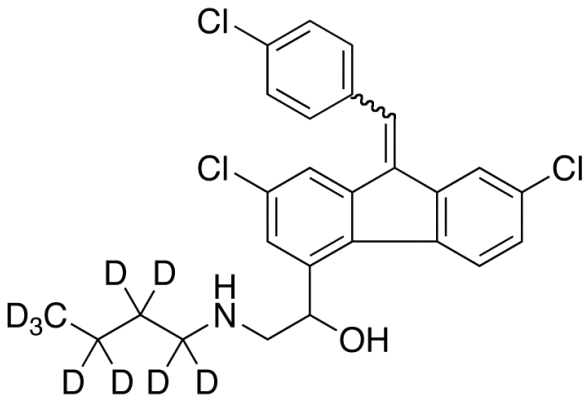


Figure 5: Chemichal structure of lumefantrine (benflumetol).

1.8.3 Quinine and its mode of action:

Quinine is a natural extract from Quinchina bark with a history of efficacy of more than 350 years. Discovered in the Northern Peru during the Spanish colonial times by the XVII Century, it was imported to Europe. For 200 years QN was only available in small quantities, as a powder, used as an anti-fever medicine for the society elites. In 1834 quinine was isolated as the main active principle, which allowed the possibility of its use as a more controlled, purified, drug (74).

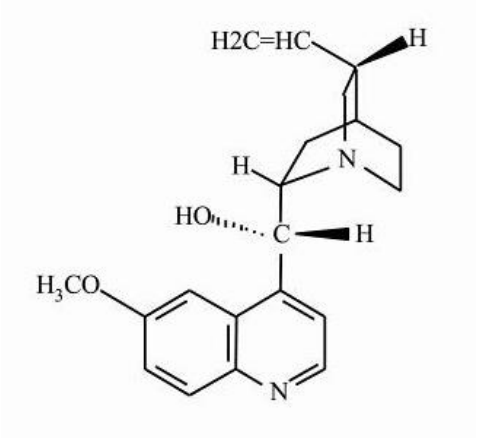


Figure 6: The chemical structure of Quinine.

Quinine is still obtained from its natural source due to the difficulty to synthetized its complex molecule (fig. 6). Quinine constituted the only treatment option for malaria until the emergence in the 1930s and 1940s of synthetic derivatives, namely mepacrine, pyrimethamine and chloroquine. Quinine is described as a blood schizonticide for all the *Plasmodium* species with some gametocytocidal activity against *P. vivax* and *P. malariae.*

1.8.3.1 Pharmacodynamis, and mode of action

The mechanism of action of quinine have not been fully resolved, albeit it is assumed that at least in part is associated with the disturbance of the haemoglobin degradation in the food vacuole, in a way similar to the observed with chloroquine (144). As with mefloquine, it has also been proposed to be able to inhibit the ingestion of haemoglobin by the parasite (59). Quinine has a narrow therapeutic index due to its association with a range of adverse events, including auditory loss ("cinchonism"), hypoglycaemia and hypotension, and a broad range of directly or indirectly associated clinical symptoms. Due to this, quinine should not be applied in any circumstance as a bolus intravenous dose (1, 194). The parasite reduction ratio of quinine has been estimated to be 1:250, leading to the necessity of steady state quinine levels with a minimum plasma concentration of *ca.* 6 µg/mL, throughout the 7 day treatment course, in order to guarantee therapeutic success. (159)

1.8.3.2 Pharmacokinetics

Quinine is a rapidly absorbed when administered orally or intramuscularly, with a peak plasma concentration (C_{max}) reached in 2-4 hours (T_{max}). The drug is characterized by an half-life of ca. 10-20 hours (100, 159). Quinine is mainly metabolized towards the less active 3-hydroxyquinoline through the action of hepatic CYP3A4. Approximately 20% of the administrated dose is unchanged and eliminated renally. The volume of distribution (V_f) of the drug is decreased during acute malaria stages, increasing as the infection is resolved and the patient recovers.

1.8.4 Artemisinin derivatives - the central components of ACT

The active principle of artemisinin is found in a natural herb, *Artemisia annua*, a plant which extracts have been claimed to have antipyretic properties for more than a Millennium in mainland China. In 1967 the Chinese government initiated a large survey programme of the local botanic resources, searching for natural products with therapeutic potential. Following this programme, the works of ancient Chinese tradition medicine were scrutinized, including the manuscripts of Ge Hong from 340 AD, where the use of teas based in "quing hao" (*Artemisia annua*, sweet wormwood) were recommended for attenuating fevers. Artemisinin ("quighaosu") (fig. 7), was isolated in 1972 by Chinese researchers as the active antimalarial constituent present in the plant (95).

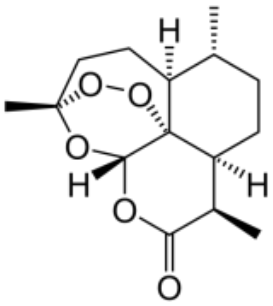


Figure 7: Chemical structure of artemisinin.

Artemisinin itself is not presently used in large scale, having been surpassed by semi-synthetic derivatives, namely the oil soluble artemether and the water-soluble ester artesunate. It is to note that one disadvantage of this presently used first generation semi-synthetic compounds is the absolute requirement of natural artemisinin as starting synthesis material (156).

1.8.4.1 Mode of action and pharmacodynamics

Artemisinin and its derivatives (ARTs), albeit most effective against the trophozoites forms have in general a broader anti-parasitic action as compared with the quinoline based antimalarials, significantly affecting most of the *P. falciparum* intra-erythrocytic cycle stages (197). ARTs selectively concentrate in infected RBCs, as compared with uninfected ones (75). It is generally accepted that the action of ARTs is dependent on their activation, through the collapse of its characteristic intra-molecular peroxide bridge. Conversely, there is no consensus on the central event of ARTs anti-parasitic action (80, 92). Several hypotheses have been put forward for mechanisms leading to fast and pleiotropic effects in the parasite. These can be classified as, (a) through a non-specific action, by generating oxygen radicals (38) which further exacerbate the oxidative stress associated with the parasite hemoglobin catabolism processes (15, 71, 79) or, (b) through the interaction of the drug with specific key molecular targets essential for the survival of the pathogen; these include, to name few, components of the mitochondrial electron transport chain (106), the parasite's SERCA pump (PfATP6/PfSERCA)(55), the redox cycling associated flavoenzyme disulfide reductase enzymes (81), the translationally controlled tumor protein (TCTP)(20, 57), or the essential *P. falciparum* exported protein 1 (*pf*EXP1), a membrane bond glutathione S-transferase (109). It is likely that the action of these drugs might actually involve several mechanisms acting simultaneously, influenced by specific contexts e.g. the genetic characteristics of the parasite, the levels of drug exposure, or the presence of synergistic factors like other antimalarials.

Artemisinins are the fastest acting antimalarials in use, with 48 hours based parasite reduction ratios (PRRs) of 1:10.000 (see section 1.9).

1.8.4.2 Pharmacokinetics

The artemisinin class drugs are rapidly but incompletely absorbed after intramuscular or oral administration. Due to differences in absorbance, significant inter-individual peak concentration (C_{max}) variation is well documented. ARTs undergo significant first pass metabolism. This class of drugs are prone to exhibit time dependent pharmacokinetics involving a phenomenon of self-induction elimination, especially concerning artemisinin, with a significant reduction in its area under the curve (AUC) during the course of 7 doses regimens (10).

Artesunate (ATS) is the most versatile artemisinin derivative in clinical use due to its water solubility. This characteristic allows its application beyond the more common oral route, including injectable and per-rectum route formulations (44). Artesunate represents an emerging alternative to quinine as a rapid treatment agent for situations of severe malaria (51, 52, 174).

Artesunate is extensively metabolized towards the pharmacodynamically active metabolite dihydroartemisinin (DHA) by the hepatic cytochrome P450 system, this being further phase II conjugated through the action of the UDP-glucuronosyltransferases isoforms 1A9 and 2B7 (89). ATS has an extremely short half-life of 15-30 minutes, a characteristic that leaves the possibility of this being essentially a pro-drug (153), with DHA being the main responsible for the antimalarial activity (103, 195). The half-life period of DHA varies inter-individually, in a range of *ca*. 30-120 minutes (93) In vitro data provide evidence for CYP2A6 as the major metabolizing enzyme for artesunate (107).

The use of artemisinin in monotherapy is associated with a high incidence of recurrent infections explaining the need of combination with other antimalarial drugs for a sustainable efficacy.

Due its key importance in the global treatment of malaria, artesunate represents a main focus of this thesis.

1.9 ARTEMISININ COMBINATION THERAPY (ACT)

The operational concept of ACTs is dependent on the extremely fast pharmacodynamics action of its artemisinin derivative partners (ARTs). With a characteristic parasite reduction ratio (PRR) of 1/10.000 in the first 48 hours of treatment (215), its action in the combination allows a fast reduction in the patient parasite load in the first hours of treatment. The remaining parasites are then eliminated by the long half-life partner drug - typically an antimalarial of the quinoline structural class - now left to handle a parasite population thousands-fold smaller than at clinical presentation. Seen in function of time, if the slow acting long standing drug, with a characteristic PRR of 1:100 (data available for mefloquine), would act alone in monotherapy, by the time that would reach the same low levels of parasitaemia (if reaching them at all) its own concentration would be significantly lower, as compared with the situation aided by ART (fig. 8). It is likely that the remaining parasites would then be exposed to sub-therapeutic levels of the drug. This not only would be non-efficacious to eliminate this parasite population, but would also set the conditions for promoting the development of resistance.

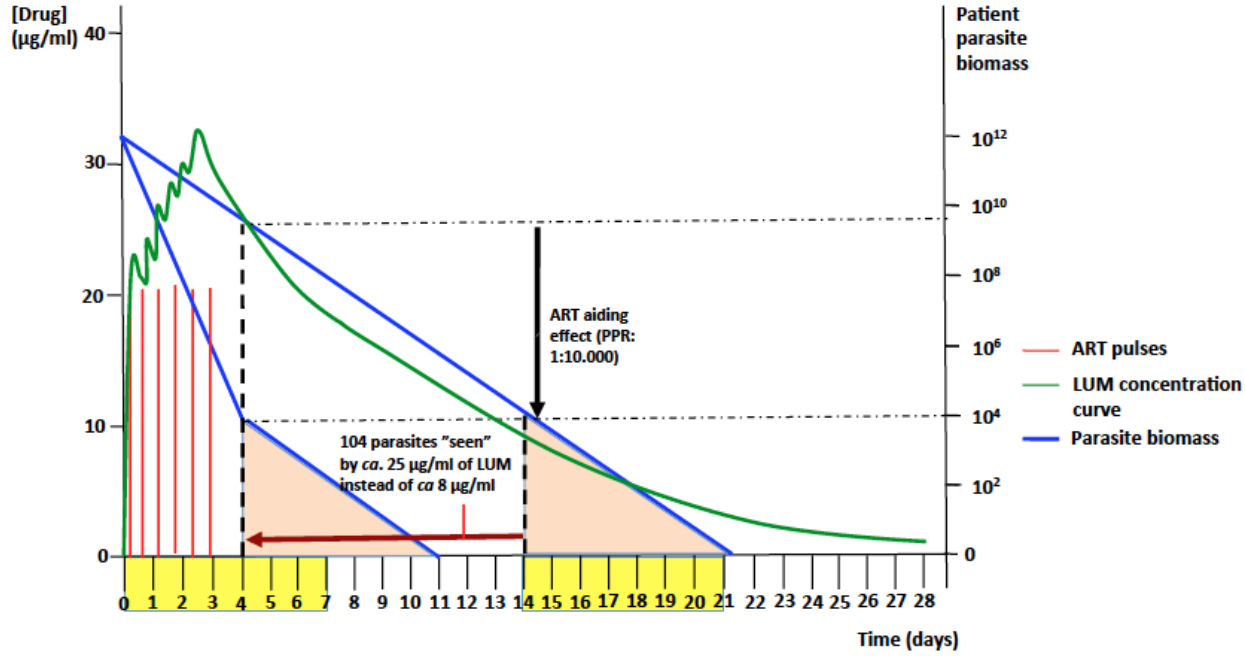


Figure 8 – The concept of ACT action herein depicted with the example of artemether-lumefantrine (AL). In this representation, lumefantrine is considered as having the same parasite reduction ratio (PRR) as mefloquine. The curve is based on published pharmacokinetic data following a standard six AL dose regimen (104). Figure adapted from Piedade and Gil (153)

The ACT partners are hoped to be associated to different mechanisms of action, promoting possible situations of synergy and an expected reduction in probability of resistance emergence. In the moment (November 2014), two major ACTs represent the backbone of most African national malaria control programs: artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ). To this adds the pioneering use of artesunate-mefloquine in Thailand, which represents the first employed ACT in a national malaria program. Two other formulations, representing second generation ACTs, are also emerging: dihydroartemisinin-piperaquine (224) and artesunate-pyronaridine (25).

The first generation ACTs have been notoriously successful, being associated with very significant decreases in malaria incidence worldwide (218). Such success has prompted the reemergence of the national malaria elimination plans in the last years, which include large mass ACT administration plans (186). Unfortunately this drug strategy has not proved to be totally resistance proof, as signs of *in vivo* decreased sensitivity to its components - including the artemisinin derivatives - are emerging, as first noticed for artesunate-mefloquine, in Thailand (29, 157). In this region, ACT came as a response to the fast declining efficacy of mefloquine monotherapy, its introduction leading to a remarkable decrease in clinical failure. But the parasite kept developing eventually becoming less sensitive even to the combination. Although the regimen has been modified and upgraded throughout the years since its introduction (29), resistance to the artesunate-mefloquine combination has been recognized since the late 1990s (155, 157). Partly this evolution can be considered as a consequence of the fact that this ACT was designed in order to increase the useful life span of a failing drug (mefloquine), a strategy no longer supported by the WHO.

In vivo and in vitro resistance to mefloquine has been strongly associated with the presence of increased *pfmdr1* copy number in the multidrug resistance gene (*pfmdr1*) (155, 157), a frequent occurrence in South East Asia and in some regions of South America, but a relatively rare event in the African continent (202).

Soon after its first implementations in Africa, AL was shown to drive the post-treatment selection of the *pfmdr1* a.a. 86N-coding allele in clinical efficacy trials performed in Zanzibar (182, 184). Soon this observation was confirmed in most settings where AL has been trialed (13, 50). Recent studies have further shown that the *pfmdr1* 86N/184F/1246D haplotype is associated with parasite survival at blood drug levels expected to be therapeutically effective (116).

Besides mefloquine and lumefantrine, in vivo resistance to amodiaquine has been observed

(86, 142), even in the context of ACT (87). Finally, concerning piperaquine, part of a second generation ACT planned to be implemented in Africa, it has been long known that the parasite is able to development resistance against these drug, at least when it was employed as a monotherapy in Southern China 25 years ago (44).

In History, *P. falciparum* parasite has never been submitted to such global and diverse drug pressure. It is conceivable that the parasite populations are being stressed to evolve towards molecular mechanisms of multidrug resistance, akin to the observed with neoplastic cells. The development of such broad range mechanisms of resistance carry the danger expressed in the classical concept of multi-drug resistance: that the exposure to one or a limited range of drugs will promote the resistance not only against these, but to a spectrum of other structurally unrelated ones (73).

With a reduction of the long-standing partner efficacy, the ART component will be under increased pressure as, operationally, the combination will progressive become more like an ART monotherapy. This, associated with the referred potential of the parasite to resist to diverse quinoline drugs, raises a concern that represents the aim of the present thesis: the future of the two critical short half-life antimalarials – the artemisinin derivatives as the central components of ACT, and quinine, still the key drug in the treatment of severe malaria.

1.10 *P. FALCIPARUM* DRUG RESISTANCE

Drug resistance has been defined to be "the ability of the parasite species to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limit of tolerance" (216). What this definition implies is that the clinical failure of a treatment should not be immediately considered as a situation of resistance by the pathogen. This could actually be due to a lack of sufficient exposure because of particular individual pharmacokinetics, situations of drug-drug interactions, lack of compliance, etc. But assumed that the exposure is the correct one, the development of resistance can be operationally understood as the collapse of the therapeutic window of the drug. The progressive necessity of ever increasing blood drug concentration because of the decreased sensitivity of the parasite pushes the exposure towards a risk of drug associated adverse events. When clinical cost-benefit of applying the drug is not acceptable anymore, a situation of full resistance has been reached (fig. 9).

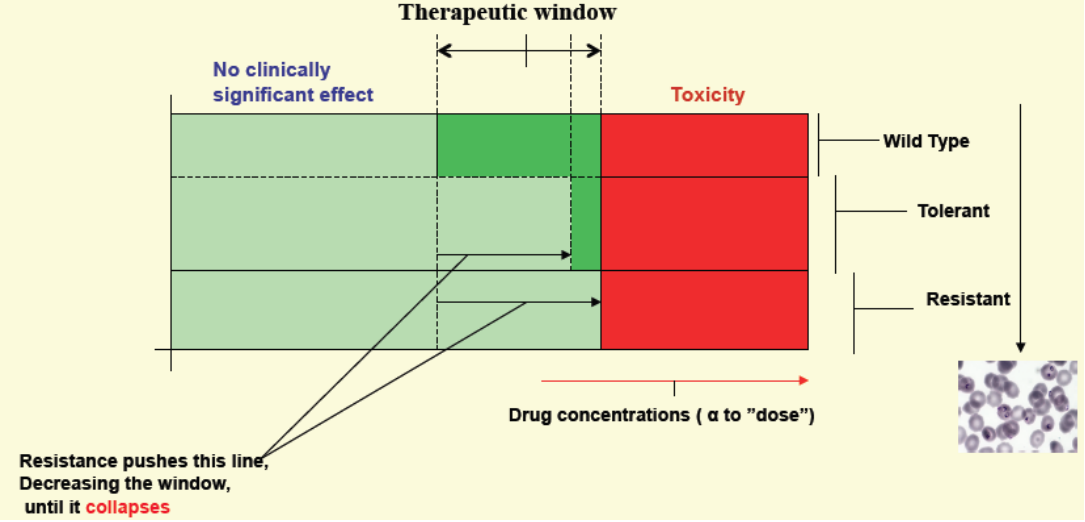


Figure 9 – Drug resistance is ultimately defined as the clinical cost-benefit balance between increasing the drug doses as a response to the decreased sensitivity of the pathogen, and the drug induced toxicity associated with these increased concentrations.

Mechanistically, resistance is associated with the inability of the drug to interact appropriately with its pharmacological target. Briefly, this can be achieved by three general mechanisms:

A. A change or alteration of the drug target.

B. The bio-transformation of the drug, rendering it inactive, an action unusual in the context of the limited parasite drug metabolism capacity, including doubts about the existence of cytochrome P450s in *P. falciparum* (152).

C. Limited access to the target: usually resulting from the action of trans-membrane transporters, which once mutated might be able to increase their capacity of efluxing the drug from its target compartment, or limit their accumulation by decreasing their active import (97, 169).

To note that still other mechanisms, like compensatory mutations increasing the fitness capacity of the parasite, can significantly contribute for its survival under drug pressure (166).

The emergence of drug resistance is dependent on the action of a stressor (generally the drug itself) upon the natural background noise of natural mutation rates in the parasite genome. Many of these rare events correspond to mutations that drive changes in essential proteins leading to a putative decrease on their functions. These populations of parasites normally do not thrive in the general population, due to the decreased fitness associated with these mutation carriers under normal (i.e. drug pressure free) environments (82, 166). But upon a sufficiently intense stressor the cost benefit can change, as the decrease in fitness is

compensated by a vast superior survival rate under drug exposure (67). This has been observed, for example, by the selection of *pfmdr1* duplications - a mutation otherwise rare in Africa - in Ghana, upon the pilot use of sub-therapeutic levels of mefloquine (123, 200).
The network of factors involved in the selection of these rare events is complex, including pharmacokinetic factors, treatment compliance, levels of immunity (influencing the parasitemia burden while allowing thrive of less fit, potentially resistant parasites), among others. Anyway, conceptually, drug resistance development strongly relies in repeated events of sub-therapeutic drug exposure. It is in this context that the aforementioned pharmacokinetic mismatch present in the available ACTs is of concern (61).

1.11 *P. FALCIPARUM* ARTEMISININ RESISTANCE

1.11.1 Origins and operational definition

The present operational concept "artemisinin resistance", assumed during the works of this thesis, is defined by the occurrence of delayed parasite clearance observed after treatment with an artesunate monotherapy, or ACTs. Essentially, the present concept of artemisinin resistance is essentially only based in the clinical/parasitological response to artesunate monotherapies. In brief, the criteria are: recrudescence inside a 28 day post treatment follow up, upon a supervised 7 day regiment, with confirmed plasma levels indicating appropriate exposure, an initial parasitemia <100.000 parasites/µL, significantly increased parasitaemia clearance time, and exclusion of reinfection events.

Artemisinin resistance was firstly suggested in the beginning of the 2000s upon several observations of treatment failures after the newly implemented ACTs (2, 205) (Table1). Due to the confounding effect of the presence of the long half-life partner, the performance of artesunate monotherapy efficacy trials with exploratory objectives was subsequently proposed (135). The first careful study with such a design was performed in the Southern Thai-Cambodian border regions (137-139). It effectively showed the presence of a fraction of infections with significantly increased clearance times, associated with clinical failure (i.e. recrudescence), as well parasites with increased DHA IC_{50}'s. This data has been subsequently confirmed with larger studies, albeit with no correlations found with ex vivo DHA IC_{50} values (53). This lack of correlation has been justified by the inadequacy of the present in vitro methods to evaluate short-term fast acting drugs as artemisinin and its derivatives (5). The issue has been recently claimed to have been resolved with a new ring stage in vitro test design (222). This method was pivotal in the recent identification of genes polymorphism

(K13 propeller) involved in the great Mekong sub region to the parasite resistance to artesunate (7, 9, 193).

Albeit many issues concerning the claims of artemisinin resistance are still to be resolved (98) and advise caution (58), the reality is that they are important for at least one reason: recent reports have shown that it is likely that the therapeutic index of artemisinin (in this specific report, artesunate) is narrower than initially thought. Bethell *et al* have shown in Cambodian subjects that a relatively small increase in the 7 day regimen dose from 4 to 6 mg/Kg gives rise to an unacceptable risk of neutropenia ((19).

Table 1: Summary of the status of artemisinin resistance in the Greater Mekong Sub-region
WHO global malaria Program, http://www.who.int/malaria/publications/atoz/update-artemisinin-resistance-jan2014/en/

	artemisinin resistance		containment activities started	AL		AS-MQ		DHA-PPQ	
	suspected year of emergence	detected		D3+	TF	D3+	TF	D3+	TF
Cambodia	2001*	2006	2009	♦	♦	♦	♦	♦	♦
Laos	2013	2013	2014	♦	–				
Myanmar	2001*	2008	2011	♦	–	♦	–	♦	–
Thailand	2001*	2008	2009	♦	♦	♦	♦		
Viet Nam	2009	2009	2011					♦	–

Legend: first-line treatment; * detected retrospectively using molecular markers or retrospective data;
♦ observed to be > 10%; – observed to be < 10%; blank = undetermined

1.11.2 Markers of resistance

Due to the global potential importance of the arteminin resistance phenotype, intense research has been conducted trying to understand the molecular basis of inter-parasite differences in artemisinin response. A number of candidates showing mutations possibly associated with such phenotypes have been proposed. These have emerged from different approaches, including in vitro or ex vivo gene/phenotype associations, animal models, and in vivo analysis of treatment outcomes. This set comprises a number of genes coding for confirmed and putative drug transporters like *pf*MDR1/Pgh (157) *pf*CRT (177), *pf*MRP1 (160) and *pf*MDR6 (209) potential targets as *pf*ATP6 (99) and *pf*TCTP (20, 56). Some of these transporters are described in further detail in the 1.12 section. Also, more general homeostasis related enzymes, essentially derived from animal models, have been considered, as *PcUBP1* (ubiquitin C-terminal hydrolase)(88). To this adds the aforementioned K13 propeller gene, associated with delayed parasite clearance times in South East Asia (7).

1.12 DRUG RESISTANCE ASSOCIATED GENES

A brief introduction to the main genes associated with antimalarial drug resistance studied in the context of the present thesis.is presented.

1.12.1 Plasmodium falciparum chloroquine resistance transporter (*pfcrt)*

This 13 exon gene located in chromosome 7, codes for a 424 a.a., 10 transmembrane domain protein localized on the parasite food vacuole membrane (63). It has been described to play a key role in *P. falciparum* resistance to chloroquine. The encoded protein *Pf*CRT is proposed to be a member of drug metabolites transporter superfamily (120). The presence of single nucleotide mutations (SNPs) in *pfcrt* can confer to its coded protein the capacity to transport the chloroquine out of the digestive vacuole (26, 119, 172). The K76T mutation has been identified as the key change in the development of chloroquine resistance (178), possibly supported in vivo by a number of other SNPs along the gene open reading frame (ORF)(47). The efflux of chloroquine (172) out of the food vacuole is expected to decrease its concentration in its lumen (26, 121, 172). Such action is consistent with the development of a chloroquine resistant phenotype as the target of chloroquine (CQ), the heme biocristalization process following the digestion of hemoglobin, is specifically situated in this organelle.

pfcrt SNPs have also been found to be associated with the in vitro and/or in vivo parasite response to LUM (183), MQ (178), quinine (60), artemisinin (33, 34, 178), and possibly PPQ (54). Consistent with these observations, recent in vitro data has pointed for the capacity of this protein to transport antimalarials besides CQ (16).

The normal (i.e. physiological) functions of this protein are still not completely understood. Recent evidences point for a potential capacity to efflux peptides and glutathione (148).

1.12.2 *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*)

The discovery of *pfmdr1* was inspired by the homology with the p-glycoptotein (Pgp), a human ATP binding cassette transporter associated with multidrug resistance in cancer. *pfmdr1* represents an intronless gene located at chromosome 5. It codes for the P-glycoprotein homologues (Pgh), a protein of 1419 amimoacids, dependent on the extension of a central polymorphic asparagine based repeat segment. Pgh is essentially located in the food vacuole FV membrane, with a small fraction present in the plasma membrane (108). It is probably oriented towards the lúmen of the vacuole (164). Polymorphisms in *pfmdr1*, including increased copy number and sequence variation (specially N86Y, 1034, 1042 and

D1246Y) have been reported to modulate the parasite susceptibility to mefloquine(37, 52, 112), halofantrine (161, 175, 176) lumefantrine(117, 182, 184), quinine (161), DHA (157), artemisinin (161, 203), chloroquine (14), amodiaquine(85, 87) and piperaquine (204). *In vitro* approches have supported the view that Pgh functions as a drug transporter (171, 173).

1.12.3 *Plasmodium falciparum* sodium/hydrogen exchanger (*pfnhe1*)

pfnhe1 codes for a putative member of the Na^+/H^+ exchanger family of transmembrane proteins. It was discovered upon a quantitative trait *loci* based analysis of the HB3 X Dd2 *P. falciparum* clone cross, previously used for the isolation of *pfcrt* (211, 212) but now taking quinine susceptibility as the phenotype of interest (60). The gene is intronless and situated on chromosome 13. It codes for a large 226 KDa transporter (*pf*NHE1), comprising 12 trans-membrane domains. The intracellular localization of *pf*NHE1 is still under discussion (134), albeit it is considered that it is primarily located in the plasma membrane (17). A complex microsatellite *locus* named ms4760 has been discovered in the 3' region of the gene. Its diversity is mainly defined by variation in the number repeats in two microsattelites, DNNND (denoted block II) and DDNHHDNHNND (block V). Significantly higher IC_{50} is observed among carriers of the ms4760-1 allele (2 DNNND copies) (60).

The physiological function of *pf*NHE1 has been proposed to be associated with the regulation of the parasite cytoplasm pH (17). This function is still under discussion (163) as it has been challenged under technical basis (134, 187). Also controversial is the association of *pfnhe1* with quinine susceptibility itself. A reasonable number of studies have been performed, both concerning culture-adapted parasites (as with M. Ferdig and colleagues seminal report) and *ex vivo* approaches. The results have been contradictory, with some studies supporting a positive association while others not (6, 12, 24, 83, 124, 143, 149, 181, 206).

The full quinine resistance phenotype is likely to be multi-genic, at least involving also the aformentioned *pfmdr1* and *pfcrt* genes, as well as others (e.g. *pfmrp1*, see section below), and the recently unveiled MAL7P1.9, coding for an HECT ubiquitin-protein ligase (170).

The mechanisms specifically associated with the contribution of the *pfnhe1* ms4760 alleles is unclear, albeit it has been proposed that the action of quinine could be modulated by changes in the parasite intracellular pH. Such changes could result from alterations in the capacity of transporting H^+ by *pf*NHE1, resulting from different conformations driven by the DNND and DDNHHDNHNND polymorphisms (17). Evidently, the validity of this hypothesis is strictly dependent on the aforementioned debate on the importance of *pf*NHE1 as a player in parasite pH homeostasis.

1.12.4 *Plasmodium falciparum* multidrug resistance associated protein (*pfmrp1*)

The *pfmrp1* gene codes for a large 12 transmembrane domain ABC-transporter, *Pf*MRP1, located in the parasite plasma membrane (70, 96). It has been proposed to act as a GSH/GSSG pump (22) involved in the REDOX stress management of the parasite. It is also expected to be able to transport a large range of drugs. These functional assumptions are supported by studies based on the targeted disruption of *pfmrp1* in the W2 clone (160). The resulting genetically modified parasites showed not only an accumulation of oxidized glutathione (GSSG), but also an increase in the sensitivity to a number of drugs, including chloroquine, quinine, piperaquine and – most importantly - artemisinin. This effect was shown for some of the tested antimalarials (chloroquine and quinine) as the result of a decreased accumulation in the parasite, indicating an efflux activity of *pf*MRP1.

pfmrp1 SNPs have been linked with the in vivo parasite response to ACT. This was concluded from the observation of significant selection patterns of the I876V a.a. position upon artemether-lumefantrine treatment (41) and K1466R with sulfadoxine-Pyrimethamine (41). In vitro based reports, including the one part of the present thesis (203)(see results section), have also provided evidence for the potential importance of *pfmrp1* SNPs in modulating *P. falciparum* drug sensitivity, namely the I876V and H191Y (128, 151) with chloroquine, as well as F1390I (128, 151) with quinine.

1.12.5 *Plasmodium falciparum* K13 propeller gene

This gene has been described in *P.faciparum* in homology to the human *KEAP1* gene. The 726 amino acids protein contains an N terminal containing a *plasmodium* specific sequence, followed by a BTB/POZ domain, and finally by the kelch propeller domain towards the C terminal. The K13 propeller has been so far studied in *Plasmodium falciparum* in *in vitro* adapted parasites that underwent several years of exposure to increasing doses of artemisinin. Throughout the process, the exposed parasites gradually accumulated a number of SNPs into the C terminal Kelch propeller domain. Some of these SNPs have showed to be correlated with the rate of survival rings after the RSA (Ring Stage Survival Assay) (7). This association was confirmed in a number of field isolates from Cambodia (fig.10). Finally, these mutations were also observed to be associated with Day 3 positivity upon ACT treatment. As a result, a set of K13 propeller domain mutations has been proposed in Cambodian plasmodium isolates to be associated to the *in vitro* and in vivo resistance to ART Four main alleles where observed as significantly involved: C580Y, R539T, I543T, and Y493H.

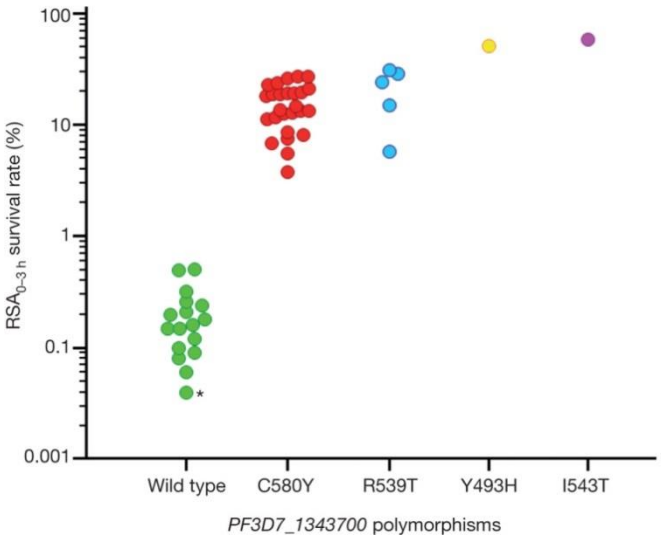
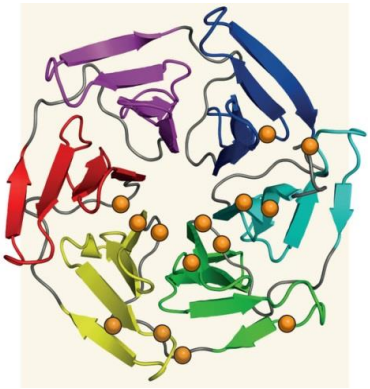


Figure 10: Survival rates of Cambodian parasites isolates in the $RSA_{0\text{-}3h,}$ stratified by K 13 propeller allele. *Reproduced from Nature 505, 50–55 (02 January 2014), with the permission from the publisher*

Importantly, the gene seems to be able to accommodate significant polymorphism, with 17 non-synonymous SNPs, having been found in Cambodia (7), showing that probably there is considerable room for structural changes in this protein. As in many Kelch proteins, mutations in the kelch domain are predicted to alter the protein structure or modify the charge altering in the same way the protein biological function. Such changes could eventually allow the emergence of a protein better suited to deal with the specific stresses associated with ART exposure.



Fugure 11: tridimensional representation of the Kelch protein K13 propeller of plasmodium falciparum with the mutations position represented in orange dots. *Reproduced from Plowe et al, Nature 505, 30-31 (02 January 2014), with permission from the publisher.*

It became as such important to better understand the origin and distribution of this biodiversity in several different settings. ACT treatment efficacy studies were assessed in different settings in Africa, India and South-East Asia. The K13 propeller mutations appeared to be significantly associated to a mean increase in parasites half-life in South-East Asia, but

not in Africa and India. In both India and Africa, the K13 propeller SNPs, when present, were different from the one previously described as artemisinin resistance potential markers (9). Further investigations aimed to find a common genetic origin to this polymorphism (mutants K13 propeller) has showed different strains background for the South African parasites and the Asian ones which also has emerged and spread independently throughout South-East Asia (193).

The function of the encoded protein (fig.11) is still under speculation. Its human homolog KEAP1 has been described in lung cancer cells as interacting with the Nrf2 by sequestration of this protein in the cytosol. Under oxidative stress, Nfr2 is liberated from the complex Nfr2/KEAP1 and induces a cytoprotective response (147). These models have been extrapolated to the *P.falciparum* K13 propeller for whom antioxidant response is high in late trophozoite stages, where the hemoglobin digestion is considerable (23) The propeller could serve the KEAP1 functions in the parasite, albeit no *P. falciparum* Nrf2 homologue has been identified yet.

1.13 DRUG RESISTANCE ASSESSMENT TOOLS

Malaria parasite resistance is assessed by several different methods. This being a complex phenotype, the several methods are expected to complement each other and being consistent with the expected phenotype. The four main approaches are briefly presented here i.e. *in vivo*, *ex vivo*, *in vitro* and subsequent molecular methods (57)

1.13.1 Resistance assessment *in vivo*

This is usually based on drug efficacy clinical trials with patient being followed up for a specific period after the therapy, typically with end points at post-treatment initiation days 28 to 52, depending on the drug under study. During the follow up, patients are checked for new infections and according to the time to recurrence during the follow up the resistance can be classified as RI (parasiteamia after day 14), RII (parasiteamia before day 14) or RIII (persistent microscopy detectable parasiteamia from day 0 infections) (150, 210, 219). Due to the difficulty of applying such classification in high transmission settings, the WHO developed in 1996 a modified protocol based on the clinical outcome targeted at a practical assessment of therapeutic response in areas with intense transmission (216). Therapeutic response is presently graded according to an outcome criteria as: (a) Adequate clinical and Parasitological Response (RCPA), (b) Early Treatment Failure = ETF, (c) Late Parasitological Failure = LPF, and (d) Late Clinical failure = LCF. All this data are validated only when the efficacy is adjusted by a molecular tool expected to distinguish recrudescent

parasites from new reinfecting ones (185). This method used alone has limitations when the drug differential pharmacokinetic in individual, the compliance to the treatment and multiplicity of infection are considered (118). As such, to this information on individual plasma or whole blood drug levels should be added. These observations can be further complemented with *ex vivo* tests (see below).

1.13.2 *Ex vivo / in vitro* assessment of drug resistance

These have been developed on the basis of a micro-test (drug test in limited volume multi-well plates) either on parasitized blood samples directly collected from the patient (*ex vivo*) or after the adaptation of parasites to long-term culture in the laboratory (*in vitro*). This allows obtaining an objective quantitative level of the parasite sensitivity to the drug of interest, most frequently expressed as different levels of inhibitory concentrations (ICs). The most common standard values are the IC_{50} (inhibiting 50% of the initial parasiteamia growth), IC_{90} and IC_{99}.

Parasite growth inhibition can be measured by different techniques, the main ones being:

(a) The WHO drug sensitivity test, based on the morphological estimation of parasite growth by the counting of the number of parasites that were able to progress until the schizont stage, after 24 hours of drug exposure. This assay is economical and simple to perform in the field, hence frequently used in *ex vivo* based evaluations. However, it is very laborious and subject to individual variability in the interpretation of the microscope readings.

(b) The DELI (Double sites Enzyme Linked Immunoassay) methods are based on the measurement by ELISA of HRP2 (Histidine Rich Protein) (136) or the pLDH (Parasite Lactate Dehydrogenase)(91), two proteins that are continuously expressed in growing parasites, being as such proxis of its metabolic activity. These two DELI methods are more sensitive than the classical WHO method, needing lower parasites densities. However they are both more expensive and difficult to set on field condition.

(c) The isotopic method which is based on the measurement of a [$_3$H]-labeled hypoxanthine (46), which is being incorporated in the course of the metabolic activity of the parasite and may be measured in a liquid scintillation counter. This method has a high degree of reproducibility and allows for the screening of a large number of *P. falciparum* strains under highly controlled conditions. However it needs well-equipped laboratory facilities, involves the handling of radioactive material, and requires high parasite densities. It is essentially not viable for large scale field work.

(d) The Sybr green method based on the measurement of parasite DNA. Sybr green is a DNA intercalating dye, the parasites growth measurement being done by quantifying the dye incorporation in the nucleic acids, as a measure of cell multiplication (11, 90). This technique is easier, faster and has a reduced cost compared to the DELI methods but it is mainly applicable to *in vitro* studies with culture adapted parasites. The presence of human DNA in field samples represents a significant confounding factor for the evaluation of the parasite growth *ex vivo* by these methods (208).

These tests reflect intrinsic antimalarial drug resistance of the parasite. They not only yield quantitative results, but also determine the phenotype of the parasite independently of the immune and physiopathological conditions of the host. On the other hand, the correlation of *in vitro* response with clinical response in patients, particularly in the definition of in *vitro* cut offs as informative parameters about *in vivo* responses, is challenging.

1.13.3 The molecular methods

Molecular tools of resistance assessment are per nature second generation technologies, as they are dependent on the definition of phenotypes and their subsequent association with specific parasite genetic characteristics. Due to its characteristics, this approach is useful for the downstream analysis of archive material, or clinical material (typically small quantities of filter paper-preserved peripheral blood) transported from remote areas (48). Most of these methods are based in the PCR amplification capacity.

Several molecular tools are used to define the parasite genotype, including PCR-RFLP, q-PCR and direct PCR amplicons sequencing, to name the most commonly used ones. The molecular approaches are potentially most relevant in terms of cost, time and ease of performance. A large advantage with molecular marker based resistance vigilance, as compared with the much more resource consuming drug efficacy clinical trials, is that it is possible to scale up the coverage of the surveillance even when the patient population is small and time points are scattered (49). Such considerations assume that the tested molecular marker is accurate enough to predict the treatment failure by direct correlation to its allele status. This represents a significant challenge, as it is difficult to define a biomarker reflecting the clinical level of the drug sensitivity *in vivo,* as many other parameters interacts especially human host factors. So far, no molecular marker is presently available with levels of specificity and sensitivity compatible with the demands of replacing phenotypic determinations of resistance, particularly in the present context of ACT. An appropriately informative molecular tool is likely to be complex and multi probe based, including molecular factors from both the parasite and the patient (69).

1.13.4 The specific case of artemisinin derivatives

The classical measurements tools above mentioned have been considered as not ideally adequate to detect an early sign of tolerance to treatment when using artemisinin derivatives, so some methodological modifications have been proposed:

(1) In vivo Parasite Clearance Estimation (PCE)

This tool has been developed to estimate Artemisinin efficacy after the emergence of resistance to these drugs threatening the survival of the currently used ACTs (137). It is based on the estimation of dynamics characteristics of the initial parasiteamia clearance upon *in vivo* artemisinin derivatives exposure. Microscopical observation during the treatment course provides the clearance profile of each patient, with these data being used to generate pharmacodynamics metrics, namely the clearance rate and clearance time. These are calculated using an online tool of PCE developed by the WWARN. This PCE allows the detection of early signs of parasite resistance to ART, for which clear characteristics for resistance are yet to be defined (e.g. cut off IC_{50}s for its main drugs, artesunate, artemether and DHA). In this approach, a significant change in the clearance characteristics leading to a longer clearance is considered to be a sign of emerging resistance to ART.

This tool is sensitive to population diversity in malaria immunity levels, as well as to several modalities of malaria microscopy (smear preparation, staining and reading), which can significantly affect the estimated parasite clearance rate.

(2) In vitro/ex vivo Ring Stage survival Assay $RSA^{0\text{-}3h}$

This approach aims to determine the percentage of viable parasites at 72 hours, following a 700-nM, 6-hour pulse of DHA. Samples suitable for testing are either adapted parasite lines (synchronized at the 0-3 hour post-invasion ring stage) in the *in-vitro* $RSA_{0\text{-}3h}$ or clinical parasite isolates (freshly obtained from patients with malaria and *P. falciparum* mono infection, which are per norm naturally synchronized in the ring stage) in the *ex-vivo* RSA. This tool is used in complement to the described *in vivo* method and cannot stand by itself as autonomously defining the resistance level of a given parasite population. It has been proposed as a step in anticipating the research of new molecular markers (7).

2 AIM OF THE THESIS

The overall aim of this thesis was focused in the early detection of *Plasmodium falciparum* decreased sensitivity to quinine and artesunate in correlation with the molecular bases of resistance.

Specific aims

Study I: To determine for the first time the role of *pfnhe1* polymorphism in *plasmodium falciparum* sensitivity *in vivo* based on a quinine efficacy trial in Mali.

Study II: To search for novel markers, particularly concerning drug transporter genes, of *In vitro* antimalarial susceptibility through the analysis of adapted *P. falciparum* isolates from one of the global *foci* of multidrug resistance in SE Asia.

Study III: To measure the Parasite clearance time in *P. falciparum* in Mali using the microscopy assessment of parasite clearance after a treatment by artesunate in monotherapy

Study IV: To determine the K13 propeller gene diversity in Mali, searching for correlations with the parasite clearance time after ART treatment.

Study V: To evaluate the use of qPCR as a second generation approach for a more sensitive evaluation of parasite clearance, and revisit the potential correlations with the identified K13 propeller polymorphism in Mali.

3 MATERIAL AND METHODS

3.1 STUDY SITES AND CLINICAL TRIALS

The clinical studies included in this thesis were all conducted in Mali, West Africa. The trials concerned four rural malaria endemic areas, Faladje, Kolle, Bougoula-hameau and Bandiagara (fig. 12) The studies took place from 2005 to 2007 for study I, in the period 2010-2011 for Study III and V. Concerning study IV, this represented two sets of retrospective samples collected in 1999-2002, 2003-2005 and one concerning the 2010-2011 period, obtained in frame of the present thesis.

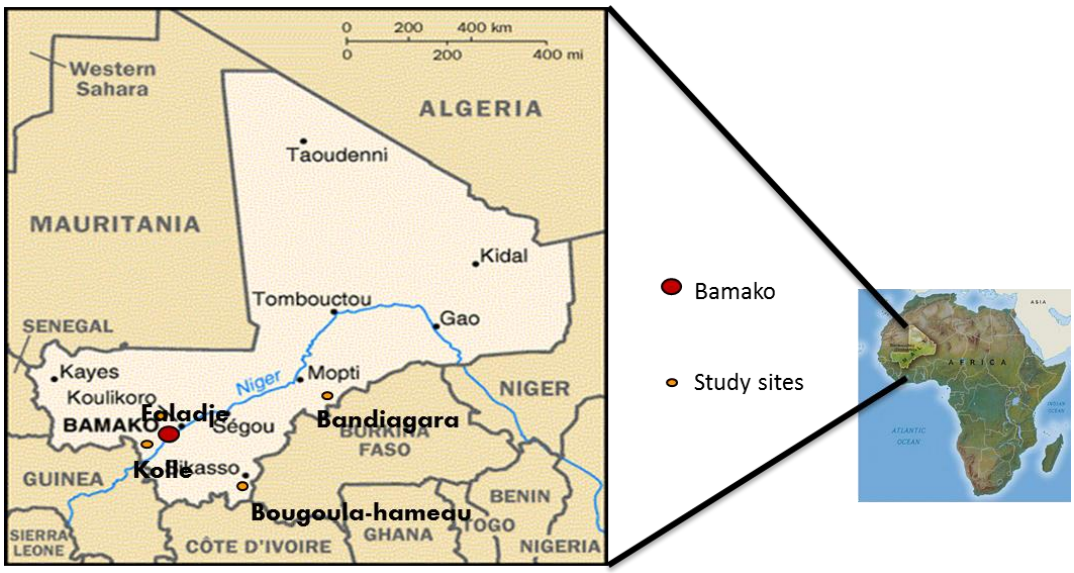


Figure 12: Study sites localization on the Mali map
Published with the kind autorisation of Professeur A.Djimde, (WANECAM)Malaria Reserch and Training Center, USTTB, Mali.

3.1.1 Faladje and Kolle

These are two rural villages at, respectively, 80 km northwest and 57 km southern of the capital city of Bamako, Mali. Malaria endemicity in both locations is seasonal mainly occurring between July and December in both areas. With maximum rainfalls in August and September, the climate is predominantly soudanian (213). *P. falciparum* infection represents the majority of clinical malaria cases (94, 196)

A longitudinal clinical study of quinine efficacy was conducted in Kolle and Faladje during the period between 2005 and 2007. Children presenting with severe case of *P.falciparum* malaria

were included for a 28 days follow-up and treated with intravenous quinine administered in 10% hypertonic glucose solution at 12.5mg/kg QN twice daily for 5 days. In 2008, the regimen was changed to 8 mg/kg 3 times daily for 7 days with a loading dose of 12.5mg/kg the first day.

3.1.2 Bougoula-Hameau

A prospective artesunate monotherapy efficacy study was conducted in Bougoula-Hameau, a peri-urban village where malaria is holoendemic. Situated in southern Mali (350 km from Bamako), climatically it represents a sudanian type zone with a landscape dominated by savanna woodland, with tall grass dotted with trees. The climate is under the influence of the humid forest zone with an annual rainy period extending for six months or more (from May to October or November) (213) The trial was performed at the Bougoula-hameau health center (fig. 13) from December 2010 to February 2011 and included 100 children with ages from 1-11 years old presenting clinically confirmed uncomplicated *P.falciparum* malaria.

Figure 13: Bougoula-hameau health center

As for the data and samples before 2010, these were provided from an artesunate monotherapy arm included in a clinical efficacy trial conducted in 2002-2005. For both periods the treatment

was a 7 days artesunate at 2 mg/kg per day with a loading dose of 4 mg/kg the first day. Participants were followed for 28 days.

3.1.3 Bandiagara

Bandiagara is a semi-rural area of approximately 13,000 inhabitants in the north-eastern of Mali at about 700 km from Bamako, situated on a rocky plain above the Dogon escarpment. It receives a mean annual rainfall of 600 mm, supporting a setting of meso to hyperendemic malaria, with highly seasonal transmission (154). In this area 97% of malaria cases are associated with *P. falciparum* infections (35, 114). Samples from uncomplicated malaria patients were collected in this location as a crosectional survey from studies conducted in 1999-2003 included in the analysis of the novel K13 propeller gene.

3.1.4 Mae-sot

The Mae Sot District is situated in the Tak province, Western Thailand, bordering Myanmar (Burma). As the south-east province near Cambodia, this region is known to be an epicenter of malaria drug resistance emergence (131, 223). *P.falciparum* malaria represents 40% of the clinical cases (217). Infected erythrocytes were collected from clinical cases between 2002 and 2008 and cryopreserved at the Sokhlo Malaria Research Unit. Parasite adaptation was performed from this clinical material at the Karolinska Institute, followed by *in vitro* drug susceptibility analysis (IC_{50} determinations).

3.2 SAMPLES AND DATA COLLECTION

Study I was based on a clinical trial conducted in Kolle and Faladje from 2005 to 2008, the purpose was to collect parasite DNA from dried blood spots realized during a 28 days follow up. Parasite genotyping could thus be realized for *pfnhe1* and *pfcrt* diversities. Clinical and parasitological parameters where also collected to assess the *in vivo* efficacy of QN treatment.

Study II samples involved culture adapted isolates collected in *P.falciparum* malaria clinical cases in Mae-sot prior to treatment. Adapted parasites were then used to assess the *in vitro* drug susceptibility.

Study III was involved the performance of thick and thin blood slides in Bougoula-Hameau in 2010-2011 to microscopy assess the clearance of parasite infections after artesunate administration; clinical parameters were also collected for the *in vivo* efficacy assessment.

Retrospective data from a study conducted in the same area by the same team in 2002-2005 for artesunate monotherapy efficacy were also considered.

Study IV used DNA of parasites collected on blood filter papers prior to antimalarial drug administration, during the artesunate monotherapy efficacy study at Bougoula in 2010-2011. For comparative objectives, filter paper preserved blood collected prior to this study in the same area, between 2002 and 2005, was also used in this investigation. Finally, samples cross sectionally collected in Bandiagara during a period between 1999 and 2002 were also included.

Study V used DNA extracted from blood preserved in filter papers obtained from the aforementioned artesunate monotherapy efficacy study conducted in Bougoula in 2010-2011. DNA was extracted from blood spots representing successive time point samples from the same patient after drug administration, and used to measure the parasite clearance using a method based on Real Time PCR.

3.3 ETHICAL CONSIDERATION

Informed consent forms were provided by an indicated field team agent in the local language. Inclusion in the trial proceeded upon signed acceptance of its terms and conditions signed by the patient or his/her guardian as appropriate.

Study I was approved by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology, University of Bamako (EC number 0522)

Study II, the collection protocols were approved by the Oxford University Tropical Research Ethics Committee (OXTREC 027-04)

Study III, IV and **V** were all approved under the same ethical approbation of the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology, University of Bamako (EC number 0520).

3.4 MICROSCOPY

Conventional microscope based procedures were used for all clinical trial in order to diagnose malaria cases, but also as a tool in study III to measure parasite clearance dynamics by sampling thin and tick slides every 8 hours. Slides were stained for 20-30 minutes with 5% Giemsa solution and were examined under oil immersion (x100 magnification). Parasite density was estimated by

counting the number of asexual parasites per 200 leukocytes and multiplying by 40, assuming 8000 leukocytes/mL (216) Gametocyte density was calculated by counting the number of sexual forms per 1,000 leukocytes and multiplying by eight (207)

3.5 WWARN TOOL FOR PARASITE CLEARANCE ESTIMATION (PCE)

The World Wide Antimalarial Resistance Network has developed a simple calculator that provides measures of parasite clearance from serial parasite count data. The PCE tool has been used in our study III to analyze the microscopy counting data according to the protocol published on the WWARN website (http://www.wwarn.org/sites/default/files/WWARN-Parasite-Clearance-Estimator-PCE-Methodology.pdf)

3.6 IN VITRO STUDIES

The *in vitro* culture adaptation of *P. falciparum* was performed for drug susceptibility analysis purposes in study II. Reference laboratory clones (3D7, Dd2) were provided by the Malaria Research and Reference Reagent Resource Center (MR4, ATCC Massanas Virginia), as well as by the Department of Animal and population genetics, University of Edinburgh, UK.

All parasites were cultured in human O+ RBCs and Malaria Culture Medium made with RPMI 1640 culture medium supplemented with 10% L-glutamine, 0.05% gentamicine (Gibco® / Invitrogen™) and 10% human AB+ serum. Parasites were incubated at 37ºC in air-tight environment achieved either by conventional candle-jar technique (199) or involving the use of gas (5% O_2 and 5% CO_2 in N_2). Parasites were grown in static manner or in suspension on an orbital shaker (50 revolutions per minute), according to the circumstances. Parasite intra-erythrocytic cell cycle synchronization, when applied, was performed by the incorporation in the parasite culture of 5% sorbitol for 10 min (101) or using magnetic columns (MACS®, Miltenyi Biotec). The later consists in passing the parasites in a magnetic column which will specifically retain the mature stages, as they contain the highest amounts of iron molecules trapped in homozoine while allowing the rings to be eluted.

Different antimalarial drugs inhibitory concentrations were determined for the laboratory strains, as well as for the fresh isolates, by relative quantification of *P. falciparum* Histidine-Rich Protein 2 (*pf*HRP2) based on a Double-Site Sandwich Enzyme-Linked Immunosorbent Assay (136), followed by nonlinear regression analysis (115).

3.7 PHARMACOKINETIC

For the study I purposes, plasma levels of QN were determined in blood samples collected at 3 time points: before QN administration on day 0, 7 days after the initiation of treatment, and 14 days after the initiation of treatment. The protocol was developed and the samples processed at the Laboratoire de Pharmacologie Analytique et Pharmacocinétique, Hôpital Saint–Vincent de Paul, Paris France.

3.8 MOLECULAR METHODS

3.8.1 DNA extraction

All DNA was extracted from dried blood spots collected on 3MM Whatman (Kent, WA 98032-1911, USA) filter papers.

Study I: *P. falciparum* genomic DNA was extracted from blood blotted onto filter paper samples by an established methanol–based method (111).

Study II: DNA was extracted from frozen isolates originally and adapted clones using the ABIPRISM®6100 Nucleic Acid PrepStation® (Applied Biosystems™, Fresno, CA, USA) and following the manufacturer recommendations.

Study IV and **V**: DNA was extracted from dried blood spots using a commercially obtained Qiagen DNA blood mini kit (Qiagen, Valencia CA, USA) according to the manufacturer's recommendations. Part of **study IV** was extracted using the BloodPrep™ Chemistry on a ABI PRISM® 6100 Nucleic Acid PrepStation® (Applied Biosystems, Fresno, CA, USA) according to an established protocol (40).

3.8.2 Recrudescence/reinfection determination through molecular correction

For study I we have adjusted the in vivo drug efficacy rate by using two *P. falciparum* highly polymorphic genes, *pfmsp, pfmsp2* and the microsatellite *Ca1* (129, 185). These biodiversity markers were used to distinguish recrudescent parasites from new infections by nested polymerase chain reaction (PCR), as described elsewhere (32, 191). A recurrent infection was classified as recrudescence if there was at least one allelic band matching with the corresponding baseline sample in all 3 genetic markers, or as a reinfection if there were no matching allelic band in at least one genetic marker (118).

3.8.3 PCR-Restriction Fragment Length Polymorphism (RFLP)

PCR-RFLP is a well-established method based on the use of restriction enzymes for SNP detection. Restriction enzymes have the capacity to cut DNA at a very specific sequence pattern. The presence of a SNP will be detected trough occurrence of restriction or not, leading to different patterns of electrophoretic band sizes by the size of restricted fragments. In study I, *pfcrt* K76T SNP was analyzed by PCR-RFLP.

3.8.4 DNA Sequencing

Sequencing of DNA was applied in order to identify novel and or consecutive SNPs, as well as for a better understanding of microsatellite diversities. This method was thus a better option for **Study I**, part of **study II** and **study IV**.

To measure the *pfnhe1* microsatellite *ms4760* diversity in Study I, a nested PCR product of approximately 350 base pairs were purified as recommended by the manufacturer (USB, Cleveland, OH). The amplicons were then sequenced with an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA) at the National Institute of Allergies and Infectious Diseases (NIAID), National Institutes of Health /NIH), Bethesda, USA.

In **Study II**, the Open Reading Frames of the *pfmdr1*, *pfcrt* and *pfmrp1* genes were fully sequenced to determine their genetic variability in laboratory strains in Thai field isolates. Amplicons were sequenced through outsourcing (Macrogen Inc., Seoul, Korea).

For **study IV**, the K13 propeller ORF SNPs were analyzed by using first obtaining an 859 bp PCR product, covering all the known polymorphic regions of this gene. The amplicons were subsequently purified on filter plates (Edge Biosystems, Gaithersburg, MD), and directly sequenced using an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA) (145).

The analysis of the resulting DNA sequence chromatograms is described in this section under the “bioinformatics analysis” section.

3.8.5 Pyrosequencing

Developed at the Royal Institute of Technology in Stockholm in 1996 (165), pyrosequencing is a method of DNA sequencing based on the "sequencing by synthesis" concept. It relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination

with dideoxynucleotides (21). This technique is particularly suited for SNP analysis with short DNA fragments sequenced directly from PCR amplified products. Also, it is an advantageous approach concerning the multiplicity of infection found during clinical trials performed in areas of high malaria transmission. As, when appropriately calibrated, this technique allows an estimate of the different allele proportions in a single infection (226).

In **study IV**, after DNA extraction using the BloodPrep™ Chemistry on ABI PRISM® 6100 Nucleic Acid PrepStation® (Applied Biosystems, Fresno, CA, USA) the Mal10-688956 and Mal13-1718319, SNPs were genotyped by pyrosequencing using a PyroMark ID according to the recommendations of the manufacturer (Biotage AB, Uppsala, Sweden). In **study II**, this technique was also the preferred choice for the determination of the Thai isolates *pfmdr1* F1226Y and *pfmrp1* F1390I polymorphisms.

3.8.6 Real-time PCR

Real-time PCR, also called quantitative PCR (qPCR) is a laboratory technique developed from the classical PCR through the addition of fluorescent probes able to hybridize and signal the accumulation of specific amplicons, allowing estimates of their accumulation, and by inference, initial amounts of starting template. It has been proven to be a reference method for accurate quantification of nucleic acids amplification since its introduction in the late 1990s.

In **study II** of this thesis the method is used to determine *pfmdr1* copy number variations in the parasite. The protocols were based in the use of specific TaqMan® probes, using an ABIPRISM® 7700 or 7000 Sequence Detection Systems (Applied Biosystems™, Fresno, CA, USA).

In **study V**, to assess parasite clearance, both parasite the PgMET (tRNA methionine) and the human HumTuBB (part of a human beta tubulin exon 4 gene) *loci* were quantified in the same sample. Sets of primers and double-labelled fluorescent hydrolysis probes specific to PgMET and HumTuBB were used to amplify and detect parasite and human DNA in a duplex quantitative qPCR, BHQ1 was the quencher used for the two probes. The amplification was performed with a thermo LightCycler® 480 System (F. Hoffmann-La Roche, Germany). For each time point (sample), parasite DNA abundance was normalized against human DNA. A relative parasite density was calculated by the delta delta C_T method ($\Delta\Delta C_T$) (18, 110). The international standard (INT) for *P. falciparum* DNA (146) which contains white blood cell DNA from the donor, was used as a positive control. A cut off of 42 cycles was used to define successful amplifications.

3.9 BIOINFORMATIC ANALYSIS

Basic genomic Information for *Plasmodium* genes for all studies was retrieved using the online databases as PlasmoDB (www.plasmoDB.org) and NCBI Entrez database (http://www.ncbi.nlm.nih.gov). In **Study I** and **II**, the Sequencher™ software versions 4.6 and 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) was used to analyze the sequence output with the 3D7 gDNA as reference. **Study IV** results were analyzed using Sequencher® v.5.0 suit (Gene Codes Corporation, Ann Arbor, MI). Genetic variation, namely the presence of SNPs was assessed by comparing each sequence to the 3D7 reference (PF13_0238).

3.10 STATISTICS

EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA), SPSS 11.0 (The Predictive Analytics; IBM, Chicago, IL), and STATA 9.1 (StataCorp, College Station, TX) were used to generate summary statistics and to compare therapeutic efficacy in **study I**. The Spearman correlation test was used to assess the relation between ms4760–1 and the *pfcrt* K76T codon mutation. The prevalence of polymorphisms before versus after QN therapy was compared with the two-sided Fisher exact test and 2 × 2 contingency table analysis.

The statistical analysis of association between the Thai isolates genotype and their correspondent *in vitro* drug susceptibility in **study II** was performed with t-test or Mann-Whitney Rank Sum Test when normality of the data failed, and adjusted for Bonferroni significance. Pearson correlation was used to check significant positive or negative correlation of the *in vitro* antimalarial drug susceptibility using rational numbers.

In **study III**, descriptive statistics were expressed as means, medians, or proportions. Categorical variables were compared using two-sided Fisher's exact test, and continuous variables were compared using the Student's t test or Mann-Whitney test, as appropriate. Multivariable models were constructed for the primary and secondary outcomes. All statistical analyses were done with Stata version 11.0 (StataCorp. College Station, TX). P values < 0.05 were considered significant. Distribution of parasite clearance rate constants and slope half-lives were generated by the new WWARN Parasite Clearance Estimator (http://www.wwarn.org/research/parasite-clearance-estimator) (64)

Study IV results were analyzed through a parametric T test for the normally distributed variable PC_{99} (time in hours for parasiteamia to be reduced by 99% of its initial value). For the non-normally distributed parasite clearance slope half-life (time in hours it takes for the parasiteamia

to decrease by half) a non-parametric Wilcoxon rank-sum test was used to compare the wild type and mutant parasites groups.

In **study V** data Cycle Threshold (C_T) values were obtained from parasite and from human DNA. The ΔC_T was obtained by calculating the difference between C_T values of the parasite target gene (PgMET) and the human reference gene (HumTuBB). The Fisher's two-sided exact test was used when searching for associations between the q-PCR data and the available K13 propeller SNP data, determined in the same sample set in a previous work included in this thesis (Ouattara *et al*. accepted). T-test was applied for comparing average PC_{99} values.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I

Potential contribution of *pfnhe1* gene to decreased quinine sensitivity of *P.falciparum* parasite

Although intra venus administered artesunate was recently shown to be superior and being rolled out (180) QN is still the central drug in the management of severe malaria and malaria during pregnancy in Africa. Every year thousands of malaria patients rely on its efficacy. But the capacity of *P. falciparum* to resist this drug has long been known (8). In 2004, Quantitative Trait Loci (QTL) investigations highlighted the *pfnhe1 ORF located* microsatellite allele ms4760-1 as associated with decreased in vitro response to quinine (60). This information prompted us to enquire if the ms4760-1 variant was selected in vivo upon a curative dose of intravenous quinine administered in severe case of *P.falciparum* malaria. For this purpose we have sequenced a *pfnhe-1* ORF region containing the ms4760 microsatellite in parasites collected from infections before quinine treatment initiation, and from any case of recurrent parasiteamia during the clinical follow up. In parallel, the *pfcrt* K76T SNP was also analyzed for the same set of sample, to seek for any contribution of this gene as reinforcement to the role of *pfnhe1* in the response to QN treatment.

Although the in vivo efficacy of the QN was 100% after molecular correction, we observed a selection of the quinine resistance associated ms4760-1 allele by the QN treatment, visible through a significant increase in its frequency between the group parasite collected before (D_0, 26.2% of 107) and after the drug administration (46.3% of 54) p=0.01 (fig. 14). In this study the *pfcrt1* mutation K76T was not selected, although a trend for an increased frequency in the subgroup of ms4760-1 carrying recurrent infections was observed. The relatively limited size of the study did not allow further conclusions concerning a possible interaction between these two genes.

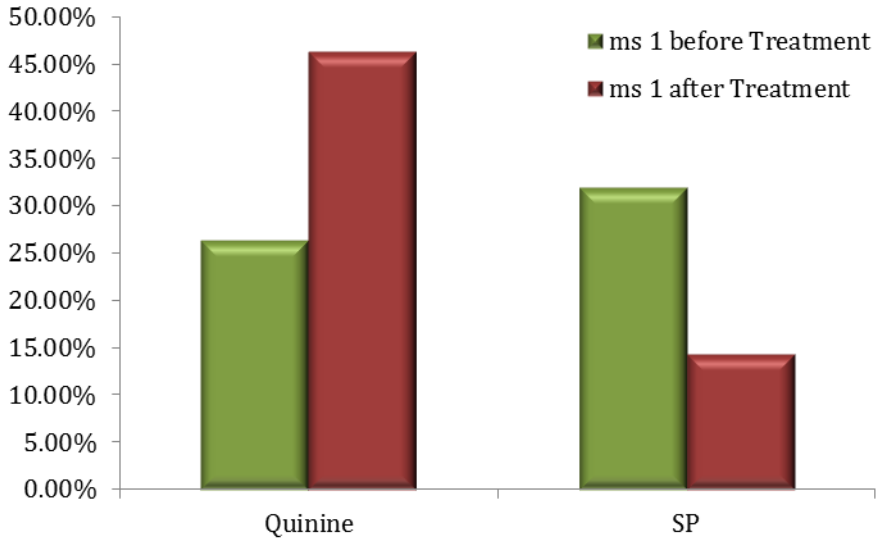


Figure 14: Selection of ms4760-1 (ms 1) by quinine but not sulfadoxine-pyrimethamine treatment with p=0.01 (1.2–4.8) and p=0.3 (.09–1.4) respectively.

Importantly, the selection of ms4760-1 observed in this study is likely to be specifically associated to quinine exposure, as no significant selection was observed among the patients receiving SP treatment. A further interesting observation is the fact that this selective effect is more concentrated in the parasiteamias detected in the bloodstream at day 14. The inability to detect residual QN in the plasma at day 14 by the technique used did not allow to make a direct link of selection by this drug, but assuming a canonical intra-hepatocyte stage period, these parasites are expected to have been released from the liver 6–10 days earlier (76). At that point in time, immediately after the end of the 5 days treatment course, an exposure of parasites to low-level QN is to be expected. Another explanatory hypothesis is that the parasites that appeared at day 14 were indeed present at day 0, but at undetectable levels even by nested PCR. In this scenario, QN might have cleared the highly susceptible parasites, while parasites with decreased susceptibility present at very low levels would have recovered and became detectable at day 14 or beyond.

The day 7 treatment course has reduced the recurrent rate and has prolonged the patent parasiteamia time by 5 days.

4.2 PAPER II

Novel potential molecular markers of *in vitro* drug resistance located in *pfmdr1* and *pfmrp1* genes

Southeast Asia is a historical epicenter of emerging *P. falciparum* drug resistance, which makes this region a resource for information on the capacities of the parasite to evade antimalarial action. The recently observed decrease in parasite artemisinin derivative susceptibility, the ACT key component, reinforces this view.

Changes in putative drug transporters have been extensively linked with decreased parasite antimalarial sensitivity. In this context, and aiming to obtain molecular information with interest beyond the SE Asian setting, we investigated the association of *in vitro* phenotypes of drug susceptibility (ICs) with the complete genotype of the aforementioned transporter genes, upon adaptation to culture of a set of parasites from the Thai-Burma border. Forty six field isolates were culture adapted and their *pfcrt, pfmdr1* and *pfmrp1* ORFs – coding for the three well documented drug resistance genes – fully sequenced. This data was then compared with IC values obtained for the long half-life partner drugs LUM and MQ, as well (and with particular interest in the context of this thesis) to artemisinin and DHA.

The 43 fully analyzed parasites for the *pfcrt, pfmdr1* and *pfmrp1* ORFs revealed new SNPs (*pfmdr1* F1226Y and *pfmrp1* F1390I). From the seven SNPs found in *pfmdr1*, the newly identified F1226Y was significantly associated with increased IC values for ART, MQ and LUM. The same pattern was observed for the *pfmrp1* F1390I, a SNP present in the transmembrane domain 11 of the protein, which has been proposed to be part of a substrate pocket in several ABC transporters, including *Pf*MDR1 (62). Even though *pfmdr1* F1226Y and *pfmrp1* F1390I alleles haves similar associated drug response profiles, they were not observed to be linked ($P>0.05$), suggesting that the actions of the respective proteins are not coordinated at the sub-cellular level. *Pfmdr1* CNV was associated with a significant decreased *in vitro* susceptibility to ART, MQ and LUM, as previously documented (155, 157, 158, 176).

All 46 field isolates carried the same *pfcrt* haplotype (Dd2-like), not allowing an evaluation of the involvement of this gene in the observed responses. By grouping the different *pfmrp1* F1390I, *pfmdr1* F1226Y and *pfmdr1* CNV haplotypes present in the Thai field isolates, we observed a progressive increase in the IC_{50} of the tested antimalarial drugs. In particular, we documented that the *pfmdr1* CNV is associated with an abrupt leap in MQ IC_{50} values, and showed a less dramatic but still significant effect with ART and LUM, confirming previous findings (36, 155, 157, 158, 176).

The molecular mechanisms associated to these mutations are unknown. A preliminarily *in silico* analysis points for the *pf*MRP1 F1390I as localized within transmembrane 11, a potential substrate pocket (45), where it might modulate the interactions between *pf*MRP1 and the tested antimalarials. As for *pfmdr1* F1226Y, it is to note that this change was frequently associated with the increased copy number of the gene, which suggests a possible enhancement of the effects already associated with the *pfmdr1* duplications. Further studies are needed for understanding its specific contribution for drug response.

DHA was tested as a proxy of artesunate action, as this drug can be essentially considered *in vivo* as a pro-drug, with the vast majority of the anti-parasitic action being attributed to the active metabolite. No significant associations were found between DHA and these markers. There was anyway an overall trend for increased IC_{50}'s for the three analyzed SNPs, a tendency that also included the successive accumulation of resistance associated alleles in the same parasite. To note that at least *pfmdr1* duplications have been already associated with decreased sensitivity DHA *in vitro* (157).

4.3 PAPER III

Delayed parasite clearance described as the phenotype of *P. falciparum* resistance to artemisinin is not observed in the sub-Saharan African country of Mali

In 2008 the first report of a potential emergence of artemisinin resistance parasites in South-east Asia was published (137). These observations, confirmed by subsequent studies with artesunate in monotherapy or in combination, raise the possibility of an eventual spreading of artemisinin resistant *P.falciparum* (7, 9, 53).

The emergence of artemisinin resistance in the African continent, or its invasion by SE Asian resistant parasites is a main concern. Early and continuous monitoring is essential to anticipate measures for the management of malaria resistance to artemisinin in Africa. In this scenario, the present study was aimed primarily to evaluate the therapeutic efficacy of artesunate in monotherapy, 6 years after the introduction of ACTs as the national policy in Mali. Secondly it aimed to identify any trends in the parasite clearance dynamics that might herald the emergence of a resistance phenotype.

The clinical trial tested the efficacy of a curative dose of Artesunate in monotherapy, administered once daily for 7 days in a group of 100 children with uncomplicated malaria, in Mali. A close follow up of the parasite clearance patterns was performed through frequent sampling and microscopy, the data being downstream analyzed using the WWARN PCE tool for the estimation parasite clearance parameters. The PCR corrected efficacy of the artesunate administered in

monotherapy was 100%, with 98.9% of the included patients having cleared 95% of their parasites by 24 hours after the treatment initiation, similar to the result observed 8 years earlier in the same area (98.3%). The median parasiteamia half-life in our setting was 32 hours. All these results were obtained after adjusting for several parameters essentially for the initial parasiteamia, age and sex. Compared with a similar trial previously performed in the same region, the *in vivo* efficacy and the proportion of participants clearing parasites by 24 hours after treatment initiation remained unchanged from 2002–2004 to 2010–2011.

The slop half-life mean of 2.07 and the median parasite clearance time (PCT) of 32 hours observed in the present work was markedly lower than the 84 hours reported in western Cambodia (53). No participants in our study had a positive blood smear at 72 hours as an *in vivo* predictor of treatment failure with ACTs (188). The efficacy observed in this study is probably due to several factors: (a) firstly, because ACTs have been implemented in Mali recently, as compared to the Asian regions, theoretically not allowing time for a full development of the phenotype among the local parasite populations (b) the fact that artemisinin have been introduced in the African health systems only in combination therapy, (c) finally, given the high intensity of *P. falciparum* malaria transmission in Mali, premunition is likely to serve as powerful delay factor to the emergence of artemisinin resistance.

Nevertheless, variation was detected, reflected in a range of clearance times

Paper IV

Non-negligible presence of K 13-propeller domain polymorphism in sub Saharan Africa *P.falciparum*

Resistance to artemisinin and its derivatives is presently assessed by measuring the parasite clearance dynamics in malaria patients, upon treatment with artesunate monotherapy. A genetic determinant of this phenotype has been proposed to be the polymorphism of the Kelch propeller domain of *plasmodium falciparum* K13 propeller gene. This gene codes for a putative homologue of the human kelch protein family.

The study IV works comes in logical sequence of **study III**, which prompts the question if artemisinin resistance phenotype is already circulating in Africa. In this work we measure the prevalence of *P. falciparum* K13-propeller polymorphisms in present-day artemisinin-sensitive parasite collected in Bougoula-Hameau, and in malaria infections that occurred before ACTs introduction in Mali in order to detect any impact of the implementation of these therapies on the biodiversity of this gene.

Samples were collected prior to all antimalarial treatment from Bougoula-Hameau artesunate monotherapy study in 2010-2011 but also in 2003-2005, as well as in Bandiagara in 1999-2002. The K13 propeller domain was PCR amplified, sequenced and compared with the described ones from SE Asia (7). From all the samples analyzed, 26 presented non-synonymous mutations on the kelch propeller domain. The remaining infections were designated "wild types" for the domain of interest, by considering the 3D7 clone as the source of the reference sequence (http://plasmodb.org/). The 3D7-type prevalence was comparable for all study sites, regardless of the study period.

All the new haplotypes found in Bougoula-Hameau in 2011 were different from the sequences derived from infections occurring before ACTs were introduced in Mali. Comparing the slope half-life of parasites harboring the mutation and the 3D7 types, no difference could be found in the slop half-life in the Bougoula-Hameau samples of the artesunate efficacy study in 2010-2011. The difference found in the PC_{99} mean of 20.6 hours for the mutant and 17.9 hours for the 3D7 types was not statistically significant.

Finally, SNPs in the Mal10-688956 and Mal13-1718319 genes, previously proposed as contributing to the resistance phenotype in SE Asia (192), were not identified in the analyzed Malian malaria infections, which harbored only the 3D7-type sequence.

In our data set none of the SNPs previously described as strongly involved in artemisinin resistance were found, which is not totally surprising as no particularly long clearance values were initially observed. However, mutations surrounding one of the key mutation or different amino acids at the same positions than Asian parasites were detected among parasites from the set corresponding to the post-ACT introduction period (2010-2011). This observation suggests a putative initial footprint of a process of selection, possibly derived from drug pressure that is unveiling previously undetected parasite populations. Assuming the importance of this gene in parasite artemisinin derivative response, this emerging variation supports the view that the possibility of *de novo foci* of artemisinin resistance in Africa should not be ignored.

4.4 PAPER V

P. falciparum polymorphism at K13-propeller domain and parasite clearance assessed by qPCR

ACT treated malaria in Mali showed to have a mean PCT shorter than observed in Asia. As mentioned, this might be related with parasites being more sensitive, while the drug action is probably aided by a stronger immune system response due to the development of premunition. The detection of differential clearance times in African infection thus needs the use of more

sensitive techniques than the microscopy based procedures. This is of importance, as improved technology can enable public health systems to early detect trends in toward parasite clearance time increase.

The final work of this thesis had as such the intention of detecting trends of differential *in vivo* clearance by using a more sensitive method. The study relied on the use of quantitative (Real time) PCR to detect residual parasiteamia not measurable by microscopy. Study III was hence revisited in order to reevaluate the infection's PCTs. This led to another issue of practical importance: with such improved phenotype, could we better define an association between the observed K-13 sequence and PCT in the Bougoula-hameau artesunate mono-therapy study?

Blood sample were collected on filter paper for each participant before treatment at day 0 and successively at day 1, day 2 and day 3 following treatment initiation. Extracted parasite DNA was analyzed using a recently published relative quantification method (18). Samples were classified as fast or slow clearer plus the group that did not clear the parasitaemia by day 3 of treatment ("non-clearers"). The results between the two techniques were compared. The role of K13-propeller polymorphism in the PCT increase with RT-PCR was investigated.

64 subjects were analyzed, corresponding to the subset where data both microscopy and RT-PCR was available. According to RT-PCR, almost half of the participant did not clear their parasite by day 3 (46.26%), i.e 72 hours after treatment initiation. The Fast clearers with no parasite 24 hours after artesunate were only 14.06% of the analyzed group.

The PCT was significantly longer by RT-PCT than microscopy, with a mean molecular PC99 (mPC_{99}) of 67.1 hours *vs* 18 hours, respectively. Interestingly, none of the fast clearer participant was infected with parasite harboring mutation at K13-propeller, a result motivating much needed follow up explorations. Otherwise, no further trends of association were observed.

The potential consequences of the emergence of artemisinin resistance in the continent are such that early surveillance is fundamental and urgent. While in our previous work, resourced by the conventional microscopy based approach, no parasites were found after day 2, in the present one, *P. falciparum* DNA was detected by day3 in near half of the analysed patients. Using a recently developed Q-PCT method to measure parasite clearance has showed that molecular methods can offer the possibility to increase detection sensitivity. This is important because albeit several genes have been pointed to be possibly involved in the parasite response to artemisinins, the fact is that no molecular marker is available with the potential of being a fully useful molecular tool of early surveillance for this phenomenon. Accordingly, for the moment it is clear that the development of more precise and complete phenotypes is a valuable alternative. Better

phenotypes also lead naturally to an improved background for the definition of future molecular markers of resistance through more robust genotype/phenotype associative approaches. This was the approach that we also piloted in this work, where the reported trend of absence of K13 propeller mutants among the fast clearers was only possible to be detected through the more sensitive q-PCT phenotyping method.

This supports the aforementioned view that while genetic markers are not available, such methods can be of use for the early detection of delayed clearance phenotype, while setting the phenotypes needed for the development of future, better field adapted molecular genetics tools.

5 PERSONNAL VIEWS AND PERSPECTIVES

This thesis was essentially articulated around the two most important drugs in the management of malaria cases in the daily practice in Africa, artesunate and quinine. Artesunate constitute one of the backbones of ACT for uncomplicated *P.falciparum* malaria management, being also increasingly recommended for the treatment of severe cases. As for quinine, in practice it remains the main compound for this purpose in the African setting, due to its wide availability and affordability.

Our results hereby show albeit a good clinical efficacy of the quinine in the initial phase of management of severe malaria cases was reported, a considerable recurrent rate of 30% also occurred inside the 28 day follow up. A non-negligible number of these recurrence happened only two weeks after a severe attack, which constitutes an issue of concern that clearly justifies the increase in the treatment duration from five days to seven days.

The observed selection of the ms4760-1 microsatellite allele of the gene *pfnhe1* by the QN treatment is the first robust evidence of the potential role of this polymorphism in the parasite sensitivity to QN *in vivo*. Unfortunately, our present results, albeit interesting, are not enough to make a direct correlation between the presence of ms4760-1 and the resistance to QN, as the allele has no predictive values regarding the occurrence of recurrent parasiteamia. In short, it seems that ms4760-1 is not central enough in the phenomenon of quinine resistance to be by itself sufficiently informative to constitute a valuable (i.e. clinically translatable) molecular marker of resistance. Resistance against quinine seems to be a particularly complex phenomenon, most likely involving an array of genetic markers, of which *pfnhe1* is only one of the actors. Accordingly, the observed selection process needs more investigation on additional genetic differences between the ms4760-1 alleles reported before the treatment which are cleared by the treatment, from the ms4760-1 carriers selected by the presence of the QN in low concentration. Another point concerns the initial pharmacodynamics of the parasite elimination - the assessment of the clearance during the first 72 hours may give different clearance profiles according to the parasite genotype.

As for artesunate, the clinical efficacy in our studies was good; in our Malian setting no cases of significantly prolonged parasite clearance could be observed after a curative regiment of artesunate in monotherapy, the reinfection rate being less than 5% for 28 days of follow-up. The lack of clinically relevant parasite clearance time increases renders the K13-propeller polymorphism unveiled in Mali as such not associated with such phenotypes. On the other

hand, one can preliminarily assume that the absence of marked increases in clearance times can be justified by the non-detection of the reported Asian resistance associated alleles.

These studies have been motivated by the documented appearance in Asia of clinical failures to the quinine as well as to ACTs. The differences observed in the parasite response to these two drugs between the two different setting, South-East Asia and Africa, are probably due in part to the history of significantly larger antimalarial availability in SE Asia as compared to Africa. Also, due to the characteristics of low transmission of the SE-Asian settings, the development of premunition is not as prevalent in the human populations as compared with the African context. This creates a situation where almost every infected individual will have clinical signs, as it is near naïve to the pathogen, which invariably leads to treatment. i.e. most parasite populations are under continuous drug pressure (the selective stressor). Additionally, the lack of premunition also means that the patient immunity is not strong enough to deal with potential residual (and potentially resistant) parasiteamia resulting from this selection process where small difference between sensitivity to the treatment differentiate several parasite populations. A probable reflection of this phenomenon has been previously reported as the intra-patient selection of *pfmdr1* duplication-carrying parasites upon mefloquine based regimens (201).

Presently, Africa is still very different from Thailand. But it is to note that with the undeniable success of ACT comes a secondary effect: the decrease in transmission will mean that premunition will be less and less prevalent among the children born in this ACT era. And with the desired availability and ready access to ACT, the drug pressure over the parasite populations is increasing. In colloquial words, we are transforming Africa in a SE Asian type of setting. The difference is that in SE Asia, ACT was used to save a failing drug, mefloquine, while in Africa the long half-life partners (amodiaquine and lumefantrine) are still highly effective. But this might be changing (117).

And finally the genetic background and geographic origin of the parasite has a big impact on the drug resistance emergence. Differences between the SE Asian and African parasite populations are likely to be significant. As an example of this view, in our works the novel *pfmdr1* and *pfmrp1* mutations associated with resistance that we found upon the analysis of the set of Thai parasites were not found in any of African samples that we ever tested. These mutations seem to be absent in Africa. Additionally, SE Asian parasites have been proposed to be genomically less stable than the ones found in the African continent, which would explain that for example that chloroquine resistance never emerged in Africa, and that the prevalence of *pfmdr1* duplications is still very low.

In conclusion, I personally believe that quinine and artesunate, as long as they are used as recommended will still have good longevity in the Malian endemic malaria regions, which can be considered as representative of this overall Western Africa region. This is supported by a clinical efficacy above expectations (100%). However a close and frequent monitoring of their efficacy is vital, as the malaria settings in these regions are changing. And with the likely increase in drug pressure one can consider a "last man standing" point of view (122), where the remaining parasite populations might be progressively less sensitive to the drugs.

Both artesunate and quinine are presently "too big to fail". Hence their useful life have to be extended as possible, as in the long term, if history repeats itself and the parasite is up to its last achievements, resistance to these drugs will eventually happen. It is more a question of "when" (that we can influence) then of "if "(as our influence is probably not strong enough to stop the parasite). Understanding the molecular basis of the parasite response to quinine and artesunate will help on creating appropriate strategies for protecting these drugs from resistance, and with that, gaining time. In my opinion this is of critical importance, as I also believe that more antimalarials will be developed and become available in the meantime.

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