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Day 7 concentration effects of partner drugs of artemisinin and derivatives on recurrent episodes of uncomplicated *Plasmodium falciparum* malaria after repetitive treatment with the same drug during two years in Mali

Referees: Prof. Dr. Walter E. HAEFELI

Dr. Ann-Kristin MUELLER

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2 Table of Contents

1 Acknowledgements III

2 Table of Contents V

3 Table of Figures IX

4 Table of Tables XI

5 List of abbreviations XIII

6 Summary XVII

7 Zusammenfassung XVIII

1 Introduction 1

1.1 Malaria epidemiology and distribution: 1

1.2 Clinical manifestations of malaria 3

1.3 Malaria biology and life cycle 4

1.4 Malaria control strategies 9

1.5 Chemotherapy of malaria 12

1.5.1 Amodiaquine 13

1.5.2 Pyronaridine 14

1.5.3 Piperaquine 15

1.5.4 Lumefantrine 16

1.5.5 Artemisinin (Qinghaosu) 17

1.5.6 Artemether 17

1.5.7 Artesunate 18

1.5.8 Preclinical antimalarial drug candidates with multiple-stage activity 19

1.5.9 Pharmacokinetic characteristics of antimalarial drugs 20

1.5.10 Analytical methods for antimalarial drugs quantification 21

1.5.10.1 Sample extraction 22

1.5.10.2 Measurement 22

1.6 Evaluation of drug efficacy 24

1.7 Antimalarial drug resistance 27

1.8 Distinction between recrudescence and new infections for monitoring the antimalarial drug therapeutic efficacy against *Pf* 30

1.9 Research question 33

1.10 Hypothesis 33
1.11 Objectives: 33
2 Methods 35
2.1. *In vivo* methods 35
2.1.1. Study sites: 35
2.1.2. Study design: 36
2.1.3. Study Population 37
2.1.4. Inclusion and non-inclusion criteria**:** 37
2.1.5. Retreatment criteria with study drugs 39
2.1.6. Temporary non-retreatment criteria with study drugs 39
2.1.7. Criteria for permanent discontinuation of receiving study drug or withdrawal from the study. 40
2.1.8. Concomitant treatment 40
2.1.9. Treatment 41
2.1.10. Samples collection 42
2.1.11. Pharmacokinetic samples collection 44
2.1.12. Statistical analysis 44
2.1.13. Ethical and deontological consideration 44
2.2. Laboratory methods 45
2.2.1. Materials 45
2.2.1.1. Materials for genotyping 45
2.2.1.2. Materials for high-performance liquid chromatography 46
2.2.2. Reagents 46
2.2.2.1. Reagents for genotyping 46
2.2.2.2. Reagents for high-performance liquid chromatography 47
2.2.3. Methods for genotyping 48
2.2.3.1. Principle of the PCR and capillary electrophoresis methods: 48
2.2.3.2. DNA Extraction by methanol: 50
2.2.3.3. Amplification of msp2 gene: 50
2.2.3.4. Amplification of msp1 gene: 53
2.2.3.5. Amplification of microsatellite ca1 gene 54
2.2.3.6. Quality control of the PCRs: 55
2.2.4. Methods for high-performance liquid chromatography 56

2.2.4.1. Chemicals and Reagents: 57
2.2.4.2. Instrumentation 58
2.2.4.3. Analytical conditions 59
2.2.4.4. Extraction 59
2.2.4.4.1. Preparation of Buffers 60
2.2.4.4.2. Extraction methods: 60
2.2.4.4.3. Preparation of standard drugs: 61
2.2.4.5. Quantification 62
2.2.4.6.1. Analytical method validation 62
2.2.4.6.2. Revalidation of N-desethylamodiaquine method 64
2.2.4.6.3. Validation of the methods during patients' samples analysis 64
2.2.4.6.4. Clinical sample quantification 64
2.2.5. Data management and analysis 65
2.3. Outcomes and statistical analysis methods 65
3 Results: 67
3.1. Clinical study findings 67
3.1.1. Study profile and baseline characteristics 67
3.1.2. Treatment response by episode at day 28 and 42 of follow-up shows the intensity of malaria exposure/recurrence by treatment arm 70
3.1.3. Treatment response by arm at day 28 and 42 of follow-up shows the intensity of malaria exposure/recurrence by site: 72
3.1.4. Distribution of the proportion of patients without subsequent episode by treatment arm and by age group at day 28 and 42 of follow-up: 73
3.1.5. Safety of the treatment arms 74
3.2. Genotype results 75
3.2.1. Genetic diversity of parasites in study sites 75
3.2.2. Treatment response by arms after molecular correction 77
3.3. Development and validation of HPLC methods for the quantification of lumefantrine, N-desethylamodiaquine, and pyronaridine in plasma. 79
3.4. Pharmacokinetic of lumefantrine and N-desethylamodiaquine at day 7 of follow-up 84
3.4.1. Number of episodes and baseline characteristics in patients with quantified drug concentrations 84

3.4.2. Accumulation of lumefantrine and N-desethylamodiaquine in study population after repetitive treatment with the same drug: AL or ASAQ 85
3.4.3. Distribution of lumefantrine and N-desethylamodiaquine day 7 concentration by age group, sex and fever status before treatment 87
3.5. Pharmacodynamics of lumefantrine and N-desethylamodiaquine..... 91
3.5.1. Relationship of lumefantrine and N-desethylamodiaquine day 7 concentrations with treatment response at day 28 and 42 91
3.5.2. Relationship between lumefantrine and N-desethylamodiaquine concentration with treatment response at day 28 by age group 93
3.5.3. Lumefantrine day 7 concentration in under five years' children was too low for post-treatment prophylaxis 95
3.5.4. Multivariable and multivariate analysis of factors associated with parasite recurrence..... 97
3.5.5. N-desethylamodiaquine day 7 concentration was positively correlated with adverse events 105
4 Discussion..... 110
5 Conclusion 121
6 References:..... 122

3 Table of Figures

Figure 1: Malaria life cycle 9
Figure 2: Metabolism of drugs used in this study 19
Figure 3: In vivo model of parasite and drugs interaction. 27
Figure 4: Gene model of the *msp2*. 31
Figure 5: Gene model of the *msp1*. 32
Figure 6: gene model of the microsatellite ca1. 32
Figure 7: Study sites 36
Figure 8: *In vivo* method 43
Figure 9: Principle of fluorescent primer amplification 49
Figure 10: The general principles of my PCR amplification methods 49
Figure 11: Summary of HPLC method 57
Figure 12: Structure of standard dugs. 58
Figure 13: Study profile. 68
Figure 14: Electropherograms of the *msp2* capillary electrophoresis. 76
Figure 15: msp2 genetic diversity in my study sites in percent. 77
Figure 16: Electropherograms of quantified drugs. 80
Figure 17: Lumefantrine calibration curve with R-Square = 0.9987 80
Figure 18: Lumefantrine and DEAQ day 7 concentration profile after repetitive treatment of a new episode of uncomplicated malaria with the same drug (AL or ASAQ). 86
Figure 19: Day 7 concentrations variation according to the time of retreatment. 87
Figure 20: Day 7 concentrations distribution by age group 88
Figure 21: Day 7 concentration distribution by sex 89
Figure 22: Day 7 concentration distribution by fever status 90
Figure 23: Association of low day 7 concentration with parasite recurrence by day 28 of follow-up. 92
Figure 24: Association of low day 7 concentration with parasite recurrence by day 42. 93
Figure 25: Collinearity between lumefantrine day 7 concentration and proportion of patients without subsequent episode at day 28 of follow-up after treatment with artemether-lumefantrine 94

Figure 26: Low day 7 lumefantrine concentration in girls under five year of age. 95
Figure 27: Lumefantrine day 7 concentration threshold for prediction of parasite recurrence at 28 days of follow-up 96
Figure 28: Lumefantrine day 7 concentration threshold for prediction of parasite recurrence at 28 days of follow-up by age group. 97
Figure 29: Positive correlation between drugs concentration QTc change. 106
Figure 30: Median QTc change and proportion of QTc change higher than 30 ms in each treatment arm. 107
Figure 31: Positive correlation between day 7 drugs concentration and ALAT change. 108

4 Table of Tables

Table 1: Cycling conditions for *msp2* the first amplification 51
Table 2: Cycling conditions for *msp2* the second amplification 51
Table 3: Cycling conditions for *msp1* first amplification 54
Table 4: Cycling conditions for *msp1* second amplification 54
Table 5: Cycling conditions for *ca1* first amplification 55
Table 6: Cycling conditions for *ca1* second amplification 55
Table 7: Characteristics of participants at inclusion into different treatment arm 69
Table 8: Number of episodes per treatment arm. 69
Table 9: Proportion of patients without subsequent episode by treatment arm and by episode at day 28 of follow-up 71
Table 10: Proportion of patients without subsequent episode by treatment arm and by episode at day 42 of follow-up 71
Table 11: Efficacy of treatment arms at day 28 and 42 of follow-up by site (ASAQ was implemented in Bougoula Hameau only) 72
Table 12: Patients without subsequent episode at day 28 and 42 of follow-up by treatment arm and by age group 73
Table 13: Level of abnormal and QTc in different treatment arms 74
Table 14: Characteristics of patients with recrudescent parasites after molecular correction in AL arm 78
Table 15: Lumefantrine Intra and inter-day precision, and accuracy 81
Table 16: Pyronaridine Intra and inter-day precision, and accuracy 81
Table 17: Validation of lumefantrine quantification method during patients' samples analysis 82
Table 18: Validation of N-desethylamodiaquine quantification method during patients' samples analysis 83
Table 19: Number of samples analyzed by episode in different treatment arm to quantify lumefantrine and N-desethylamodiaquine 84
Table 20: Episodes' baseline characteristics of patients with drug concentration 85
Table 21: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 28 in AL arm 99

Table 22: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 28 in ASAQ arm..... 101
Table 23: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 42 in AL arm..... 102
Table 24: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 42 in ASAQ arm..... 104
Table 25: Effect of day 7 concentration on ALAT 109

5 List of abbreviations

ABC	ATP-binding cassette
ACT	artemisinin-based combination therapy
ACPR	adequate clinical and parasitological response
AE	adverse event
Ag	Antigen
ALAT	Alanine aminotransferase
AL	artemether-lumefantrine
AMA1	Apical membrane antigen 1
AMP	antimicrobial peptides
APL1	*Anopheles Plasmodium-responsive leucine-rich repeat 1*
AQ	Amodiaquine
ASAQ	artesunate-amodiaquine
ASAT	aspartate aminotransferase
ASMQ	artesunate-mefloquine
ASSP	artesunate-sulfadoxine-pyrimethamine
AUC	area under the curve
CI	confidence interval
CQ	Chloroquine
CRF	case report form
CRT	chloroquine resistance transporter
CSP	circumsporozoite protein
CV	coefficient of variation
CYP	Cytochrome P
DAD	diode array detector
DEAQ	N-desethylamodiaquine
DHA-PPQ	dihydroartemisinin-piperaquine
DHFR	Dihydrofolate reductase
DHPS	dihydropteroate synthase
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
DNDi	drugs for neglected diseases initiative

dNTP	dinucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
EIR	entomological inoculation rate
EMA	European Medicines Agency
ETF	Early treatment failure
FDA	food and drugs administration
G6PD	glucose-6-phosphate dehydrogenase
GAP	genetically attenuated parasites
GPI	Glycosylphosphatidylinositol
Hb	Haemoglobin
HPLC	high performance liquid chromatography
HR	hazard ratio
IL	Interleukin
Imd	immune deficiency
IPTi	intermittent preventive treatment in infants
IPTp	intermittent preventive treatment in pregnancy
IRQ	interquartile range
IRS	indoor residual spraying
IS	internal standard
ITN	insecticide-treated mosquito net
JNK	Janus kinase
Kg	Kilogram
LC	liquid chromatography
LCF	Late clinical failure
LLOD	lower limit of detection
LLOQ	lower limit of quantification
LPF	Late parasitological failure
Mg	Milligram
MRTC	Malaria Research and Training Center
MS	mass spectrometry
Ms	Millisecond
msp1	*merozoite surface protein 1*

msp2	*merozoite surface protein 2*
NMCP	national malaria control programme
PA	pyronaridine-artesunate
PAMPs	pathogen-associated molecular patterns
P.	*Plasmodium*
PCR	polymerase chain reaction
Pf	*Plasmodium falciparum*
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfNHE1	*Plasmodium falciparum* sodium hydrogen exchanger 1
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
PRR	parasite reduction ratio
Pv	*Plasmodium vivax*
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
QC	quality control
RDT	rapid diagnostic test
ROC	receiver operating characteristic
RON	rhoptry neck
SAE	serious adverse event
S.D	standard deviation
SMC	seasonal malaria chemoprevention
SPZ	Sporozoites
STAT	signal transducers and activators of transcription
TAE	tris acetate EDTA
TBME	terbuthyl methyl ether
TEA	Triethylamin
TEMED	-N-N-N-N tetramethyl-ethylene diamine
TRAP	thrombospondin-related anonymous protein
ULN	Upper limit of normal
UV	ultra-violet
WANECAM	West African Network for Clinical Trials of Antimalarial Drugs
WHO	World Health Organization

WWARN	WorldWide Antimalarial Resistance Network

6 Summary

The recommendation of artemisinin-based combination therapy (ACT) as a first line treatment of malaria continues to be based only on data obtained from single episode treatment rather than repetitive treatment. However, malaria episodes are frequent and there is thus a need to understand the long-term impact of repeated use of ACT to treat consecutive episodes of malaria over successive seasons. My study aimed to define the risk of parasite recurrence as a function of the pharmacokinetic and pharmacodynamic characteristics of ACT partner drugs in patients re-treated for multiple malaria episodes. Participants from Mali were randomized into three treatment arms: dihydroartemisinin-piperaquine (DHA-PPQ), pyronaridine-artesunate (PA), and first-line ACT (either artemether-lumefantrine [AL] or artesunate-amodiaquine [ASAQ]). Participants received the same ACT for each new episode of malaria for two years. Clinical and parasitological data were collected at each visit. Plasma samples were collected at day 7 of follow-up for quantification of drugs using high-performance liquid chromatography methods. In total, study participants experienced 5,260 episodes of malaria during the two-year follow-up period. Major findings were: i) accumulation of desethylamodiaquine (DEAQ), the main and active metabolite of amodiaquine (AQ), in the study population after early (between 25 to 45 days) retreatment with ASAQ; ii) no association of DEAQ concentration on day 7 with treatment outcome; iii) an association between day 7 lumefantrine concentrations and a reduced risk of re-infections within day 28 follow-up (hazard ratio, HR = 0.605, CI (0.50 – 0.74), $p < 0.001$). This protection of lumefantrine was concentration dependent; a concentration below a threshold of 380 ng/ml did not protect against subsequent re-infection by day 28. Importantly, the majority of the children under five years (84 out of 140; 60%) had lumefantrine day 7 concentrations (median (interquartile range): 305 ng/ml (207 – 490 ng/ml)) below this threshold. In conclusion, my results demonstrated an accumulation of DEAQ in the study population after early re-treatment with ASAQ, and suggest a need of lumefantrine dose optimisation in under five years age group. My analyses also showcase the value of re-treatment studies for improving treatment recommendations.

7 Zusammenfassung

Die Empfehlungen für die Artemisinin-basierten Kombinationstherapien (artemisinin-based combinations, ACT) basieren nach wie vor auf Daten von einzelnen Malariaepisoden. Jedoch sind Malariaepisoden häufig und ein besseres Verständnis von wiederholten Behandlungen auf Wirksamkeit und Nebenwirkungen ist wünschenswert. Meine Analyse zielte daher auf eine Definition des Risikos von Reinfektionen als Funktion der pharmakokinetischen und pharmakodynamischen Eigenschaften von ACT Partnerwirkstoffen in Patienten mit multiplen Malariaepisoden ab. Patienten mit unkomplizierter *P. falciparum* Malaria (>6 Monate) von Mali wurden in drei Behandlungsarme randomisiert: Dihydroartemisinin-Piperaquine (DHA-PPQ), Pyronaridin-Artesunat (PA) und eines der beiden gegenwärtigen Frontline ACT (entweder Artemether-Lumefantrin (AL) oder Artesunat-Amodiaquin (ASAQ). Studienteilnehmer erhielten die gleiche ACT für jede neue Malariaepisode über 2 Jahre. Blutproben vom Tag 7 nach Beginn einer Behandlung wurden für die Bestimmung von Plasmakonzentrationen mittels High-Performance Liquid Chromatography verwendet. Insgesamt wurden 5.260 Malariaepisoden registriert. Meine wichtigsten Ergebnisse sind: 1) Akkumulation von Desethylamodiaquin (DEAQ), dem Hauptmetaboliten von Amodiaquin (AQ), in der Studienpopulation nach früher (25-45 Tage) Neubehandlung mit ASAQ; 2) keine Assoziation von DEAQ Konzentration am Tag 7 mit der Zeit bis zur nächsten Infektion; 3) Tag 7 Konzentration von Lumefantrin korrelierte negativ mit dem Risiko von Reinfektionen bis Tag 28 (hazard ratio, HR = 0.60; CI, 0.50-0.74; $p<0.001$). Dieser Schutz war abhängig von der Konzentration von Lumefantrin; eine Konzentration unter 380 ng/ml schützte nicht vor Reinfektionen bis Tag 28. Insbesondere Kinder unter 5 Jahren (84 von 140; 60%) zeigten Tag 7 Lumefantrin Konzentration unter diesem Schwellenwert: Median von 305 ng/ml (interquartile range, 207-4.900 ng/ml). In Zusammenfassung, meine Ergebnisse zeigen eine Akkumulation von DEAQ in der Studienpopulation bei früher Neubehandlung mit ASAQ und legen eine Optimierung der Dosierung von Lumefantrin in Kindern unter 5 Jahren nahe. Meine Analysen unterstreichen auch den Wert von Studien mit wiederholten Behandlungen für verbesserte Behandlungsempfehlungen.

1 Introduction

1.1 Malaria epidemiology and distribution:

Malaria is a parasitic disease transmitted to human and other animals by infected female *Anopheles* mosquito's bites. Malaria can also rarely be transmitted through blood transfusion, and intrauterine transmission from mother to child (congenital malaria). Human malaria is caused by protozoan parasites of the genus *Plasmodium. Plasmodium falciparum (Pf), P. malariae (Pm), P. ovale (Po) (curtisi and wallikeri), P. vivax (Pv),* and *P. knowlesi* are the species that are known to cause human malaria. Of these six species, *Pf* and *Pv* are the most prevalent accounting for more than 95% of malaria cases worldwide. In 2016 the World Health Organization (WHO) estimated that *Pf* accounted for 99% of malaria cases in sub-Saharan Africa. *Pv* was the most dominant in the WHO region of the Americas, responsible for 64% of malaria cases, and more than 30% of recorded cases in the WHO South-East Asia and 40% in the Eastern Mediterranean regions [1].

In Mali, which is part of the WHO defined region of sub-Saharan Africa, except for *P. knowlesi*, all five human malaria species are encountered. *Pf* is the dominant parasite species with a prevalence fluctuating between 63.7 to 99% [2, 3]. *Pv* is present in north and north-eastern Mali and its prevalence can reach 30% in some locations [3, 4]. The presence of *Pv* is also described in central Mali in Duffy antigen negative people with a prevalence of 2 to 2.5% [5].

Malaria transmission is seasonal in Mali. The low transmission period coincides with the dry season (January to May), with an incidence rate of malaria infection close to zero, but this period is characterized by persistent infections. The high transmission period begins with the start of the rainy season in June and lasts until December. Peak incidence of malaria infection occurs at the end of the rainy season (between September and October) [6, 7].

Despite an 18% global reduction in number of cases of malaria between 2000 and 2016 (from 76 to 63 cases per 1,000 persons at risk [1]), malaria infection remains a critical public health challenge. The estimated annual number of malaria cases in 2016 was 216 million, with 445,000 deaths due to malaria, of which 91% occurred in the sub-Saharan region of Africa [1]. Children under five years represented 7 out of 10 cases of death due to malaria [8].

In the sub-Saharan region of Africa, a report by Bhatt *et al.* in 2015 estimated that from 2000 to 2015 malaria incidence rate (case per 1,000 per annum) declined from 321 to 192 cases [9]. Another report by Gething *et al*. in 2016 showed a drop in malaria mortality from 12.5 to 5.5 cases (per 10,000 population per year) during the same period [10]. The reduction in malaria mortality observed across all sub-Saharan Africa was less pronounced in Mali where the incidence rate dropped only from around 490 to 463 cases from 2000 to 2015 [9], while malaria mortality fell from around 32 to 23 cases during the same period [10].

In malaria endemic countries, particularly in *Pf* endemic countries, patients can suffer more than 10 episodes of clinical malaria per year [11], resulting in frequent use of antimalarial drugs. The development of partial immunity can occur after repeat exposure to infection. A naturally acquired immunity (premunition) can protect against severe malaria and later, also against mild malaria but never fully protects against infection [12, 13]. The key challenge for controlling or eventually, eliminating malaria, particularly *Pf* malaria, is the capacity of the parasite to evade sterilising immunity and to cause life-long re-infections.

The life-threatening character of *Pf* malaria has exerted a strong selective pressure on the malaria-exposed population. This has given rise to haemoglobinopathies, which can provide almost full protection against severe malaria but at the same time can cause disabling conditions with significantly reduced life expectancies [14, 15]. The most prominent example of haemoglobinopathy is sickle cell or haemoglobin S (HbS), a well-studied structural variant of the β-globin chain of haemoglobin (substitution of glutamic acid with valine at position 6). HbS is frequent in malaria-exposed populations with allele frequencies of up to 30% [15, 16], despite the debilitating consequence of its homozygote form [17]. Carriers of homozygote and heterozygote forms of HbS are protected against severe malaria [18, 19] with a 10-fold reduction of risk [20]. Another mutation at the same position (replacement of glutamic acid by lysine) of the same *hbb* gene, called HbC, does not cause debilitating conditions, but also protects against severe malaria whether in homozygote or heterozygote form [21, 22]. HbC is more frequent in West Africa and is a characteristic of the Dogon people, an ethic group in Mali, from severe malaria [21]. Haemoglobin E (substitution of glutamic acid with lysine at position 26) is another variant of β-globin chain. It is a common variant in Southeast Asia, and is assumed to

protect against invasion of erythrocytes by *Pf* [23]. Other genetic variants in the human host which are protective against malaria and involve modifications to erythrocyte biology include: G6PD deficiency [24-26], α-thalassemia [27, 28], ovalocytosis [29, 30], Duffy-negative blood group [31] and glycophorins [32, 33].

1.2 Clinical manifestations of malaria

Human malaria infection is a potentially life-threatening disease characterised by fever and other unspecific symptoms. Following the bite of an infected mosquito, the first symptoms appear in the human host after 7 to 30 days (incubation period), and are caused by the erythrocytic schizogony in the blood. During the erythrocytic stage, malaria parasites consume and degrade host haemoglobin to produce hemozoin pigment waste. This hemozoin pigment and other malaria toxins like glycosylphosphatidylinositol (GPI) moieties are dumped into the blood stream during schizont rupture and merozoite release from infected red blood cells. These induce the synthesis and release of tumour necrosis factor and others cytokines like interleukin-1 (IL-1), IL-6, IL-12, interferon-gamma and soluble factors like nitric oxide intermediates and reactive oxygen intermediates, resulting in chills, fever and other symptoms associated with malaria infection [34-37].

Depending on symptoms, malaria can be classified as uncomplicated or complicated. Uncomplicated *Pf* malaria is defined by WHO as symptomatic *Pf* malaria parasitaemia without signs of severity or evidence of vital organ dysfunction [38]. The clinical manifestations of uncomplicated *Pf* malaria are usually: fever or history of fever, chills, headache, body aches, dizziness, vertigo, altered behaviour, weakness, nausea, vomiting, diarrhea, jaundice, hepatosplenomegaly. Patients can often be found with different combinations of the cited symptoms above.

Complicated (severe) *Pf* malaria is defined by WHO as an acute falciparum malaria with signs of severe illness and/or evidence of vital organ dysfunction. The more common symptoms found in the patients with complicated *Pf* malaria are: hyperparasitaemia; where the parasite count is higher than 500,000 per microliter of blood, hyperpyrexia; where the temperature is higher than 40˚C, hypotension, hypoglycaemia, severe aneamia; where the haemoglobin level is under 5 g per decilitre, jaundice, generalized convulsions, impaired consciousness, coma, metabolic

acidosis, acute respiratory distress syndrome, pulmonary oedema, acute kidney failure, and disseminated intravascular coagulation [39, 40].

Pf is responsible for the majority of severe malaria cases. But, it has also been reported in patients with *Pv* malaria, where except for hyperparasitaemia, the clinical manifestations of *Pv* versus *Pf* severe malaria are the same including symptoms of severe anaemia, acute respiratory distress [41], and acute kidney failure [42]. Severe *knowlesi* malaria also has been described. Here symptoms similar to those of *Pf* malaria are typical with two differences, being hyperparasitaemia, which for severe *knowlesi* malaria is defined as a parasite density higher than 100,000 per microliter, and jaundice, characterised by a parasite density higher than 20,000 per microliter [39]. The other two species infecting human (*malariae* and *ovale*) rarely cause severe malaria.

1.3 Malaria biology and life cycle

Plasmodium spp. are single-celled eukaryotic protozoan parasites. They have a complex life-cycle which alternates between an invertebrate (female Anopheles mosquitoes) and a human host.

Infected mosquitoes carrying malaria sporozoites in their salivary gland, during a blood meal, deposit them into the skin of the human host. At this stage, a large proportion of sporozoites remain in the skin and are eliminated. Others travel to the lymph nodes, where some are engulfed and degraded in dendritic leucocytes, and some can partially differentiate into exoerythrocytic stages before destruction [43-45]. The remaining sporozoites glide to enter the dermal capillaries and are drained by blood flow into the liver. In the liver, they are arrested by binding to the sinusoidal cell layer, where they glide and pass through the Kupffer cells. They traverse several hepatocytes, killing them, before settling in one for differentiation and multiplication [43-45]. The thrombospondin-related anonymous protein (TRAP), a protein which is expressed on the surface of sporozoites, is indispensable for gliding motility [46]. TRAP and other major surface proteins of sporozoites including the circumsporozoite protein are implicated in sporozoites binding to the sinusoidal cell layer of hepatocytes [45]. In hepatocytes, the parasites develop inside a parasitophorous vacuole (PV), the formation of which is induced by sporozoites during the process of invasion [47]. Sporozoites replicate asexually to form schizonts (a multinucleate stage of the parasite), and then several thousand of merozoites (the parasite stage that can

invade and infect red blood cells). After hepatocyte infection *Pv* and *Po* sporozoites can enter a latent, non-replicative state called hypnozoites [48]. These dormant hypnozoites can resume growth and complete liver-stage development, and then cause relapsing blood-stage infection months to years after the initial infection, in the absence of a new infective mosquito bite. The last step of the hepatic stage of parasite infection is egress from hepatocytes. Rupture of the PV membrane releases merozoites inside the hepatocytes cytoplasm followed by initiation of host cell detachment and death [49]. During this process of detachment and death vesicles filled with merozoites, known as merosomes, bud into the sinusoid lumen [50]. To avoid detection by the host immune system the parasites manipulate the hepatocyte and inhibit the exposure of phosphatidylserine on the outer leaflet of plasma membranes [50]. The merosomes migrate and deliver merozoites directly into the bloodstream.

Very few antimalarial drugs are active on the hepatic forms of the parasite, particularly the latent hypnozoites. The main antimalarial drugs active on hypnozoites are primaquine and tafenoquine [51-53].

Merozoites are the smallest cell of the *Plasmodium* life cycle. They have all the conventional organelles of eukaryotic cells, including a nucleus, mitochondrion, Golgi apparatus, endoplasmic reticulum, and an inner membrane complex underlying the plasma membrane, sub-pellicular microtubules, and an apicoplast. Micronemes, rhoptries and dense granules constitute the apical complex of secretory organelles.
The merozoite stage is immunologically important. It is the stage where the parasites are briefly extracellular and exposed to host antibodies between egress and new invasion. Released merozoites invade red blood cells and start the erythrocytic cycle. During the invasion process the initial interaction involves deformation of the erythrocyte after attachment of merozoites to the cell surface. Some studies suggested the implication of merozoites surface protein 1 (MSP-1) and other surface proteins in this attachment [54, 55]. The initial attachment is followed by apical reorientation, bringing into contact the apical pole with the erythrocyte surface. At this stage, the parasite is irreversibly attached to the cell surface and is ready for invasion [56]. The parasites move inside the red blood cells under the action of an actomyosin motor and the formation of a tight junction between parasite and erythrocyte plasma membranes [57, 58]. The tight junctions are established using apical membrane antigen1

(AMA1), a microneme derived protein associated with the parasite membrane and rhoptry neck (RON) complex of proteins; a rhoptry derived protein anchored into the red blood cell [59]. As the merozoites move inside, the PV and membrane are formed from rhoptries and cell membrane components. The last step of invasion is echinocytosis of the red blood cell, after which the parasites are free and mobile in the cells [60].

In the infected erythrocyte, parasite asexual development is complex, and passes through a succession of three morphological stages, being ring, trophozoite and schizont. At the end of the erythrocytic cycle, the rupture of the schizont leads to the release of 16 to 32 daughter merozoites, which can enter new red blood cells to start the cycle again. Parasites develop inside the parasitophorous vacuolar membrane (PVM). For the survival inside the erythrocytes, parasites modify the host cell through the export of proteins. The majority of parasite proteins are exported during the ring stage of development [61]. The trophozoite stage is characterised by rapid growth of the parasites and the appearance of haemozoin (malaria pigment) a bio-crystal remnant of haemoglobin digestion [62]. Many antimalarial drugs interfere with this bio-crystallisation of haems to haemozoin. Free haems are toxic for malaria parasites.

Pf is detectable by microscopy in the blood stream only in the first half of its erythrocytic cycle. During the second half, trophozoites and schizonts are sequestered in the deep vasculature [63], a principal cause of morbidity and mortality induced by malaria [64]. Parasite proteins, including *Pf* erythrocyte membrane protein 1 (PfEMP1), expressed on the surface of host cell are involved in the sequestration and parasite virulence [65-67]. These *Pf* proteins are believed to be trafficked to the host cells surface trough parasite-induced vesicular structures named Maurer's clefts [62, 68]. The display of these *Pf* proteins, necessary for the sequestration of infected erythrocytes, is facilitated by host cell surface structures named knobs [69].

In addition to parasite multiplication, the blood-stage parasite asexual replication results in the generation of the sexual stage, the gametocytes. The commitment of the asexual form to sexual development starts at some point in the preceding schizogony [70]. The asexual schizont committed for gametocytogenesis releases merozoites which invade new red blood cells and develop through the trophozoite stage to produce gametocytes [70]. Each dedicated schizont produces progeny of only one sex: either male or female gametocytes [70]. The maturation of

gametocytes passes through five stages. Only stage I, the early one and stage V, the mature one, are found in the blood circulation. Stages II – IV sequester in the bone marrow, and are absent from the blood circulation [71]. Many factors have been associated with increased gametocyte production including administration of the antimalarial drug chloroquine. Other antimalarial drugs, such as the 8-aminoquinoleine primaquine, have gametocytocidal activity [72].

The pre-erythrocytic phase lasts for around 5 – 7 days for *Pf*, 9 - 10 days for *Pv*, 9 days for *Po*, 13 days for *Pm* and 8 – 9 days for *P. knowlesi* [73]. Antimalarial drugs with a short half-life which do not have any action on pre-erythrocytic parasite stages cannot protect patients against rapid new infection.

Most of the available antimalarial drugs act on the erythrocyte stage of the parasite's life cycle (Figure 1). This stage takes around 24 hours to be completed for *Plasmodium knowlesi*, 48 hours for *Pf, Pv and Po* and 72 hours for *Pm* [74-76].

Female mosquitoes, during they blood meal, take up the mature gametocytes. In mosquitoes' midgut, a gametogenesis is initiated with the rapid transformation of mature gametocytes into male and female micro and macro gametes, respectively. One female gametocyte transforms into one macrogamete, while each male gametocyte undergoes three rounds of endomitotic replication to release eight haploid and motile microgametes after exflagellation [77, 78]. Fertilisation of macrogametes by microgametes results in the formation of diploid zygotes [77], which undergo endomeiotic replication to produce a single tetraploid zygote, without nuclear or cellular division [77]. Transformation of zygotes results into motile ookinetes, which glide over the midgut epithelium apical surface and enter epithelial cells. The presence of ookinetes in epithelial cells results in the cell death and extrusion in the midgut lumen. During cell extrusion, ookinetes egress and then migrate intercellularly to the basal surface of the epithelium [79-81]. Under the basal lamina, ookinetes cease movement and continue their differentiation into oocysts [81]. Immediately after oocyst formation, several mitotic replications of the four haploid products of the meiosis occur within the nucleus with an upregulation of the synthesis of some proteins. This results in significant asexual amplification of parasite numbers, termed sporogony [82]. Oocyst transformation continues until the formation of sporoblast, and is completed with the production of thousands of sporozoites, which bud out of the sporoblast. Free sporozoites circulate passively in the haemolymph [83] of the

mosquito and then actively invade the salivary glands by gliding motility [84]. In the salivary duct, mature sporozoites are ready for inoculation into the vertebrate host during subsequent blood-feeding by the infected mosquitoes [85, 86].

The sexual cycle of malaria parasites in mosquitoes is not a smooth passage. Indeed, parasites are exposed to the mosquitoes' defenses mainly during the ookinete stage. It is this stage where the parasite is extracellular and motile in the midgut. The mosquito immune system utilizes innate barriers and effectors, but lacks adaptive immunity. The main physical barriers are the peritrophic matrix, the midgut epithelium and the basal membrane. These barriers provide the first lines of the defense against parasites [87] in the invertebrate host. Mosquitoes' humoral and cellular immune responses also contribute significantly to defence against parasite infection [88]. Hemocytes are the major immune cells involved in mosquito innate immune response [88] and both hemocytes and fat bodies are involved in the release of immune effectors implicated in phagocytosis, melanization, nodule formation, agglutination, encapsulation and secretion of antimicrobial peptides (AMPs) [89, 90]. Hemocytes and other cells are implicated in nitric oxide and reactive oxygen species production, which are toxic for malaria parasites [91]. In the humoral immune response, mosquitoes' resistance to malaria parasites, specifically to *Pf*, has been attributed to *Anopheles Plasmodium-responsive leucine-rich repeat 1* (*APL1*) in *Anopheles gambiae* [92]. Each of these humoral and cellular components are connected by signaling pathways. These pathways include immune deficiency (Imd), Toll, Janus kinase (JNK), and signal transducers and activators of transcription (STAT). Toll and Imd pathways are activated when they recognize pathogen-associated molecular patterns (PAMPs). This activation is important for the nuclear translocation of the NF-κB transcription factors Rel1 and Rel2, respectively, which activate transcription of immune effector genes such as AMPs and other factors. The main classes of AMPs are defensins, cecropins, attacin, and gambicin [89, 93].

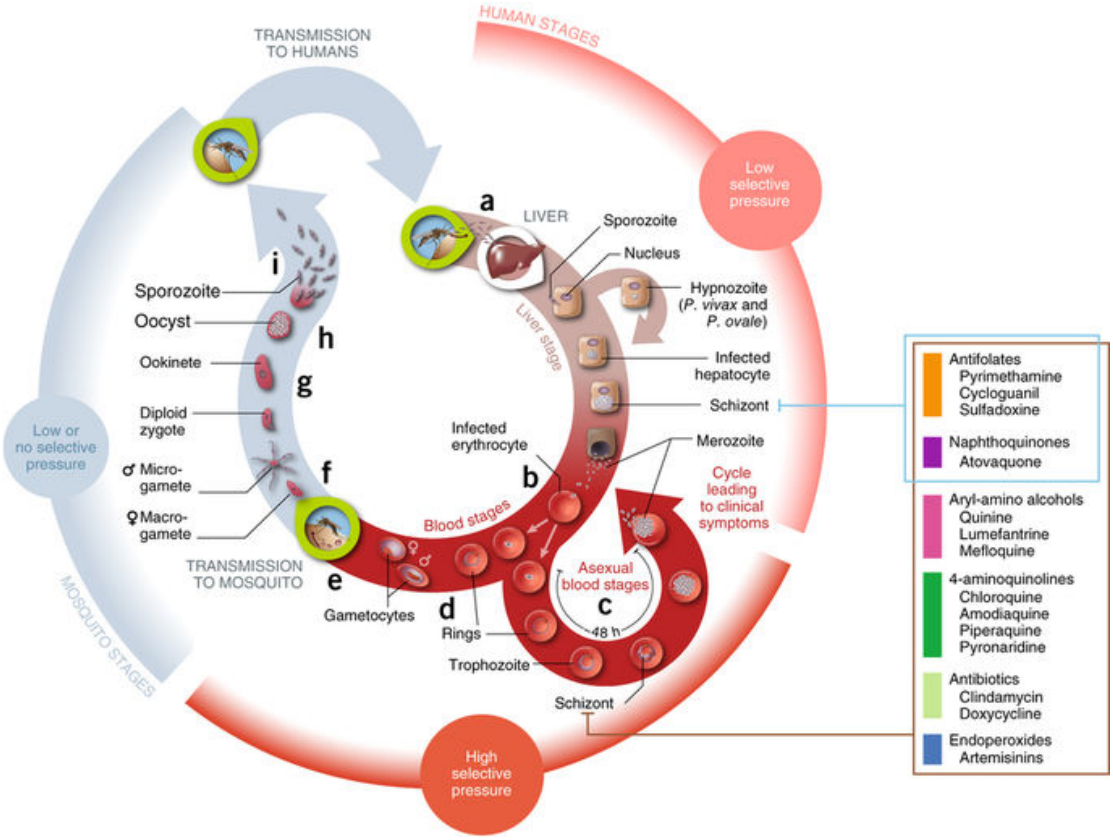


Figure 1: Malaria life cycle (Blasco *et al.* 2017) [94]

1.4 Malaria control strategies

In terms of malaria disease control strategies, WHO recommends the following:

- Management using rapid diagnostic test (RDT) or microscopy for confirmation of infection;
- Treatment with ACT,
- intermittent preventive treatment for pregnant women (IPTp) and infants (IPTi),
- Seasonal malaria chemoprevention (SMC) for children aged 3 – 59 months in the sub-Sahel region of Africa with highly seasonal malaria i.e. Chad, the Gambia, Guinea, Mali, Niger and Senegal; and
- Use of insecticides to control vector populations including insecticide-treated mosquito nets (ITN), indoor residual spraying (IRS) and larval control [8].

It should be noted that with the exception of larval control, the majority of these intervention and management strategies have been implemented in Mali.

One of the ways to avoid malaria progression to life-threatening complications is early diagnosis as well as effective treatment. As a result of global resistance of *Pf* to antimalarial monotherapies (chloroquine, amodiaquine and sulfadoxine-pyrimethamine), WHO has recommended the use of ACT for the treatment of uncomplicated *Pf* malaria since 2001 [95]. The recommended ACTs are: AL, ASAQ, artesunate-mefloquine (ASMQ), DHA-PPQ, PA, and artesunate-sulfadoxine-pyrimethamine (ASSP)[39, 96, 97].

By the end of 2009, ACTs were adopted as national policy for first line treatment of uncomplicated *Pf* malaria in most of the malaria endemic countries [98]. In Mali, where *Pf* malaria is responsible for 42% of hospital admissions, the national malaria control program (NMCP) has recommended the use of AL or ASAQ for the treatment of uncomplicated cases of malaria since 2006 [8].

WHO advises monitoring the efficacy, safety, and resistance to antimalarial drugs using its standard protocols. The first-line antimalarial drug should be changed if the proportion of total treatment failures after molecular correction is ≥ 10%, after monitoring (*in vivo)* for therapeutic efficacy [39]. A number of studies have evaluated the efficacy and safety of ACTs, particularly in sub-Saharan Africa, and report cure rates of more than 90% after molecular correction as recommended by WHO [99-103]. Other studies specific to Mali have shown that ASAQ, ASSP, ASMQ and AL are safe and efficacious, with corrected cure rates of more than 95% after 28 days of follow-up [102, 104-107].

While these ACTs are still efficacious in sub-Saharan Africa, there is concern about documented parasite resistance to antimalarial drugs, mainly to artemisinin and its derivatives, in Southeast Asia [108-110]. Resistance to the partner drugs of artemisinin and derivatives have also been reported in Southeast Asia [111-113]. Despite maintenance of good efficacy of ACTs in Africa, one study showed a declining response of *Pf* to DHA-PPQ, and to AL in Kenya [114] and other studies have identified a persistence of sub-microscopic *Pf* parasitaemia until day 14 after AL treatment [115, 116]. These two studies did not show a clear relation between these phenomena and resistance to ACT. However, they emphasise the need for continued surveillance of ACT efficacy as recommended by WHO to rapidly detect the emergence of parasite resistance in case it does appear in Africa. This

recommendation mandates the clinical and parasitological assessment of therapeutic efficacy which should include [39]:

- Confirmation of the quality of the antimalarial medicines tested;
- Molecular genotyping to distinguish between re-infections and recrudescence, and to identify genetic markers of drug resistance;
- Studies of parasite susceptibility to antimalarial drugs in culture; and
- Measurement of antimalarial drug levels to confirm adequate exposure in the case of slow therapeutic response or treatment failure.

To these existing anti-malarial tools, vaccines would be a perfect addition. For the moment, there is no vaccine for prevention, control, elimination and eradication of malaria. The parasites causing malaria have a complex biology that complicates the research of vaccines. There are many vaccine candidates, the most advanced to date is a pre-erythrocytic stage vaccine, a recombinant circumsporozoite protein vaccine, RTS,S/AS01. The target of this vaccine is the *Pf* circumsporozoite protein (CSP) present on the surface of the sporozoites and exported in the cytoplasm of hepatocytes. CSP is a major surface protein of the sporozoites. CSP was the key target of protective immunity induced by irradiated sporozoite vaccines in animal models [117]. The first vaccine candidates targeting PfCSP failed to generate significant protection [118]. RTS,S/AS01 was created based on experience during the development of the genetically engineered hepatitis B vaccine. RTS,S designate the presence of the CSP repeat region (R), T-cell epitopes (T) fused to the hepatitis B surface antigen (HBsAg) (S) and assembled with unfused copies of HBsAg (S) [119]. The efficacy of this candidate vaccine against clinical malaria, over a period of 14 months after first vaccination, varied from 30.1% in infants in the younger age category (6 to 12 weeks of age at the time of first vaccination) [120] to 50.4% in children in the older age category (5 to 17 months of age) [121]. In July 2015, RTS,S received a positive scientific opinion from European Medicines Agency (EMA), for use outside the European Union.

The only malaria vaccines which have a vaccine efficacy higher than 90% in malaria-naïve volunteers, are based on the whole-parasite vaccine approach. The potency of this approach was first shown in 1973 with the use of radiation-attenuated *Pf* sporozoites (PfSPZ) inoculated by mosquito bites [122, 123]. To date, substantial progress has been made with the development of this approach. Chemoprophylaxis

with sporozoites (CPS) is a second approach, where infectious PfSPZ are inoculated by mosquito bites to participants who are under chloroquine (CQ) [124, 125] or mefloquine [126] chemoprophylaxis. CPS induces potent, long-lasting immunity. Another approach is to use genetically attenuated parasites (GAP), using knockouts of P52, P36, sap1 or B9 genes [127-130]. Parasites deficient in these genes can develop normally from the asexual erythrocyte stage to the sporozoite stage; however, their development is arrested after hepatocyte invasion. The most advanced GAP in clinical development is the double-mutant parasite PFSPZ-GA1 produced by the biotechnology company, Sanaria, where attenuation is achieved by deletion in *b9* and *sap1* genes, which are indispensable for successful liver-stage development of the parasite [130]. In the PfSPZ-chemoprophylaxis vaccine (PfSPZ-CVac): non-irradiated, aseptic, purified, cryopreserved PfSPZ (PfSPZ challenge) are inoculated intravenously in healthy volunteers under CQ chemoprophylaxis. This approach protected 100% of malaria-naïve volunteers against controlled human malaria infection using the vaccine strain (NF54) [131].

Proprietary methods have been developed to manufacture, purify, cryopreserve and intravenously inject aseptic whole PfSPZ, minimizing the use of the complex model of mosquito bites.

The whole parasite vaccine model is also efficacious in malaria-exposed adults, albeit with a substantially reduced degree of protection [132]. A study of the safety and efficacy of the PfSPZ vaccine in Mali showed a vaccine efficacy of around 48% against first infection and 29% against all infections for 6 months during the malaria transmission season [132]. Thus, the key goal for malaria vaccine development, the generation of robust and long-lasting protection against heterologous natural challenge, remains elusive.

1.5 Chemotherapy of malaria

Anti-malarial drugs constitute one of the critical elements in malaria control and prevention. Drugs used for chemotherapy or chemoprevention can be classified according to their chemical structure, their use for treatment or chemoprophylaxis, or stage-specificity of action. For stage-specificity of action, the recommended antimalarial drugs can be sub-grouped into three categories. The majority of anti-malarial drugs available on the market are drugs with activity against the asexual

blood stages only, e.g., CQ, amodiaquine, mefloquine, artemisinins and its derivatives, quinine and quinidine, lumefantrine, piperaquine and pyronaridine. These drugs are used to treat, or prevent, symptomatic malaria.

A second group of drugs targets both liver stages and asexual erythrocytic forms of *Pf*, and is comprised of atovaquone, proguanil, pyrimethamine, sulfadoxine and all antibiotics with antimalarial action, e.g., azithromycin, doxycyclin and clindamycin.

Primaquine and tafenoquine are representatives of a third group of drugs, with activity against liver stages (even latent liver stages) and gametocytes.

1.5.1 Amodiaquine

AQ is a 4-aminoquinolone compound with schizonticidal activity. Like other quinolone-containing drugs, AQ accumulates in the acid-food vacuoles of the intraerythrocytic-stage malaria parasite [133, 134]. *Pf*, during its asexual reproduction phase, digests host cell haemoglobin and releases heme, which is toxic for the parasite [135, 136]. To detoxify the heme, the parasite polymerizes it to hemozoin. It is hypothesised that AQ and related malaria schizontocides act by inhibiting the heme polymerase enzyme activity [137].

WHO recommends the use of AQ in combination therapy with an artemisinin derivative. AQ is combined with artesunate in the treatment of uncomplicated *Pf* malaria. The fixed-dose combination of ASAQ was the first treatment made available by Drugs for Neglected Diseases *initiative* (DND*i)* in 2007 through a partnership with Sanofi and has been included on the WHO Essential Medicines List in 2011. DND*i* estimated the distribution of 320 million treatments of ASAQ in Africa by the end of August 2014 [138].

AQ is administered at a dose of 10 mg per kg body weight once a day for a period of three days. In combination with artesunate, the body weight-adjusted dose of AQ ranges between 7.5 – 15 mg per kg per dose [139].

Since 1990, AQ is no longer advised for prophylaxis due to cases of agranulocytosis which were observed in some patients who took the drug for malaria prevention [140]. As a result of this toxicity issue, the use of AQ plummeted over the late 1980s until 2000s, however, emerging resistance to CQ and sulfadoxine-pyrimethamine has renewed the interest in AQ as a partner drug for ACT.

AQ, with its short terminal half-life of around 5 hours, is metabolised rapidly to its main metabolite N-desethylamodiaquine (DEAQ) (Figure 2) [141]. Even at a therapeutic dose, AQ concentration is low in plasma, and it is not detectable 8 h after administration. AQ and DEAQ exhibit first order pharmacokinetics, meaning that the amount of drugs excreted in the urine in a set amount of time is in a linear relationship with the amount of drug in the body [142]. DEAQ is an active metabolite of AQ with a long half-life of 1 to 3 weeks [143, 144]. AQ and DEAQ bind to plasma proteins with a proportion of over 90%.

AQ is metabolised to DEAQ in the liver mainly by CYP2C8 [145]. This cytochrome (CYP) is highly polymorphic, with the variant CYP2C8*2 being the most frequent among the mutant forms in the African population. CYP2C8*2 showed slow metabolism of AQ (threefold higher Km and six-fold lower intrinsic clearance) [146]. The activity of CYP2C8 is not known to be influenced by age or gender [147].

AQ toxicity, mainly its role in neutropenia, may be due to its metabolite quinoneimine [148]. AQ and DEAQ are also metabolised by extrahepatic CYP (CYP1A1 and CYP1B1) to an intermediate metabolite called M2 [145, 149]. This intermediate is responsible for the generation of toxic quinoneimine by the action of CYP1A1, CYP1B1 (found in peripheral blood leucocytes [151]) and myeloperoxidases [150].

1.5.2 Pyronaridine

Pyronaridine is a schizonticide antimalarial drug, first synthesised in 1970 in China, where it has been used alone in the treatment of uncomplicated *Pf* malaria for more than 30 years [152]. The structure of pyronaridine is related to 4-aminoquinolines, but it is more active than CQ. It is highly active *in vitro* and *in vivo* on *Pf* multidrug-resistant parasites, particularly CQ resistant parasites [153-156].

The mechanism of action of pyronaridine is explained by its capacity to inhibit the formation of β-hematin [157]. It forms a complex with hematin, to enhance hematin-induced red blood cell lysis. It also acts in inhibiting glutathione-dependant hematin degradation [157-159]

Pyronaridine is used in a fixed-dose combination with artesunate in a ratio of 3:1 in the treatment of uncomplicated *Pf* malaria. The combination PA is available in

two formulations: PA granules (60:20 mg) for children and infants between 5 and 20 kg, and tablets (180:60 mg) for patients weighing more than 20 kg.

Few methods have been developed to quantify pyronaridine concentrations in human whole blood or plasma [160-163]. Pyronaridine concentrations in blood cells in rabbits demonstrate blood:plasma ratios which vary from 4.9 to 17.8 [161]. Another study in rabbits found blood:plasma ratios ranging from 3 to 6 after intramuscular dosing [164]. The blood:plasma distribution of pyronaridine was also evaluated *in vitro* using whole blood in rabbit (2.5 to 3.8) and in human (1.2 to 1.7) [152, 165]. Between 92-96% of pyronaridine is bound to plasma proteins [165].

After 3-day treatment of malaria patients with a 12 mg/kg daily dose of pyronaridine, the estimated time to reach the maximum plasma concentration was 80.0 ± 79.9 hours, with an elimination half-life of 194.8 hours [166, 167].

Cytochrome P450 enzymes, mainly CYP1A2, CYP2D6 and CYP3A4, metabolise pyronaridine [165]. Morris CA and *al*. in 2015 identified nine primary and four secondary metabolites of pyronaridine [168]. The routes of excretion of pyronaridine and its metabolites are urinary and fecal [168].

1.5.3 Piperaquine

Piperaquine is a bis-4-aminoquinoline antimalarial drug, synthesised independently in 1966 in China and in France [169]. It has been evaluated in China for prophylaxis and treatment of uncomplicated malaria [170]. It also has been assessed in Africa [171]. The development and use of piperaquine was pursued in China until the 1980s. Piperaquine received renewed consideration during the last two decades because of *Pf* resistance to CQ, and due to its long half-life.

Piperaquine is available in combination with dihydroartemisinin in two different strength tablets: 160:20 mg for patients weighing less than 13 kg and 320:40 mg for patients weighing at least 13 kg. It is administered orally once daily for 3 days to treat uncomplicated *Pf* malaria. Following administration to malaria patients, the maximum plasma concentration of piperaquine is observed at around 4 hours, with a median terminal elimination half-life of 23 days. The median day 7 capillary plasma concentration is around 64 ng/ml with high inter-individual variation [172, 173]. Piperaquine is probably metabolised by CYP3A4 [174].

Various methods of detection have been developed to quantify piperaquine in different biological matrices such as venous whole blood, plasma, serum, urine and capillary whole blood, including the use of HPLC with UV detection or tandem mass spectrometric detection [175-179]. Extra care is required during piperaquine quantification because it is heavily adsorbed on glass surfaces. To avoid this issue, plastic material or silanized glassware has to be used during analytical methods development and its quantification [180].

The plasma protein binding fraction of piperaquine is estimated to be 97% [175].

1.5.4 Lumefantrine

Lumefantrine, or benflumetol, is an arylaminoalcohol antimalarial drug used exclusively in combination with artemether for the treatment of malaria. AL is indicated for the treatment of uncomplicated malaria in patients of 5 kg bodyweight and above. A 3-day treatment schedule with a total of 6 doses (after the initial dose subsequent doses are scheduled at 8; 24; 36; 48 and 60 hours) is recommended according to body weight as below:

5 kg to less than 15 kg: One tablet per dose (total course of 6 tablets).

15 kg to less than 25 kg: Two tablets per dose (total course of 12 tablets).

25 kg to less than 35 kg: Three tablets per dose (total course of 18 tablets).

35 kg body weight and above: Four tablets per dose (total course of 24 tablets).

Lumefantrine has a relatively long half-life (3 – 6 days) and is metabolised to desbutyl-lumefantrine. Both lumefantrine and it's metabolite are active and the proportion of desbutyl-lumefantrine found in the body is only 10% of lumefantrine.

Lumefantrine is a schizonticidal drug. Its mechanism of action is not well understood, but some studies show that it binds to hemin to inhibit the formation of ß-hematin [181]. More than 99% of lumefantrine was found to be plasma protein bound [182].

Lumefantrine is metabolised by CYP3A4, and it inhibits CYP2D6 [183]. CYP3A4 is the most abundant CYP expressed and accounts for approximately 30 to 40% of the total CYP content in human adult liver and small intestine. CYP3A4 activity is higher during infancy (around 120%) than that of adults. Its activity and

expression are also higher in women than in men [184, 185], which can have an impact on the pharmacokinetic profile of its substrates like lumefantrine.

Analytical methods have been developed to quantify lumefantrine and its metabolite in plasma; and in whole blood spotted onto filter paper using HPLC with UV detection or tandem mass spectrometric detection [186, 187]. Precaution has to be taken to avoid evaporation of the processed eluates containing lumefantrine in plastic tubes. Lumefantrine can be adsorbed on plastic surfaces [186]. Whole blood spotted onto filter paper is a simple method for sample collection and storage prior to the quantification of antimalarial drugs. Lumefantrine extraction recovery from filter paper is weak. To salvage extraction recovery, pre-treatment with 0.75 M tartaric acid and storage at 4ºC is required [188]. Another method is based on the use of 1.6 mol/L phosphoric acid for pre-treating sampling paper [189].

1.5.5 Artemisinin (Qinghaosu)

Artemisinin, isolated from *Artemisia annua* is a sesquiterpene lactone with an endoperoxide bridge. Artemisinin and its derivatives are the most potent antimalarial drugs currently in use [63]. Their use as mono-therapy is not advised; however, use of artemisinin and its derivatives in combination with other antimalarial drugs with different mechanisms of action is the recommended first-line treatment in all malaria endemic countries. Artemisinin activation is heme-dependent [190]. It acts by binding to many proteins which are implicated in essential biological processes of the parasite [190]

1.5.6 Artemether

Artemether is a semi-synthetic derivative of artemisinin used in the treatment of *Plasmodium spp*. It is administered in combination with lumefantrine to treat uncomplicated malaria caused by *Pf* or any case of uncomplicated malaria due to other *Plasmodium* species. Artemether, with its short half-life, has a high parasite reduction ratio (PRR), which leads to a rapid symptomatic relief. The function of lumefantrine in this combination is to eliminate the remaining parasites.

Artemether is metabolised by cytochrome P450 3A4 and 3A5 to the active metabolite dihydroartemisinin (Figure 2). Artemether and its active metabolite have short half-lives of 2 – 4 hours and 1 -2 hours respectively.

Artemether and its metabolite are endoperoxide antimalarial drugs, which like other endoperoxide antimalarials, are activated by heme or ferrous ions to generate a cytotoxic radical species (oxygen and carbon-centered radicals).

Artemether and its metabolite bind to plasma protein with different degrees, 92 to 98% for artemether and 47 to 76% for dihydroartemisinin [191].

Methods have been developed to quantify artemether and its active metabolite in human plasma by high-performance liquid chromatography after derivatization or by liquid chromatography-tandem mass spectrometry with electrospray ionisation and atmospheric pressure chemical ionisation [160, 192-194]. To date, there is no method to quantify artemisinin and its derivative from samples collected onto filter paper.

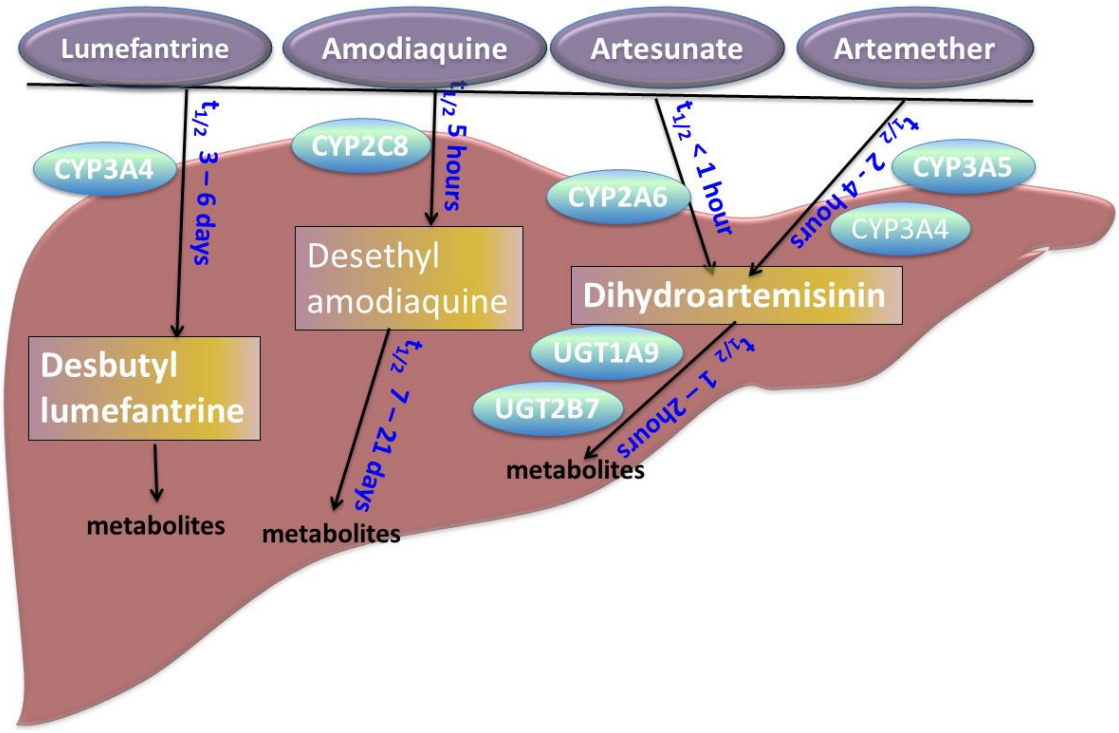
1.5.7 Artesunate

Artesunate is also a semi-synthetic derivative of artemisinin used in combination for the treatment of malaria. Because of its solubility in water, artesunate is used for parenteral administration in the treatment of severe malaria.

For the treatment of uncomplicated malaria, particularly *Pf* malaria, artesunate is combined with AQ or pyronaridine.

Artesunate has a short half-life of less than one hour. CYP2A6 metabolises it to the active metabolite dihydroartemisinin, which is further metabolised by glucuronosylation to an inactive metabolite. Its mechanism of activation and mode of action mirror those of artemisinin and its other derivatives.

Methods have been developed to quantify artesunate and its active metabolite in plasma.



<u>Figure 2:</u> Metabolism of the Anti-Malarial Agents Lumefantrine, Amodiaquine, Artesunate and Artemether

1.5.8 Preclinical antimalarial drug candidates with multiple-stage activity

Most of the antimalarial drugs in preclinical development are derived from screening of many thousands of compounds, using diversity-oriented synthesis with high-throughput phenotype-based screens.

For malaria control, elimination and eradication, new antimalarial drugs need to meet requirements such as a mechanism of action different from the currently used antimalarial drugs, they must cure liver stages, cure the asymptomatic blood infections, prevent transmission by killing or preventing development of sexual stages, be suitable for mass administration, and ideally achieve this with single dose administration[195]. Current preclinical antimalarial drug candidates which meet these requirements include DDD107498, bicyclic azetidine series, and NITD609.

DDD107498 is a new multiple-stage activity antimalarial agent that inhibits protein synthesis. Beatriz Baragana and colleagues discovered DDD107498 after screening 4,731 compounds. DDD107498 is derived from a 2,6-disubstituted quinoline-4-carboxamide scaffold and contains a fluorine atom, an ethyl-pyrrolidine group, and a morpholine group. DDD107498 has demonstrated activity against liver

stage parasites, blood stage parasites, and both male and female gametocytes. Baragana and colleagues identified translation elongation factor 2 (eEF2) as the molecular target of DDD107498. This factor is essential for protein synthesis [196].

Bicyclic azetidine series: These series are also novel multiple-stage antimalarial inhibitors, discovered after screening 100,000 compounds using a phenotypic blood-stage growth inhibition assay. The resulting compound, BRD3444, was modified to improve its physicochemical and pharmacokinetic properties, with the synthesis of BRD1095 and BRD7929 by replacement of hydroxymethyl at position C2 with aminomethyl and dimethyl aminomethyl substituents. These compounds are active against all parasite life stages, after a single oral exposure, and they act by inhibition of *Pf* cytosolic phenylalanyl-tRNA synthetase [197].

NITD609 is a potent antimalarial drug active against *Pf* and *Pv* blood stages at a low nanomolar concentration [198]. It can be orally administered once daily. NITD609 (spirotetrahydro-β-carboline or spiroindolone) was discovered after screening 12,000 both pure natural products and synthetic compounds [198]. NITD609 acts by rapid inhibition of protein synthesis in *Pf* [198]. Point mutations in the gene of P-type cation-transporter ATPase4 (PfATP4) are responsible for parasite resistance to this drug [198].

1.5.9 Pharmacokinetic characteristics of antimalarial drugs

The pharmacokinetic characteristics of antimalarial drugs are evaluated by using a classic pharmacokinetic study. The area under the curve (AUC) is the best pharmacokinetic parameter for the determination of treatment outcome in uncomplicated malaria [63, 199]. However, the determination of AUC necessitates collection of samples at multiple time points from the same patient, making it time-consuming, painful for small children, work-intensive due to the requirement for large numbers of samples for processing and analysis. Studies have found a good correlation between the day 7 concentration of lumefantrine, sulfadoxine and pyrimethamine and the AUC of these drugs [200]. This observation implies that sample collection at one time point (day 7) may be used as a surrogate measure of total exposure [201].

To date, the day 7 concentration of long half-life antimalarial drugs is increasingly used as a surrogate of AUC and a predictor of treatment failure [191,

202-205]. Validating the use of day 7 plasma concentrations of lumefantrine and other partner drugs of artemisinin and its derivatives as a good parameter for the determination of treatment outcome in uncomplicated malaria, is needed for repeat treatment episodes.

Blood drug concentration does matter when treating patients. A blood drug concentration that is too low can lead to treatment failure and/or induction of parasite resistance, whereas excessive blood drug concentrations may be toxic. Achieving the appropriate drug concentration in circulating blood is multifactorial. This can be linked to both genetic and non-genetic factors. Genetic factors include the human enzymes implicated in drug absorption, metabolism and elimination. Non-genetic factors include age [206], sex, disease status [207], ethnicity, food intake [206], and concomitant treatment [208]. Because of these factors, there is high inter-individual variability in the pharmacokinetic profile of antimalarial drugs leading to treatment failures and drug-related adverse events in patients.

1.5.10 Analytical methods for antimalarial drugs quantification

High-performance liquid chromatography methods and mass spectrometry methods have been developed to quantify lumefantrine, AQ and DEAQ in plasma and whole blood [160, 188, 209, 210]. Fewer methods have been developed for the quantification of pyronaridine [160, 161].

Mass spectrometry is more specific and sensitive than HPLC, its drawback is that the instruments are expensive and the cost of analysis per sample is more than 4 times the costs compared to HPLC. This kind of device is not affordable in most of the malaria endemic countries in sub-Saharan Africa.

Whole blood spotted onto filter paper for quantification of drugs is a simple technique and requires less blood volume compared to plasma or serum. For quantification of lumefantrine, pre-treatment of filter paper is required to achieve acceptable extraction recovery. To date, there is no filter paper method validated for quantification of pyronaridine.

The majority of analytical techniques used for the quantification of antimalarial drugs in biological fluids are HPLC and mass spectrometry, particularly liquid chromatography coupled with tandem mass spectrometry.

These techniques can be divided into two parts, sample extraction, and measurement of the given analyte (separation stage and detection stage) [211].

1.5.10.1 Sample extraction

Sample extraction consists of separating the analyte from the other constituents of the matrix. Many techniques are used for this purpose, including protein precipitation, liquid-liquid extraction, and solid phase extraction.

Liquid-liquid extraction consists of extracting the analyte by using two different immiscible liquids, generally one aqueous phase and one organic solvent phase. The analyte is partitioned between the two phases according to its solubility.

This technique is widely used to extract a broad range of analytes, and it has a good extraction recovery. Like protein precipitation, it is a non-specific method. Hydrophobic constituents of the biological fluids can contaminate the final extract. Compared to the two other techniques, it is time-consuming and generates a lot of organic solvent waste.

1.5.10.2 Measurement

In biological fluids, this step is divided into separation of mixtures and detection.

Separation

The most commonly used laboratory technique for the separation of mixtures containing antimalarial drugs is liquid chromatography, mainly HPLC. The technique is based on the differential partitioning of the components of the mixtures between the stationary and mobile phase. In HPLC, the stationary phase is embedded inside a tube (column). Inside the column, the mixtures pass through the stationary phase driven by the mobile phase at high pressure. Depending on the polarity of the stationary and mobile phase there is a normal-phase liquid chromatography and reversed-phase liquid chromatography.

In reversed-phase liquid chromatography the stationary phase is non-polar while the mobile phase is polar. The silica particles of the stationary phase are modified by the addition of straight-chain alkyl group having 8 or 18 carbon atoms. The non-polar molecules (analytes) are retained while the polar one passes through more quickly on the stationary phase. The non-polar molecules are retained because

of their hydrophobic interactions with the non-polar stationary phase. The more the contact surface area between the non-polar segments of an analyte and the stationary phase is high, the more the analyte is retained. The solution mixtures, driven by the mobile phase under high pressure, are separated on the stationary phase of the column according to their polarity.

Reversed-phase HPLC is easier to use than a normal-phase, it is robust and can be applied to a wide range of analytes especially organic analytes.

Reversed-phase HPLC methods have been developed to quantify the majority of antimalarial drugs.

Besides normal and reversed-phase HPLC, methods of separation include ion-exchange chromatography, size-exclusion chromatography, affinity chromatography, chiral chromatography and supercritical fluid chromatography.

Detection

The most commonly used detectors for the identification of antimalarial drugs are ultraviolet (UV), mass spectrometry (MS), fluorescence, and electrochemical detection.

Ultraviolet detection technique is the frequently used detection method for quantification of antimalarial drugs by HPLC. Analytes are detected because they absorb light in the UV wavelength range (100nm to 400 nm). The most commonly used solvent in HPLC-UV mobile phase is water, which absorbs UV light at 190 nm. Thus, to avoid false reading, UV wavelengths higher than 210 – 220 are used for detection. For HPLC-UV detection, in addition to water, UV transparent solvents are used as a mobile phase.

In the range of these UV wavelengths, analytes absorb the light because of the presence of the functional groups (chromophores), which absorbs UV light. The absorbance (A) of an analyte is proportional to its concentration *(C)* and the path length *(l)* of the solution as stipulated in Beer-Lambert law.

$$A = \varepsilon lC$$

Where ε is absorption coefficient of the analyte.

UV detection is the cheapest form of detection; it is robust, easy to manipulate and sensitive. The disadvantage of this type of detection with antimalarial drugs is its inability to quantify artemisinin and its derivatives because these drugs do not have a chromophore group to absorb UV light. The other disadvantage is that it is not sensitive enough to quantify long half-life antimalarial drugs at their terminal elimination phase.

Accurate, precise, and reproducible HPLC-UV methods have been developed to quantify antimalarial drugs from samples collected from malaria patients.

UV detectors, with variable wavelength, are preferred compared to a fixed wavelength. Variable wavelengths are more sensitive because they quantify analytes at peak absorption wavelength. Variable wavelength detectors include a diode array detector (DAD or PDA), which can scan a wide range of wavelengths simultaneously and quantify the analyte at its maximum absorption wavelength. Diode array detection can also be used to identify unknown metabolites and to verify the purity for an analyte.

For quantification of artemisinin and its derivatives, and to resolve the problem of sensitivity during quantification of antimalarial drugs at their terminal elimination phase many laboratories use MS detection.

1.6 Evaluation of drug efficacy

Control and elimination of malaria requires the use of effective antimalarial drugs. WHO recommends bi-yearly monitoring of the first- and second-line antimalarial drugs adopted by the national malaria control programmes. These medicines have to be changed if their molecular corrected cure rate falls below a threshold of 90% [39]. To better evaluate the efficacy of adopted and new antimalarial drugs, and to allow comparability between trials, WHO recommends standardized protocols to determine PCR-corrected adequate clinical and parasitological response cure rates [212, 213]. Besides these *in-vivo* standard protocols, there are *ex-vivo*, and *in-vitro* methods to guide the evaluation of drug sensitivity.

For an *in-vivo* test (Figure 3), patients that receive treatment are followed during 28 or 42 days according to the elimination half-life of the drug, to assess clinical and parasitological outcomes of the treatment [214-216]. During these follow-

up periods clinical and parasitological parameters are collected at different time points. Responses to treatment are classified as follows for all levels of malaria transmission according to WHO [213]:

- Early treatment failure (ETF)
  - Danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia;
  - Parasitaemia on day 2 higher than day 0, irrespective of axillary temperature;
  - Parasitaemia on day 3 with axillary temperature ≥ 37.5 ˚C; and
  - Parasitaemia on day 3 ≥ 25% of count on day 0.
- Late clinical failure (LCF)
  - Danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and
  - Presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature ≥ 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure.
- Late parasitological failure (LPF)
  - Presence of parasitaemia on any day between day 7 and day 28 (day 42) with axillary temperature < 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.
- Adequate clinical and parasitological response (ACPR)
  - Absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

With artemisinin and its derivatives and ACTs, parasite clearance half-life is used for identification and surveillance of resistance to artemisinin derivatives. Delayed or slow parasite clearance time defined by an elevated parasite clearance half-life is considered to be an indicator of resistance to artemisinin and its derivatives [217].

In-vitro and *ex-vivo* methods monitor the sensitivity of parasites to antimalarial drugs. These methods are not affected by human immunity. In an *ex-vivo* test, parasites collected from malaria patients are exposed to increasing drug concentrations to evaluate their susceptibility. In an *in-vitro* test, parasites are adapted in the laboratory before evaluating their sensitivity to a drug. Many *ex-vivo* and *in-*

vitro assays have been established for screening and evaluating the sensitivity of antimalarial drugs. There are methods based on schizont maturation [218, 219], radioisotope assays [220-223], immunoassays [224-227], high throughput screening [228], and fluorescence-based assays [222, 229]. The ring-stage survival assay (RSA) [230] is considered to be the best method for evaluating the sensitivity of parasite isolates or clones to artemisinin and derivatives *in-vitro* and *ex-vivo*.

All *in-vitro* tests except the macrotechnique of Rieckmann *et al.* [218] are based on the *in-vitro* method for continuous cultivation of *Pf* of Trager and Jensen [231]. In addition to *in-vivo*, *ex-vivo* and *in-vitro* assays, molecular methods have also been developed for the surveillance of *Pf* resistance to antimalarials drugs. Mutation in *Pf* CQ resistance transporter gene is a marker of resistance to CQ [232] and can be used as a molecular tool for surveillance of CQ resistance *in-vivo* [233]. Dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) quintuple mutations predict resistance to sulfadoxine-pyrimethamine *in-vivo* [234, 235]. The presence of two mutations (DHFR Arg-59, and DHPS Glu-540) also predicts sulfadoxine-pyrimethamine failure [235].

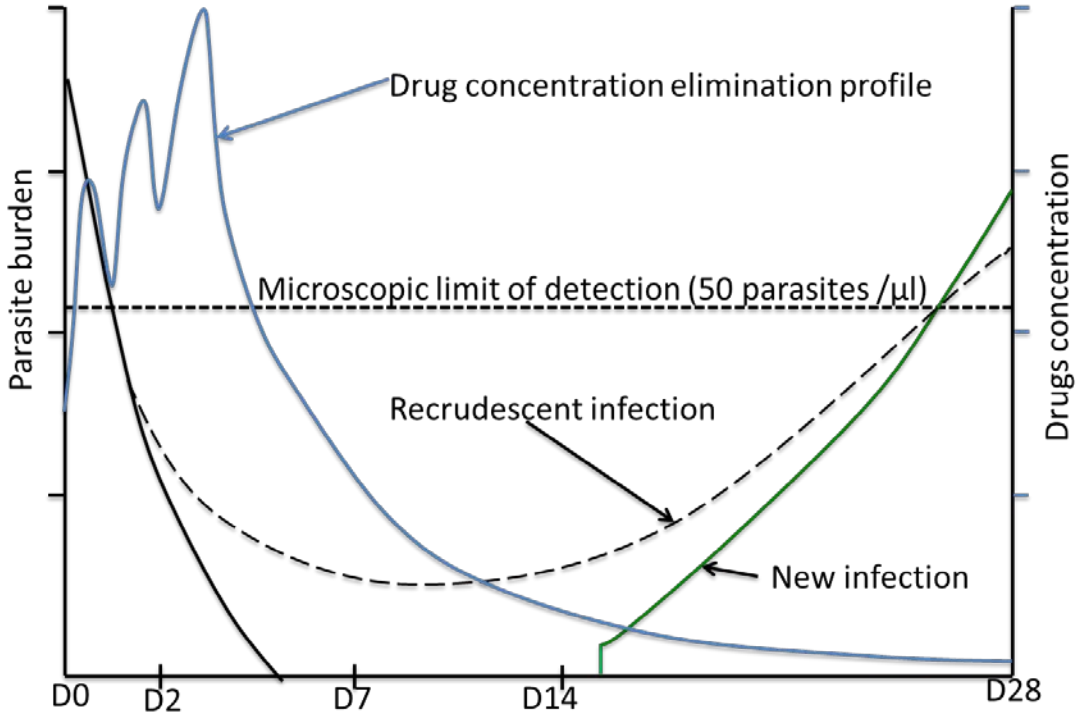


Figure 3: In vivo model of parasite and drugs interaction.

(black line) Infection with high parasitaemia which declines after treatment and becomes undetectable at day 2 - these parasites may be completely cleared if they are sensitive or they may recrudesce if they are resistant to the drugs.

(black dot line) Recrudescent infection with undetectable parasitaemia from day 2 to day 25 - parasitaemia becomes apparent after day 25.

(green line) New infection unrelated to the first one can appear and become detectable when the drug concentration falls below the inhibitory concentration.

(blue line) Concentration profile of the drugs administered once a day during three days.

1.7 Antimalarial drug resistance

Resistance to a drug is defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of medicine given in doses equal to or higher than those usually recommended" [38]. Resistance has been described to almost all of the classes of antimalarial drugs. Resistance can be due to mutations and/or amplification of specific genes encoding drug targets, transporters, or proteins involved in maintaining cellular homeostasis.

The first antimalarial drug to which resistance was documented worldwide was CQ. Resistance to CQ was first described in 1957 in Southeast Asia, particularly in Thai-Cambodian border. At least six origins of *Pf* CQ-resistant parasites have been

documented [236]. CQ resistance spread to reach Africa in 1978 [237], reaching Mali in 1987 [238].

In 1967, SP replaced CQ as the first-line treatment of uncomplicated malaria in Thailand. Cases of resistance to those drugs have since been documented in Thailand [239, 240]. SP resistance spread from Thailand to the other South-East Asian countries. Resistance to SP arose and became widespread in the late 1990s in Africa [241, 242].

Atovaquone was introduced in 1996, and resistance was described in the same year [94]. Bark extracts, which contains quinine were used since 1632 for the treatment of malaria. The first case of resistance to quinine was described in 1910 [94]. For mefloquine, the first case of resistance was described in 1982 after its introduction in 1977 [94].

Mutations in the gene coding for a protein termed *Plasmodium falciparum* CQ resistance transporter (PfCRT) are responsible for the resistance of *Pf* to CQ. A single point mutation, resulting in the substitution of lysine by threonine at amino acid position 76 of the PfCRT is the key mutation that confers resistance [232, 243]. The gene, which codes for the protein PfCRT, is located on chromosome 7. PfCRT is a 424 amino acid transmembrane protein in the digestive vacuole of malaria parasites. Other mutations in PfCRT, which confer resistance to CQ in association with K76T are C72S, M74I, N75E, A220S, Q271E, N326S, I356T, and R371I [243].

These PfCRT mutations have also been implicated in resistance to quinine, AQ and piperaquine [244-247]. However, the presence of PfCRT K76T mutation enhances the susceptibility of *Pf* to lumefantrine [246].

Many studies showed the role of *Pf* multidrug resistance protein 1 in antimalarial drug resistance. The gene (*Pfmdr1*), which codes for the protein with the same name, is located on chromosome 5. PfMDR1 is a transmembrane protein located in the digestive vacuole of the parasite. It belongs to the ATP-binding cassette (ABC) superfamily [248]. Mutations in this gene (N86Y, Y184F, S1034C, N1042D and D1246Y) have been implicated in drug susceptibility to CQ, quinine, mefloquine, halofantrine, piperaquine, lumefantrine and artemisinins [249, 250]. Lumefantrine, mefloquine and dihydroartemisinin are slightly more active against parasites harbouring the N86Y mutation, while these parasites are resistant to CQ and DEAQ [250]. Copy number variation of *Pfmdr1* has been associated with resistance to

quinine, mefloquine, lumefantrine, halofantrine and artemisinin [112, 251, 252]. Copy number variation of *Pfmdr1* is not common in Africa [250].

Decreased susceptibility of *Pf* to quinine is associated with polymorphism of *Pf* sodium hydrogen exchanger (PfNHE1). *Pfnhe1* is located on chromosome 13 and codes for protein, which is a transmembrane protein of *Pf* plasma membrane [253]. Polymorphism of the microsatellite ms4760 consisting of a DNNND repeat unit in *Pfnhe*1 is associated with decreased susceptibility to quinine [253, 254].

Resistance to pyrimethamine and sulfadoxine is associated with mutations in *Pf* dihydrofolate reductase-thymidylate (*Pfdhfr*) synthase and *Pf* dihydropteroate synthetase (*Pfdhps*), respectively. *Pfdhfr-ts* is located on chromosome 5 of *Pf* and codes for the PfDHFR and thymidylate synthase proteins. PfDHFR catalyzes the reduction of dihydrofolic acid to tetrahydrofolic acid in the tetrahydrofolate synthesis pathway, which is required for the essential synthesis of purines. DHPS is an enzyme, which catalyzes the synthesis of dihydropteroate from para-aminobenzoic acid (PABA) during folate synthesis. Mutations on *Pfdhfr* at positions (S108D, N51I, C59N, and I164L) and *Pfdhps* at positions (S436A/F, A437G, L540E, A581G, and A613T/S) are associated with sulfadoxine-pyrimethamine resistance [255-258].

PfDHFR mutations at codon position A16V and S108T are associated with cycloguanil resistance [259].

Resistance to atovaquone, which acts against the malaria parasite by inhibiting the mitochondrial electron transport chain, is associated with point mutations in the gene coding for the CYPbc1 protein. The mutation at position Y268N/S/C of CYPbc1 is associated with atovaquone resistance [260, 261].

Treatment responses to artemisinin and derivatives are evaluated by determining parasite clearance parameters. Parasites with slow or delayed clearance after artemisinin treatment are considered as tolerant. In Southeast Asia, mutations in the gene coding the Kelch propeller (K13-propeller) domain were associated with delayed parasite clearance after artemisinin treatment [110]. The K13-propeller is a protein encoded by a gene situated on chromosome 13, and it has many functions [262]. It is implicated in protein-protein interaction [263]. Among K13-propeller mutations, C580Y, R539T, and Y493H are the three most commonly associated with delayed clearance after treatment of malaria patients with ACTs [110].

Drug-resistant parasites have also been reported in *Pv*. *Pv* resistance is hard to evaluate because of relapses. Dormant liver stage (hypnozoites) can recur as early as 3 weeks after initial infection depending on environmental conditions [264]. Cases of CQ resistance of *Pv* were reported from Papua New Guinea in 1989 and from Indonesia in 1991 [265, 266]. *Pv* resistance was also reported for mefloquine and sulfadoxine-pyrimethamine.

The study of the molecular mechanisms of *Pv* resistance is based on identifying homologues of key genes implicated in *Pf* resistance. *Pvcrt,* the homolog of *Pfcrt* in *Pf,* is not involved in *Pv* resistance to CQ. A mutation of *Pvmdr1* conferring a change at codon position Y976F was strongly associated with *Pv* resistance to CQ [267, 268]. *Pv* resistance to mefloquine, as with *Pf* resistance, is associated with copy number variations of *Pvmdr1*. Parasites with an amplification of *Pvmdr1* were less susceptible to mefloquine [269].

Analogous to *Pf,* a set of mutations of *Pvdhfr* and *Pvdhps* has been reported to affect the susceptibility of *Pv* to pyrimethamine and sulfadoxine, respectively [270-273]. Four mutations have been reported in *Pvdhfr* (F57L/I, S58R, T61M, and S117T) and three in *Pvdhps* (S382A, A383G, and A553G).

1.8 Distinction between recrudescence and new infections for monitoring the antimalarial drug therapeutic efficacy against *Pf*

In malaria endemic countries, WHO recommends the use of molecular genotyping in the assessment of therapeutic efficacy of antimalarial drugs against *Pf* to distinguish between recrudescence and reinfections. To differentiate recrudescent infections from reinfections, polymorphic genes such as merozoite surface protein 2 (*msp2*), merozoite surface protein 1 (*msp1)*, glutamate-rich protein (*glurp*), and microsatellites such as ca1 and ta99 are used. One of the limitations of this technique, in a high-endemicity area, however, is the multiplicity of infection at the baseline before treatment. Minority parasite population genotypes may not be detected at baseline, but if these parasites are resistant to the treatment, they could be detected during follow-up and then classified as reinfection [274].

Merozoite surface protein 2 is a 45-kDa merozoite surface antigen of *Pf.* It is encoded by a single exon on chromosome 2. Structural diversity in this protein allows the classification of *Pf* into two families (FC27 and 3D7). The protein can be divided

into five regions including two highly conserved regions (block 1 and 5) flanking two semi-conserved regions (block 2 and 4) and a highly variable and repetitive region (block 3) [275, 276].

The two families have two conserved regions in common: the 43 N-terminal residues and the 74 C-terminal residues. In the 74 C-terminal residues, the FC27 sequence differs from 3D7 sequences by a single nucleotide polymorphism at position 89 bases from the terminal codon. The consequence of this mutation is the substitution of a serine in the MSP2 protein of FC27 by asparagine in 3D7.

Semi-conserved blocks are characterised by non-repetitive variable regions. The variations are specific to each family.

For variable region (block 3), FC27 isolates contain two identical copies of a 32-amino acid repeat, starting at base pair number 178. The 3D7 contains multiple copies (5 copies) of a 12-bp repeat starting at position 169. The 3D7 isolates contain other multiple copies of a 9-bp repeat that code for the amino acid threonine (poly-threonine region). Figure 4 represents a gene model for msp2 showing FC27 and 3D7 families.

Because of these size variations, parasite clones can be differentiated by size polymorphism in each family.

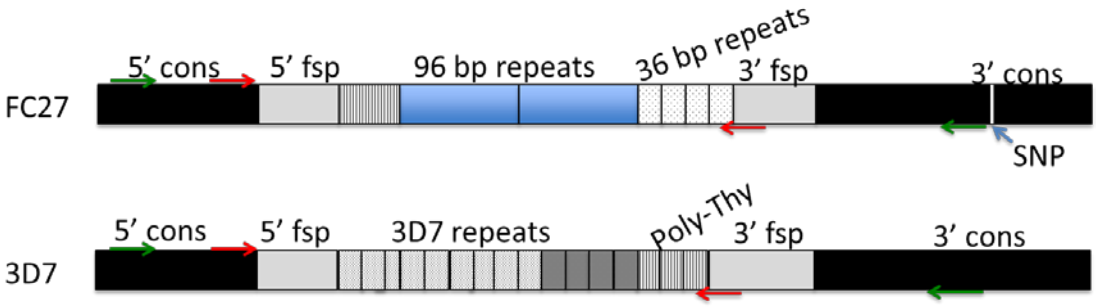


Figure 4: Gene model of the *msp2*.

Green arrows show the annealing position of primers of the first amplification. Red arrows show the annealing position of primers of the second amplification. 5' and 3' represent the five and three prime end of DNA. (fsp) flanking the semi-conserved part. Black blocks are the conserved (cons) part between the two families except one single nucleotide polymorphism (SNP) in 3' cons of FC27. (bp) base pair

MSP1 is a surface protein of the *Pf* merozoite, anchored in the membrane by a glycosylphosphatidylinositol moiety [277]. It is an 185-195 kDa glycoprotein. The gene coding for MSP1 is localised on chromosome 9 and is used for the

discrimination of parasite clones. This gene can be divided into 17 blocks: 5 conserved blocks (block 1, 3, 5, 12 and 17), 5 semi-conserved blocks (block 7, 9, 11, 14 and 15) and 7 highly variable blocks [278, 279].

Figure 5 depicts the gene model for *msp1*.

The variable block 2 is characterised by tripeptide (9 base pairs) repeats in the MAD20 and K1 families, while there are no tripeptide repeats in the RO33 family. The repeat numbers vary between 5 to 25 for K1 and 5 to 16 for MAD20. In K1 there is glutamine and proline rich region in block 8, which is absent in MAD20 and RO33. Apart from block 2 repeats, RO33 is similar to MAD20 [279-282].

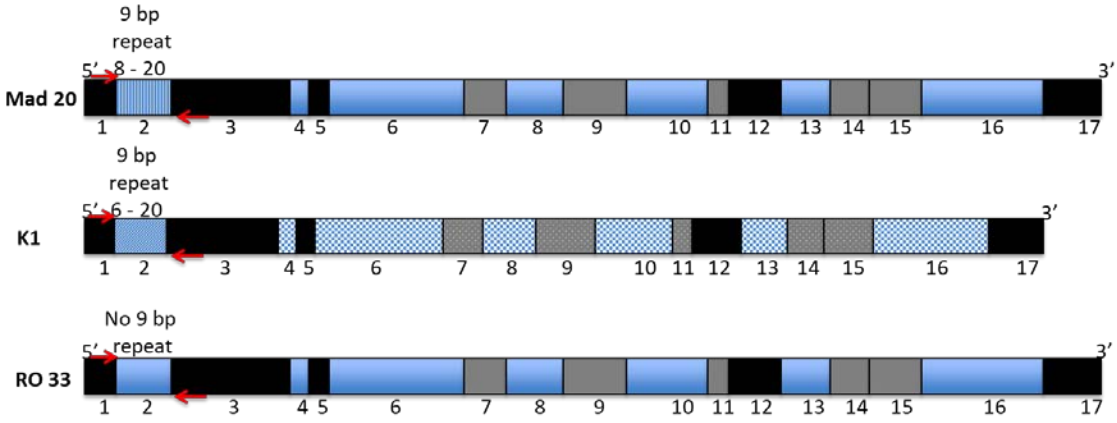


Figure 5: Gene model of the *msp1*.

Arrows show the annealing place of primers of the second amplification

Microsatellite *ca1* is a non-coding sequence used for the genetic analysis of *Pf*. It is characterised by the repetition of the simple nucleotide sequences at and atatt (Figure 6). These repetitions vary between parasites and can be used for discrimination. This microsatellite is located in the intron of the calmodulin gene of *Pf* [283, 284]

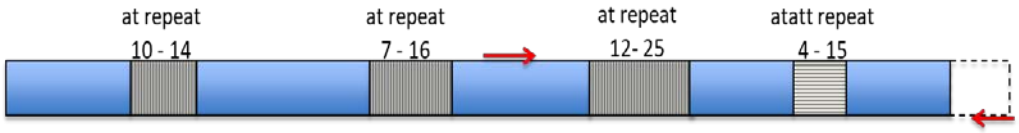


Figure 6: gene model of the microsatellite ca1.

Arrows show the annealing place of primers of the second amplification. (at repeat) repetition of adenine and thymine. Numbers below at repeat are the number of time of at or atatt.

Research question

The human populations of endemic countries pay a heavy price to malaria, mainly in sub-Saharan Africa where incidence of disease remains high. Children can suffer more than five malaria episodes per year in areas where the transmission is seasonal or perennial [11, 104, 285]. The consequence is more than 5 repetitive treatments with an ACT. The efficacy, safety, as well as pharmacokinetic properties of these ACTs have mostly been studied for the management of single episodes of malaria. The question posed in this thesis is a focus on the relationship between the day 7 concentration of lumefantrine, desethylamodiaquine, piperaquine and pyronaridine and parasite recurrence, drug-related accumulation and safety following repeat treatment for multiple malaria episodes with the same drug (AL or ASAQ or DHA-PPQ or PA) during a period of two years of follow-up.

1.9 Hypothesis

It was hypothesized that day 7 concentrations of lumefantrine, desethylamodiaquine, pyronaridine and piperaquine are implicated in parasite recurrence and drug-related safety after repeated treatment with the same ACT during a period of two years of follow-up.

1.10 Objectives:

Main objective: To help to define the risk of parasite recurrence and drug-related safety as a function of the pharmacokinetic and pharmacodynamic characteristics of the slowly eliminated artemisinin combination partner drugs after repetitive treatment with the same ACT during two years.

Specific objectives:

- Determine the genotypes of *Pf* infections at baseline and at day of recurrence after treatment with these ACTs using the polymorphic parasite genes *msp2*, *msp1* and the microsatellite *ca1*.
- Develop and validate a HPLC method to quantify lumefantrine, desethylamodiaquine, pyronaridine and piperaquine in plasma.
- Determine the day 7 concentration of lumefantrine and DEAQ in the study population after repetitive treatment with AL or ASAQ

- Determine the threshold of day 7 plasma concentration of ACT partner drugs (lumefantrine and desethylamodiaquine) that can identify patients at high risk of rapid recurrence
- Determine the relationship between day 7 concentration of the partner drugs (lumefantrine and desethylamodiaquine) and incidence of adverse events

2 Methods

2.1. *In vivo* methods

2.1.1. Study sites:

Samples included in this sub-study were from three sites in Mali (Sotuba, Bougoula-Hameau and Kollé). At these three sites, the dominant malaria species is *Pf* occurring with an incidence of more than 95% of malaria cases.

Sotuba

Sotuba is a peri-urban area of Bamako capital city of Mali where malaria is mesoendemic with seasonal transmission. The entomological inoculation rate (EIR) is less than 4 infected bites per person per month during transmission season [286]. The peak of malaria transmission is between August and September. The population is estimated to be around 5665 inhabitants. It is a mixed population with no dominant ethnic group. The study started in Sotuba first in November 2011, where PA was compared to AL in adult patients (above 15 years).

Bougoula Hameau

Bougoula Hameau is a peri-urban village next to Sikasso, at 375 km southeast from Bamako. The population is estimated to be around 5,000 inhabitants. Senoufo is the major ethnic group. Malaria is hyper-endemic and seasonal. Bougoula-Hameau has a longer and intense transmission season (June to November), with an EIR of around 300 infected bites per person per month during the transmission season. The peak of transmission is between August and September.

Kollé

Kollé is a rural village situated in the southwest at around 57km of Bamako. The transmission is hyper-endemic and seasonal (June-October) with an EIR of around 100 infected bites per person per month. The peak of malaria transmission is between August and September. In Kollé area the population is estimated to 20,000 people. The major ethnic group is Malinké.

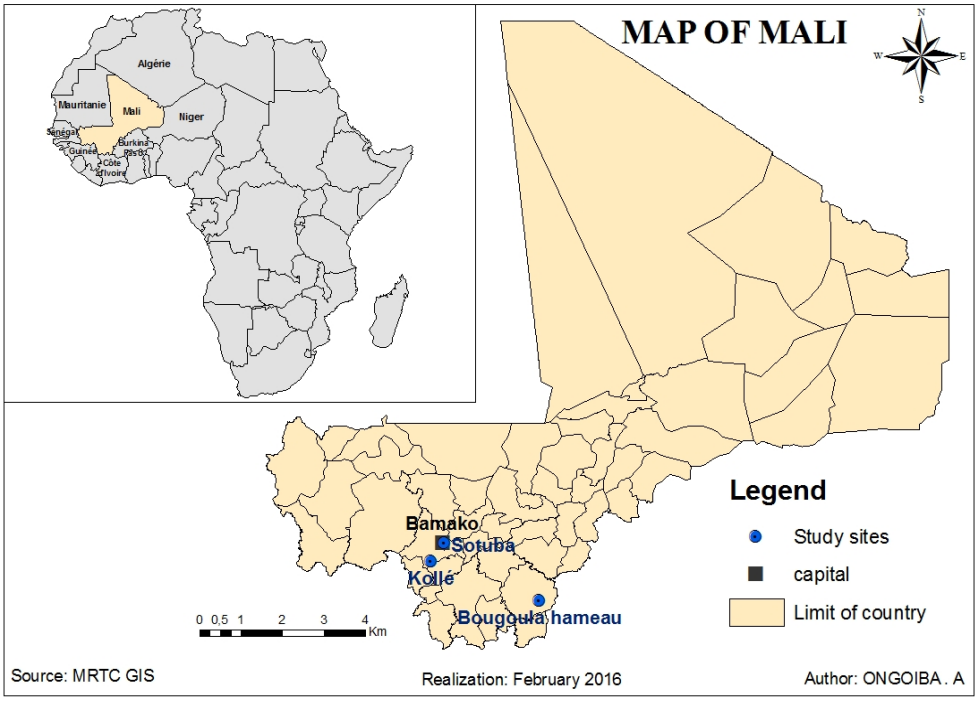


Figure 7: Study sites

2.1.2. Study design:

This sub-study is part of the WANECAM study, a large clinical trial which is a comparative, randomised, multicenter, open-label, parallel 3 arm study to assess the safety and efficacy of repeated ACT therapy over a period of 2 years in children and adults with uncomplicated *Plasmodium* sp. malaria at enrolment. At each site, eligible participants were randomised to one of 3 treatment arms DHA-PPQ, PA, and first line treatment of the ACT (either AL or ASAQ) [287].

Data from this sub-study was collected from participants in Mali.

PA, DHA-PPQ and AL were administered at all of the three studies sites (Sotuba, Kollé and Bougoula Hameau), while ASAQ was used in Bougoula Hameau only (according to the main study plan).

At all of the three study sites, PA was compared to AL. In Sotuba and Kollé DHA-PPQ was compared to AL and in Bougoula Hameau DHA-PPQ was compared to ASAQ. During this sub-study, a formal comparison between treatment arms was not made.

Once a patient was randomised to a treatment arm, he/she received the same study drug for the subsequent uncomplicated malaria episodes for up to 2 years after

the first randomization. Complicated malaria or treatment failure cases (early treatment failure or late clinical) occurring before day 28 of follow-up were treated with quinine or artesunate, and then for safety reasons, the follow-up was completed at day 42 and considered as treatment failure and reported as such. Subsequent uncomplicated clinical malaria occurring at least 4 weeks apart received the same ACT and followed for ACPR assessment over the two-year study period.

Symptomatic malaria recurrences between 28 days and day 42 were treated with the same initially randomised study drug as an uncomplicated malaria case and followed up to day 42 or 63.

The period of administration for each of the study drugs was three days. At the baseline visit, patients that met study eligibility criteria were randomly assigned to one of the above treatment arms. Local guides or a health worker performed a monthly scheduled home visit, to document the presence and health status of the patient, and to monitor the patient's movements from the immediate locality. Participants were reminded to come to the study clinic for any health issue. All malaria episodes during the study period were treated and followed by the study team.

All of the treatments were administered under the supervision of the study teams. Participants were hospitalised during the treatment period.

Participants were actively followed during the treatment period, and also specifically on day 7, day 14, day 21, day 28, day 35 and day 42 to assess treatment efficacy and safety.

Passive follow-up was continued after the 42 days for safety assessment throughout the two-year period of the study.

Plasma samples were collected at day 7 of follow-up after treatment with study drugs for pharmacokinetic study.

2.1.3. Study Population

The study population consisted of all eligible patients at the study sites with microscopically confirmed acute, uncomplicated *Plasmodium sp* malaria.

2.1.4. Inclusion and non-inclusion criteria**:**

Patients had to satisfy the following inclusion criteria to be eligible for the study:

- Male and female patients aged from 6 months and above
- Body weight equal or above 5 kg
- Microscopically positive cases of *Pf* with parasite density less than 200,000 parasites per μl, and fever, as defined by axillary temperature ≥ 37.5°C, or history of fever in the previous 24 hours (not needed at reinclusion)
- Ability to swallow oral medication
- No documented malaria treatment during the last two weeks
- Ability to stay permanently in study area with missing periods less than 3 months
- Ability and willingness to participate in the study and to comply with all scheduled follow-up visits.
- Written informed consent or assent provided by the patient and/or parent/guardian/spouse.
- Haemoglobin level ≥7 g/dl, no sign of other illness or malnutrition, no pregnancy

Patients, fulfilling the following criteria, were not eligible for this study:

- Patients with signs and symptoms of severe/complicated malaria requiring parenteral treatment according to the World Health Organisation Criteria 2000 (16).
- Severe vomiting described as more than three times in the 24 hours before inclusion in the study or inability to tolerate oral treatment or severe diarrhoea defined as 3 or more watery stools per day.
- Known history or evidence of clinically significant syndromes such as cardiovascular (including arrhythmia, QTc interval greater or equal to 450 milliseconds (QTC of ≤ 450 msec with either Bazett or Fridericia's correction was acceptable)), history of jaundice, hepatic, respiratory (including active tuberculosis), renal, gastrointestinal, immunological (including active HIV-AIDS), neurological (including auditory), endocrine, infectious, malignancy, psychiatric (active depression, generalised anxiety, psychosis, recent history of

depression, schizophrenia or other major psychiatric disorders), history of convulsions or other abnormality (including recent head trauma).

- Hb < 7 g/dL.
- The presence of febrile conditions caused by diseases other than malaria at the first inclusion as well as cases for whom oral treatment is not possible for the subsequent episodes.
- Known history of hypersensitivity, allergic or adverse reactions to study drugs.
- Documented use of any other antimalarial agent, including traditional medicines, within 2 weeks before the start of the study.
- Female patients of child-bearing potential (≥12 year-old) must be neither pregnant (as demonstrated by a negative pregnancy test) nor lactating, and must not be planning on becoming pregnant during each 42 day period after treatment.
- Received a trial drug within the past 4 weeks.
- Known or suspected chronic alcohol abuse, more than three units/day in men and more than two units/day in women.
- Known active Hepatitis A IgM (HAV-IgM), Hepatitis B surface antigen (HBsAg) or Hepatitis C antibody (HCV Ab).
- Known positive for HIV antibody.
- Known significant renal impairment as indicated by serum creatinine of more than 1.5 x ULN.

2.1.5. Retreatment criteria with study drugs

Participants fulfilling the above inclusion/exclusion criteria at the subsequent malaria attack during the passive follow-up period, or at, or after the day 28 scheduled active follow-up, were re-treated with the same ACT allocated at the initial randomization.

2.1.6. Temporary non-retreatment criteria with study drugs

Participants were not re-treated with the same study drugs for new malaria episode if they met the following criteria:

- One of the above non-inclusion criteria

- *Pf* parasite density greater than 200,000 parasites/µl
- Ongoing serious adverse events not related to study drug
- Parasite infection before Day 28 scheduled follow-up visit
- Use of any other antimalarial agent, other than the one used for malaria rescue treatment or severe malaria
- Significant arrhythmia or prolonged QTc > 450 milliseconds during previous treatment or QTc > 450 milliseconds at the time of presentation or re-treatment.

2.1.7. Criteria for permanent discontinuation of receiving study drug or withdrawal from the study.

Patients, which had any additional study drug treatment, or were withdrawn from the survey, if any of the following criteria were met:

- SAE related to study drug
- Hypersensitivity, allergy to study drug
- Sustained prolongation of QTc (>450 msec) related to treatment
- Active chronic Hepatitis B or Hepatitis C.
- Known positive for HIV.
- Liver function [Alat levels] abnormality related to the study drug, an isolated increase of Alat more than 5 x ULN or Hy's law (Alat >3 x ULN AND Total bili >2 x ULN).
- Travel outside the study area for more than 3 months period.
- Any other medical condition in the opinion of the investigator that may jeopardize the patient safety if she/he continues receiving the study drug. Such condition should be documented in details, and the study monitor should be notified immediately.
- Consent withdrawal

2.1.8. Concomitant treatment

The administration of paracetamol/acetaminophen was allowed if the patient's condition warranted it and was recorded in the Case Report Form (CRF).

When early treatment failure or late clinical treatment failure occurred within 28 days, an alternative treatment was used as rescue therapy (quinine for 7 days). Late

clinical treatment failure occurring within 28 days scheduled visit were treated with the randomized ACT.

Molecules with antimalarial activity (such as co-trimoxazole, macrolides, tetracycline or doxycycline), were avoided if possible. When during follow-up, infections other than malaria required the administration of these antibiotics, they were clearly recorded and taken into account in the statistical analysis. On the other hand, beta-lactamines were recommended.

All concomitant medications taken by the patient during the study, from the date of signature of the informed consent were recorded in the appropriate section of the CRF.

2.1.9. Treatment

PA, DHA-PPQ, ASAQ and AL were used during this sub-study. Each dose was administered with clean water under the supervision of study clinicians. As a precaution, patients were hospitalized during drug administration period. Doses were administered at fixed time point +/- 1 hour during the three-day administration period.

Participants, who vomited one dose within 30 minutes of study drug administration, received an additional dose. If vomiting occurred between 30 and 60 minutes, half a dose was administered. During the treatment phase, no more than two doses could be replaced. Each dose was administered according to body weight and the assigned treatment.

The study drug administration scheme was as follows:

Pyronaridine-artesunate (Pyramax®)

PA was presented as a sachet (60:20 mg) for children and tablet (180:60 mg) for adult patients. It was administered once daily for 3 days as follow.

- 1 sachet from 5 to < 8 kg
- 2 sachets from 8 to < 15 kg
- 3 sachets from 15 to < 20 kg
- 1 tablet from 20 to < 24 kg
- 2 tablets from 24 to < 45 kg
- 3 tablets from 45 to < 65 kg
- 4 tablets from 65 to < 90 kg

Dihydroartemisinin-piperaquine (Eurartesim®)

DHA-PPQ was available as a tablet with two strengths (20:120 and 40:320 mg) and was administered as follow.

- 20:160 mg ½ tablet from 5 to < 7 kg
- 20:160 mg 1 tablet from 7 to < 13 kg
- 40:320 mg 1 tablet from 13 to < 24 kg
- 40:320 mg 2 tablets from 24 to < 36 kg
- 40:320 mg 3 tablets from 36 to < 75 kg
- 40:320 mg 4 tablets from 75 to 100 kg

Artemether-lumefantrine (Coartem-Dispersible® and Coartem®)

AL was available as dispersible and crushed tablets with one strength 20:120 mg. It was administered twice daily for 3 days. The doses were administrated as follow: dose 2 was given 8 hours after dose 1 administration. The administration time window for dose 2 was not higher than ± 1 hour. For the following doses at Hours 24, 36, 48 and 60 (twice daily), the time window was not higher than ± 2 hours.

- 1 dispersible tablet from 5 to <15 kg
- 2 dispersible tablets from 15 to <25 kg
- 3 tablets from 25 to <35 kg
- 4 tablets for ≥ 35 kg 40:320 mg 2 tablets from 24 to < 36 kg

Artesunate-amodiaquine (ASAQ-Winthrop®/Coarsucam®)

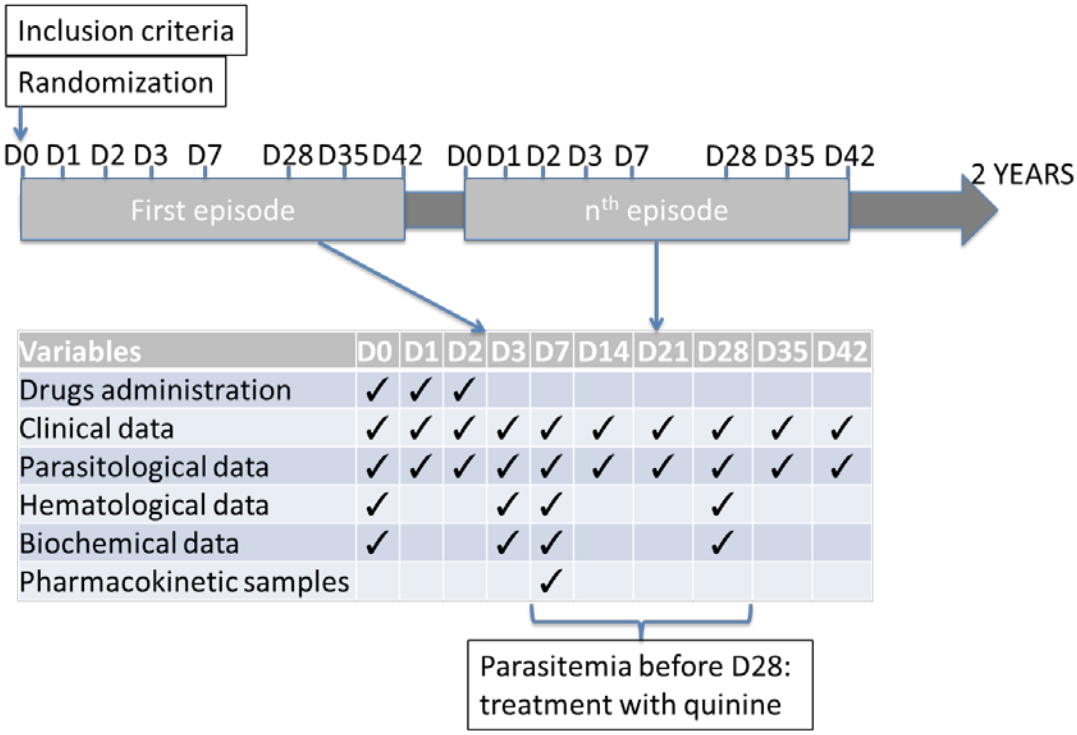
ASAQ was used in 3 strength tablets and was administered once daily during three days as follow.

- 25:67.5 mg: 1 tablet for ≥5 kg to <9 kg
- 50:135 mg: 1 tablet for ≥9 kg to <18 kg
- 100:270 mg: 1 tablet for ≥18 kg to <36 kg
- 100:270 mg: 2 tablets for ≥36 kg

2.1.10. Samples collection

After enrolment on study, each patient was followed for 42 days or 63 days, depending on the study site. Patients were actively monitored (clinical and laboratory data were recorded) at day 0 (day of inclusion) day 1; 2; 3; 7; 14; 21; 28; 35; 42; and

63, and passively (Clinical and laboratory data were recorded in case of unscheduled visit) during the other days until day 63. Antimalarial drugs were administered from day 0 to day 2. Genotype samples were collected at day 0 and day of recurrence (if a patient came with parasitaemia before completing 63 days of follow-up). Pharmacokinetic samples were collected at day 7 (Figure 8).



Variables	D0	D1	D2	D3	D7	D14	D21	D28	D35	D42
Drugs administration	✓	✓	✓							
Clinical data	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Parasitological data	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Hematological data	✓			✓	✓			✓		
Biochemical data	✓			✓	✓			✓		
Pharmacokinetic samples					✓					

Parasitemia before D28: treatment with quinine

Figure 8: ***In vivo*** **method**

Socio-demographic parameters: The variables measured for socio-demographic characteristics were: weight, height, age, gender, ethnicity.

Clinical parameters: Variables, which were measured as clinical parameters included temperature, spleen size, blood pressure, heart rate, adverse event and concomitant treatment. These variables were measured at each active follow-up day. Electrocardiogram was done before initiation of the treatment at day 0 and day 2.

Parasitological parameters consisted of asexual parasite (trophozoites) and sexual parasite (gametocytes) count.

Hematological parameters measured were: total white blood cells and red blood cells count, haemoglobin, hematocrit, platelets, lymphocytes, monocytes, neutrophils, eosinophils and basophils count.

Biochemical parameters: they were represented by creatinine, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and total bilirubin

2.1.11. Pharmacokinetic samples collection

Plasma samples were collected from all the patients at any episode, on day 7 of the ongoing follow-up (four days after administration of the last dose of the study drug).

Briefly, around 1.2 ml of venous blood samples were collected in EDTA tube from patients treated with study drug (one of the ACTs) for uncomplicated malaria. S-Monovette®, hematology (potassium EDTA), 4.9 ml, diameter 13 mm, length 90 mm, (SARSTEDT) tubes were used to avoid hemolysis during samples collection. Tubes were gently inverted for homogenisation and were centrifuged at 1,650 x g for 10 minutes at room temperature within 15 minutes after sample collection. At least 500 µl of plasma were stored in cryo-tube for drug quantification. Samples were kept directly at 4°C and transferred in -80°C within 24 hours on study sites or in our main laboratory in Bamako. Samples were shipped from Bamako to Heidelberg Germany on dry ice and kept at -80°C until analysis at the laboratory of the Department of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg.

2.1.12. Statistical analysis

Data were entered and double-checked on Access. The data analysis was done using Stata version 12.1. Diagrams were designed using GraphPad Prism version 5.0d.

Chi-square test or Fischer exact test were used as appropriate to compare proportions. Mann-Whitney test or Kruskal-Wallis were used to compare medians of abnormally distributed variables. Spearman's rank correlation test was used for correlation between two continuous variables.

2.1.13. Ethical and deontological consideration

The clinical protocol and the participant consent forms were approved by the ethics committee of the Faculty of Medicine, Pharmacist and Dentistry of Bamako before the start of the study. Each participant was free to participate in the study, and they were also free to withdraw for the study at any time. Written consent was to be given by each patient, or parent/guardian in the case of minors, after the receipt of detailed information on the study. Community permission was obtained before the start of the study.

2.2. Laboratory methods

2.2.1. Materials

2.2.1.1. Materials for genotyping

- 0.5 µl Eppendorf tube
- 0.2 µl PCR tube
- 1.2 ml Eppendorf tube
- 2 ml Eppendorf tube
- 0.1 - 2.5 µl pipette and tips
- 0.5 - 10 µl pipette and tips
- 2 - 20 µl pipette and tips
- 10 -100 µl pipette and tips
- 20 - 200 µl pipette and tips
- 100 - 1000 µl pipette and tips
- 0.5 – 10 µl multichannel pipette
- 10 - 100 µl multichannel pipette
- Permanent marker
- Gloves
- Rack for PCR and Eppendorf tube
- Filter paper
- Vortex mixer
- Neoblock (Neolab)
- Mastercycler Eppendorf
- Gel tank
- Tank cover
- Electrodes
- Power supply
- Casting tray
- Combs
- Erlenmer flasks 200 ml
- Gel Doc XR imaging system (Bio-Rad)
- Microamp optical 96 well reaction plate
- 96 well plate retainer
- 96 well plate base

- 96 well plate SEPTA
- 4 capillaries 36 cm
- 0.5 – 10 µl multichannel pipette
- 10 – 100 µl multichannel pipette
- ABI 3130 genetic analyzer
- ABI 3130 and 3100-*Avant* capillary array 36 cm

2.2.1.2. Materials for high-performance liquid chromatography

- Ps- tube, screw cap, 12 ml, 16.8/100mm
- Test tube, starwandig, ca. 100 x 16 mm
- NeoSpenser 1 to 5 ml
- Magnetic stirring bars
- Ultrasonic Baths, mechanical timer with heater, M2800h-E
- 0.1 - 2.5 µl pipette and tips
- 0.5 - 10 µl pipette and tips
- 2 - 20 µl pipette and tips
- 10 -100 µl pipette and tips
- 20 - 200 µl pipette and tips
-100 - 1000 µl pipette and tips
- PH-meter-Set with electrode
- Centrifuge with refrigeration
- Permanent cryomarker
- Gloves
- Rack for tubes

2.2.2. Reagents

2.2.2.1. Reagents for genotyping

- Methanol
- Distilled water
- 10x Taq buffer with $(NH4)_2SO4$ (Thermo Scientific)
- 25 mM MgCl2 (Thermo Scientific)
- diNucleotide TriPhosphate dNTP mix at 2 mM (Thermo Scientific)
- Primers (Eurofins MWG/Operon)
- Taq DNA polymerase 5 u/µl (Thermo Scientific)

- DNA
- 70° alcohol
- Distilled water
- Ultrapure agarose
- Tris base
- Glacial acetic acid
- EDTA (PH 8,0)
- Deionized water
- SYBR Safe (or Ethidium bromide)
- 100 bp DNA ladder plus
- DNA loading dye
- Pop polymer (POP-7^{Tm} 3130)
- HiDi^{Tm} Formamide
- Rox size standard
- DNA
- Water HPLC grade
- Buffer (10x) with EDTA (3130 machine)
- Matrix standard

2.2.2.2. Reagents for high-performance liquid chromatography

- Internal standard
- Standard drug
- Acid Boric
- Water Cromasolv for HPLC
- TEMED (-N,N,N,N TetraMethyl-Ethylene diamine)
- Orthophosphoric acid
- Dimethyl sulfoxide
- 2-propanol HPLC grade
- TerButyl Methyl Ether
- Triethylamine
- Potassium Hydrogen Phthalate
- Sodium hydroxide solution
- pH buffer solution pH7
- pH buffer solution pH4

2.2.3. Methods for genotyping

A validated high-throughput capillary electrophoresis with fluorescence detector was used to differentiate recrudescence from reinfection by analyzing *msp2* polymorphism. The method can detect a difference of two base pairs of genotype size [288]. It is also able to distinguish the different *msp2* families. With this sensibility and specificity, it was possible to differentiate recrudescence from reinfection with a higher degree of accuracy.

Parasite DNA extraction from filter paper was done by methanol extraction method [260]. After first amplification, five microliters of this product was used for nested amplification using family specific reverse primers coupled with different fluorescence dyes. The non-fluorescent–labeled forward primer is modified at the 5' end by adding a 7-bp tail to avoid non-template–directed addition of a single nucleotide to the 3' end of a blunt-end double-stranded DNA (**"**plus-A-artefact**"**)[289].

According to second amplifications' DNA concentration, different dilutions were done before capillary electrophoresis. Results were interpreted according to the elaborated and validated criteria.

Recrudescent samples with *msp2* were reanalyzed with *msp1*. For specimens remaining recrudescent, a third amplification was done using the size polymorphic microsatellite *ca1*. These two last genes were amplified using simple nested PCR without capillary electrophoresis. The size polymorphism was detected by eye after agarose-gel electrophoresis.

Samples deemed to be recrudescent with these three techniques were considered as true recrudescent samples in this study.

2.2.3.1. Principle of the PCR and capillary electrophoresis methods:

Fluorescent primers were designed for the second amplification of *msp2*. The reverse primers were coupled with a fluorescent dye. 6-FAM (6-Carboxyfluorescein) with its blue color after emission at wavelength 517 nm was combined with FC27 family-specific primer, while VIC, with its green color, was coupled with 3D7 family-specific primer.

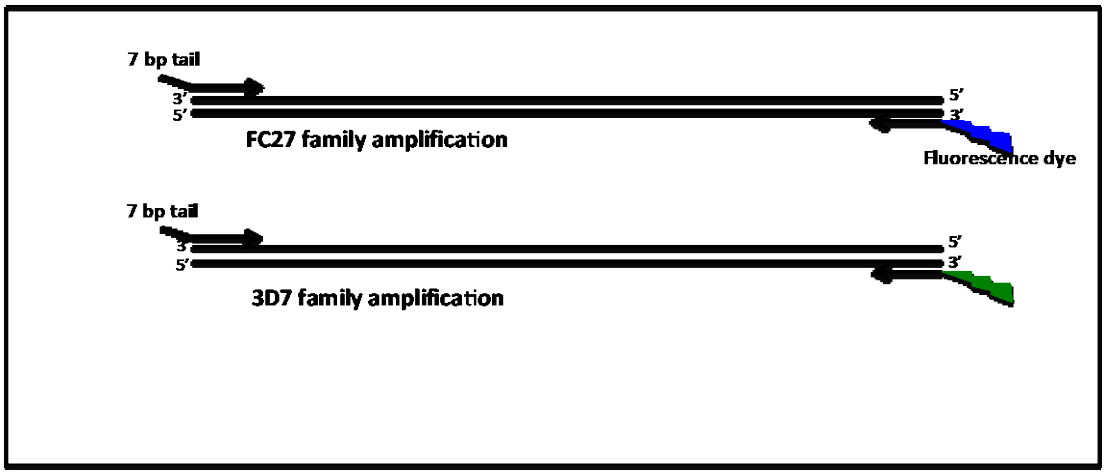


Figure 9: Principle of fluorescent primer amplification

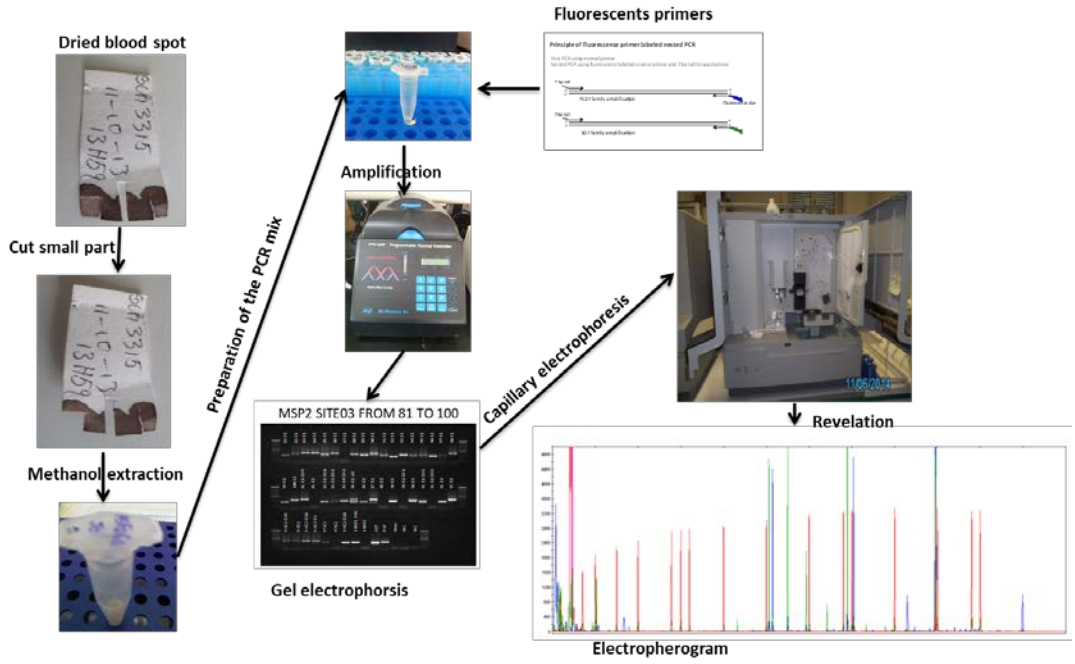


Figure 10: The general principles of my PCR amplification methods

2.2.3.2. DNA Extraction by methanol:

DNA was extracted from whole blood spotted and dried onto filter paper using the methanol extraction method [260]. The principle is to fix other constituents of the blood onto the filter paper with methanol and elute the DNA in sterile water under the action of the heat. Briefly, a small piece of filter paper was cut and embedded with whole blood (around 3 mm), then incubated in methanol for 15 min. The methanol was discarded and allowed to dry. A 50ul volume of distilled water was added; then boiled at 100°C for 15 min and vortexed each 5 minutes.

Use the extracted DNA in distilled water for PCR or keep it at -20 or lower temperature until need.

2.2.3.3. Amplification of msp2 gene:

Nested PCR, coupled with capillary electrophoresis, was used to amplify merozoite surface protein 2 (*msp2*) gene.

The process consisted of first amplifying the gene with primers which bind to the 3' and 5' conserved parts of the gene, resulting in amplification of all of the semi-conserved and variable regions of *msp2*. A second amplification step used inner primers that are family specific. After running a gel of the second amplification, samples, which gave a band, are used for capillary electrophoresis.

Protocol for amplification:

Primers sequence for *msp2* amplification:

First amplification:

S2 (forward primer): GAAGGTAATTAAAACATTGTC

S3 (reverse primer) : GAGGGATGTTGCTGCTCCACAG

Second amplification:

S1 (forward primer): 7 bp tail-GCTTATAATATGAGTATAAGGAGAA

M5 (reverse primer FC27 specific): 6FAM-GCATTGCCAGAACTTGAA

N5 (reverse primer 3D7 specific): VIC-CTGAAGAGGTACTGGTAGA

For PCRs, I used 10X Taq buffer with $(NH4)_2SO4$, 25 mM Mgcl2, dNTP mix concentrated at 2 mM each, primers at 10 µM, Taq DNA polymerase (5 u/µl), (the final concentration of all the reagents was 1X). I used 5 µl of extracted DNA at first

amplification, and 1 µl of amplicon of the first amplication was used for second amplification. The final volume of the reaction was set at 50 µl.

Mastercycler Eppendorf was used for DNA amplification and the cycling condition are in table1 and table 2 bellow.

Table 1: Cycling conditions for *msp2* the first amplification

1	**Initial denaturation**	**94 °C**	**5.00 minutes**
2	Denaturation	94 °C	30 seconds
3	Annealing	55 °C	2 minutes
4	Extension	70 °C	2 minutes
5	Final extension	72 °C	7 minutes
6	Keep at	4 °C	
Repeat step 2 to 4 for 30 times			

Table 2: Cycling conditions for *msp2* the second amplification

1	**Initial denaturation**	**94 °C**	**2.00 minutes**
2	Denaturation	94 °C	30 seconds
3	Annealing	50 °C	45 seconds
4	Extension	70 °C	1.30 minutes
5	Final extension	70 °C	10 minutes
6	Keep at	4 °C	
Repeat step 2 to 4 for 44 times			

Gel revelation:

To monitor the result of the amplification a gel was run of the amplified products. Positive samples were selected for capillary electrophoresis.

Preparation of the gel:

It consists of making 1% of agarose gel in 1x Tris Acetate EDTA (TAE)

Preparation of 1x TAE: I prepare a stock solution of TAE concentrated at

50X.

- Dissolve 242 g of Tris base in deionized water
- Add 57.1 ml of glacial acetic acid
- Add 100 ml of 500 mM of EDTA (PH: 8) solution
- Bring the final volume up to 1 liter.
- To have 1X, you dilute it with deionized water

Preparation of 1% agarose gel:

- Weight 1 g of agarose powder in 200 ml Erlenmeyer flask
- Add 100 ml of TAE 1X
- Boil
- Add 10 µl of SYBR Safe or Ethidium bromide
- Mix
- Pour it into the tray with combs
- When dried take out the combs
- Put the tray with the gel in tank filled with 1X TAE
- Mix the DNA with loading dye and load it in the well let by the combs
- Cover the gel tank and run the samples for 30 at 100 millivolts.
- Make the revelation under the gel photo machine

Capillary electrophoresis:

The principle is the separation of DNA fragment according to their sizes in the capillary filled with polymer. And the revelation is done according to the fluorescent dye link to each DNA. It is a high throughput technique capable of detecting a difference of two base pairs.

Samples preparation for capillary electrophoresis:

Dilution of samples: According to the intensity of the band of the second amplification on the gel, I make a dilution of sample (1/200) in HPLC grade water.

Preparation of samples:

For each sample:

- Put 10 µl of Rox size standard in the well of the Microamp optical 96 wells reaction plate.
- Add 2.5 µl of the diluted product of the second PCR amplification

- Add 10 µl of HiDi
- Cover the plate with the 96 wells plate SEPTA
- Make sure that all of the liquid are at the bottom of the well not on the wall
- Put the plate in the 96 wells plate base
- And retains it with the 96 wells plate retainer
- Put it into the capillary electrophoresis machine or keep it at -4°C until needed.

2.2.3.4. Amplification of msp1 gene:

Samples that remain recrudescent after *msp2* analysis were reanalysed with *msp1*. The method consists of the amplification by nested PCR of the variable region (bloc 2) of the gene. The discrimination was done by size polymorphism and the visualization by eye.

Primers sequence for *msp1* amplification:

First amplification:

O1 : CACATGAAAGTTATCAAGAACTTGTC

O2 : GTACGTCTAATTCATTTGCACG

Second amplification:

N1 : GCAGTATTGACAGGTTATGG

N2 : GATTGAAAGGTATTTGAC

Amplification of msp1 sequence was done with following reagents: 5X (Taq buffer + 7.5 mM Mgcl2), 10X dNTP mix concentrated at 2 mM each, 100X primers at 10 µM, Taq DNA polymerase (5 u/µl), (the final concentration of all the reagents was 1X). Five microliter of extracted DNA was used at first amplification, and 1 µl of amplicon of the first amplication was used for second amplification. The final volume of the reaction was set at 25 µl.

Mastercycler Eppendorf was used for DNA amplification and the cycling condition are in table 3 and table 4.

Table 3: Cycling conditions for *msp1* first amplification

1	**Initial denaturation**	**94 °C**	**3.00 minutes**
2	Denaturation	94 °C	25 seconds
3	Annealing	50 °C	35 seconds
4	Extension	68 °C	2.50 minutes
5	Final extension	72 °C	3 minutes
6	Keep at	4 °C	
	Repeat step 2 to 4 for 30 times		

Table 4: Cycling conditions for *msp1* second amplification

1	**Initial denaturation**	**94 °C**	**2.00 minutes**
2	Denaturation	94 °C	30 seconds
3	Annealing	50 °C	45 seconds
4	Extension	70 °C	1.30 minutes
5	Final extension	70 °C	10 minutes
6	Keep at	4 °C	Forever
	Repeat step 2 to 4 for 44 times		

2.2.3.5. Amplification of microsatellite ca1 gene

Samples that are still recrudescent after *msp2* and *msp1* correction were analyzed by the microsatellite *ca1*. That means that the discrimination was done using two size polymorphic genes that are under immune pressure (*msp1* and *msp2*) and one without immune pressure *ca1*.

The reagents and materials are the same like for *msp1* except for the primers' sequences and the cycling conditions.

Primers sequences for *ca1* amplification:

First amplification:

ca1-1L : GCTGTAAAACGTGAACAACAAA

ca1-1R : CAATTCTGCTTCAGTTGGATT

Second amplification:

ca1-L : ATTATGAACAATTCAGAC

ca1-R : GTTGTTATAGCTAATGAG

Table 5: Cycling conditions for *ca1* first amplification

1	**Initial denaturation**	**95 °C**	**5.00 minutes**
2	Denaturation	92 °C	30 seconds
3	Annealing	45 °C	30 seconds
4	Extension	65 °C	30 seconds
5	Final extension	65 °C	5 minutes
6	Keep at	4 °C	
	Repeat step 2 to 4 for 44 times		

Table 6: Cycling conditions for *ca1* second amplification

1	**Initial denaturation**	**95 °C**	**5.00 minutes**
2	Denaturation	92 °C	30 seconds
3	Annealing	45 °C	30 seconds
4	Extension	65 °C	30 seconds
5	Final extension	65 °C	5 minutes
6	Keep at	4 °C	
	Repeat step 2 to 4 for 19 times		

2.2.3.6. Quality control of the PCRs:

A negative control was included in all PCR samples to control for false positives and contamination. The negative control consisted of a tube without dried blood spot that otherwise replicated all of the extraction processes used for sample preparation. Briefly, I added methanol in this tube, evaporated the methanol, I added 50 µl of water I used for the extraction and boiled it like the samples. This negative control was used during all of my amplifications, and it should remain negative after amplification.

For control of the quality of my PCR amplifications, I used the reaction mix without the addition of DNA for the first amplification. This negative control was amplified at the second amplification too. I also used a second negative control for the

second amplification that was constituted with the reaction mix of the second amplification without DNA.

In addition to the negative controls, positive controls for the PCR amplification were also included. These positive controls were from *in vitro* culture samples *Pf* 3D7 and D10 (FC27) from our lab. Each control had specific characteristics according to the gene amplification. For example, for *msp2* capillary electrophoresis, 3D7 electropherograms have a green color, which came from the fluorescent dye coupled with the primer, its size after second amplification is 267 bp. While D10 (FC27), with its blue color, which came from the coupled fluorescent dye, has a size of 358 bp after second amplification.

2.2.4. Methods for high-performance liquid chromatography

A developed and validated sensitive and specific high-performance liquid chromatography (HPLC) method with ultra-violet (UV) light detection (335 nm for lumefantrine and 340 nm for DEAQ) was used to quantify the day 7 concentration of these drugs in plasma. The limits of quantification were set at 20 ng/ml for lumefantrine and 24 ng/ml for DEAQ.

The method was developed and validated for quantification of pyronaridine in plasma with UV detection at 275 nm and a limit of detection of 25ng/ml. After analyzing 40 patients' samples, I found that my method was not able to quantify pyronaridine day 7 concentration in patients plasma, which is around 37ng/ml in whole blood[290]. Also a published data in rabbit showed that the blood plasma ratio of pyronaridine varies from 4.9 to 17.8. [161].

Briefly, i used a liquid-liquid extraction method to extract the drugs (pyronaridine, lumefantrine, and DEAQ) from 250 µl of plasma collected from malaria patient in Mali. After extraction, the upper organic phase (where the drug was) was dried under nitrogen gas. Two hundreds (200) µl of mobile phase were added to the dried drug, mixed, and 50 µl were injected in the HPLC machine (Figure 11).

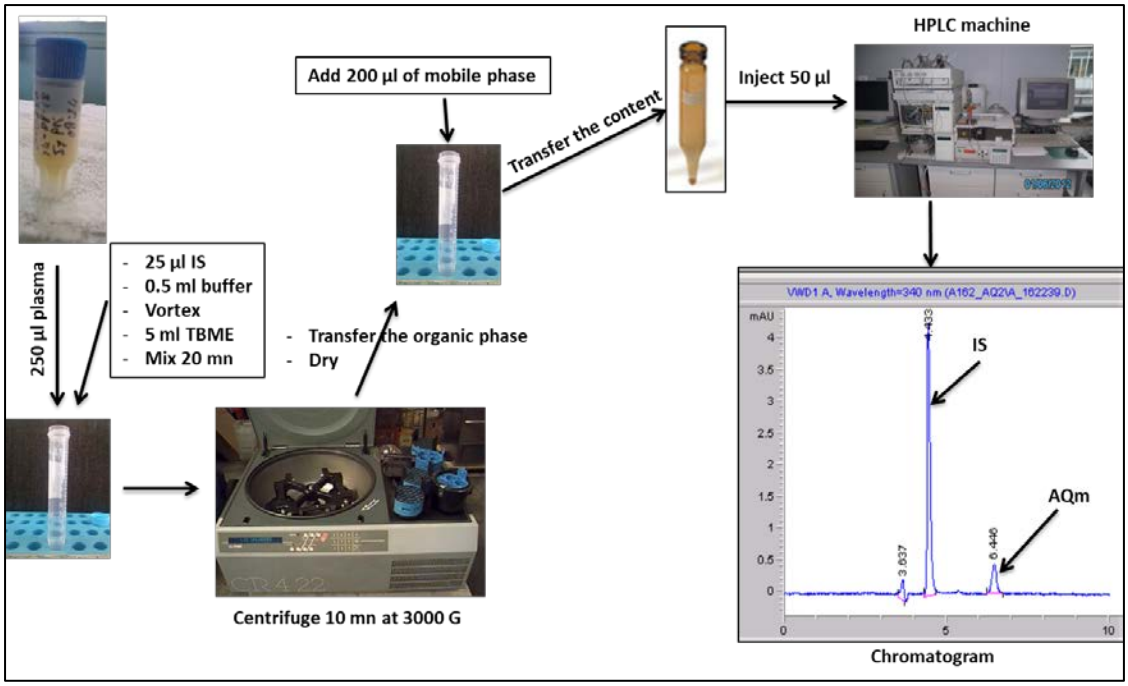


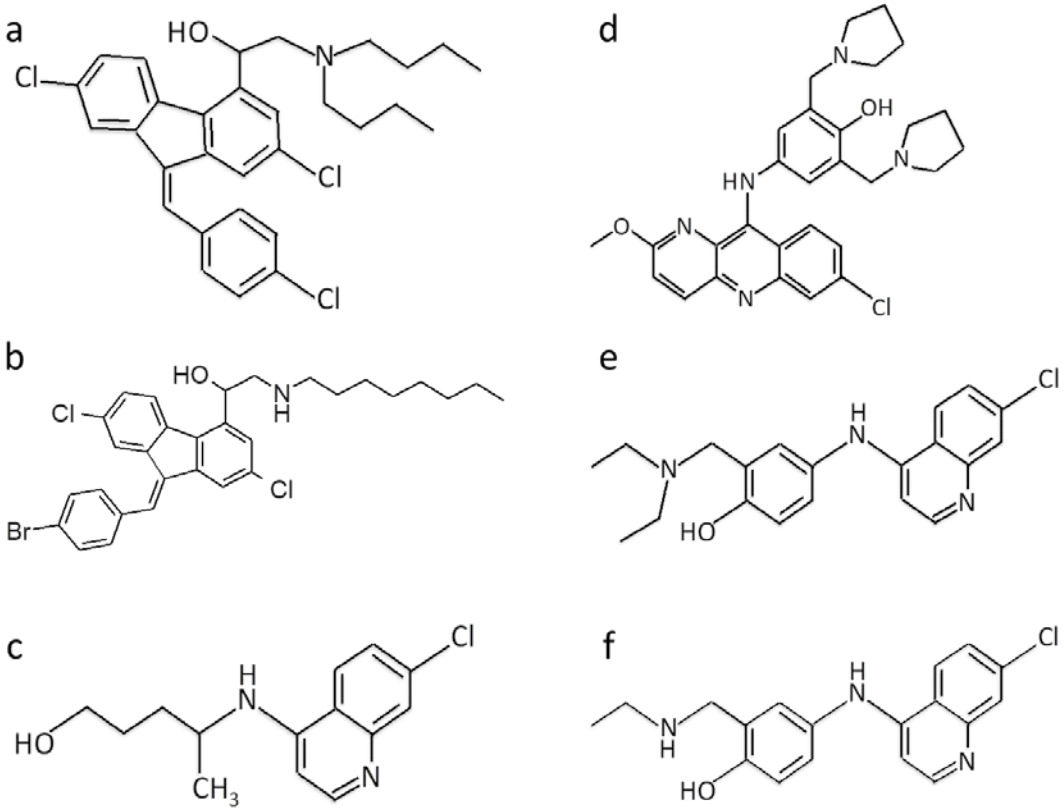
Figure 11: Summary of HPLC method

2.2.4.1. Chemicals and Reagents:

Pyronaridine, lumefantrine and DEAQ (AlsaChim) were gifts from WorldWide Antimalarial Resistance Network (WWARN), lumefantrine internal standard (a lumefantrine analogue TA3039, formula: C30H32BrCL2NO, molecular weight = 573.41; was a single diastereomer: E configuration of double bond see structure) is a gift from Novartis Pharma AG CH – 4002 Basel Switzerland. (4-((7-chloro-4-quinolinyl) amino)-1-Pentanol), formula C14H17ClN2O, molecular weight = 264.75 was pyronaridine and DEAQ internal standard purchased from Toronto Research Chemicals Inc. High-purity water was prepared in our laboratory using TKA lab HP 6UV/UF system with a conductivity of 0.055µS/cm and a total organic carbon (TOC) of 3 – 10 ppb. TEMED (-N-N-N-N tetramethyl ethylene diamine) was purchased from SIGMA-ALDRICH (Germany). Orthophosphoric acid from NeoLAB (Germany), triethylamine (TEA) was purchased from MERCK. Acetonitrile gradient grade for liquid chromatography was from LiChrosolv. Acid Boric for analysis was purchased from EMSURE (MERCK); Potassium hydrogen phthalate for analysis from MERCK; tertbutyl methyl ether (TBME) from ENSURE, 0.1N Hydrochloric

acid, dimethylsulfoxide for analysis, 0.1N Sodium hydroxide and 1N sodium hydroxide TitriPUR were purchased from VWR (Merck Millipore) (Germany).

For the preparation of the calibration curve standard, I used blank plasma from the outdated blood donation unit.



<u>Figure 12</u>: Structure of standard dugs.

(a) lumefantrine, (b) lumefantrine internal standard TA3039, (c) 4-((7-chloro-4-quinolinyl) amino)-1-Pentanol, (d) pyronaridine, (e) AQ, (f) DEAQ.

2.2.4.2. Instrumentation

The chromatographic analysis was carried out using a Hewlett Packard HP 1050 Series HPLC system, equipped with a 1050 Series quaternary pump model 79852A, 1050 series ultra violet detector mode 79853C, 1100 series vacuum degasser model model G1322A for HPLC systems HP, 1050 series autosampler model 79855A.

Separation of the analytes was carried out using Phenomenex Luna C18 100A column (250 X 4.6 mm, 5µm; USA), protected by phenomenex security guard

cartridges C18 (4 X 3 mm ID). The chromatographic data were analyzed on HP 1050 Agilent ChemStation for LC and LC/MS system-Rev.A.10.02 software.

2.2.4.3. Analytical conditions

The mobile phase for pyronaridine quantification was constituted by a mixture of acetonitrile - of TEMED buffer at PH3.5 (82.5 : 17.5, volume/volume) at a flow rate of 0.7 ml/minute for 16 minutes.

For lumefantrine quantification, the mobile phase was constituted with a mixture of acetonitrile – (TEMED buffer PH3.5 + 300 µl of TEA) (64 : 36, volume/volume) at a flow rate of 1 ml/min for 21 minutes.

TEMED buffer preparation:

- Take 950ml of HPLC grade water
- Add 1500 µl of TEMED
- Adjust the PH at 3.5 with orthophosphoric acid
- Complete at 1000ml with HPLC grade water
- Add 300 µl of TEA
- Add 50 ml of Acetonitrile (to avoid bacterial contamination)
- And mix

The mobile phase for DEAQ quantification was constituted with the mixture of acetonitrile – TEMED buffer PH3.5 (14 : 86, volume/volume) at a flow rate of 1 ml/min for 10 minutes

TEMED buffer preparation:

- Take 950ml of HPLC grade water
- Add 1500 µl of TEMED
- Adjust the PH at 3.5 with orthophosphoric acid
- Complete at 1000ml with HPLC grade water

2.2.4.4. Extraction

The following method was used to extract pyronaridine, lumefantrine, AQ and its metabolite DEAQ from plasma samples.

2.2.4.4.1. Preparation of Buffers

Preparation of borate buffer PH 10:

- Acid Boric: molecular weight 61.83g/mol
- 1N NaOH
- 0.1N NaOH
    - Solution A: to do 1liter (weigh 12.37g of boric acid, add 100ml of 1N NaOH and complete to 1liter with HPLC grade water)
    - Solution B: 0.1N NaOH
    - You can do:
        - 59.6ml of solution A + 40.4 ml of solution B that give you PH10 (control with the PHmeter)
        - 240 ml of solution A + 162 ml of solution B (control with the PHmeter)
        - 298ml of solution A + 202 ml of solution B (control with the PHmeter)
- The final concentration of Boric acid is 0.2M

Preparation of phthalate buffer PH 3:

Potassium Hydrogen Phthalate: C8H5KO4; molecular weight 204.23g/mol

- To do 1liter of 0.1M
- Weight 20.425g and dissolve it in HPLC grade water
- Adjust the PH at 3 with 1N HCL.

2.2.4.4.2. Extraction methods:

- Put 250 µl of plasma samples in 12 ml tube
- Add 25 µl of internal standard
- Add 0.5 ml of Borate buffer pH10 for pyronaridine and DEAQ (0.3 ml of phthalate buffer pH3 for lumefantrine)
- Vortex for 1 minute
- Add 5 ml of TBME
- Mix by tumbling for 20 minutes at 7rpm with the tumbling machine

- Centrifuge at 3000 G for 10 minutes for pyronaridine and DEAQ, and 20 minutes for lumefantrine at 10 degree Celsius.
- Transfer 4.5 ml of the upper organic phase in new tube
- Dry under nitrogen gas at 40 ºC during 20 minutes
- Add 200 µl of mobile phase
- Vortex and inject 50 µl into the column.

2.2.4.4.3. Preparation of standard drugs:

- Pyronaridine standard was dissolved in water + 0.5% of 1N HCl to have the standard stock solution. Working solutions were obtained by serial dilution of the standard stock in water + 0.5% of 1N HCl to have a concentration range of 250 to 10000 ng/ml. To make the calibration standard, 25 µl of these working solutions were spiked in 250 µl of blank plasma to have a calibration samples with the concentration range from 25 to 1000 ng/ml.
- Pyronaridine stock solution for quality control (QC) was dissolved in water + 0.5% of 1N HCl and then serially diluted to have the QC working solutions. 25 µl of these solutions were spiked in 250 µl of blank plasma to have a final concentration of 80, 425 and 850 ng/ml
- Lumefantrine standard was dissolved in methanol/HCl (90/10, v/v) to have a standard stock solution, which was kept frozen until need. The standard stock solutions were serially diluted with the suitable volume of methanol to obtain an individual working solution at a concentration range of 0.2 to 200 µg/ml and kept them at +4°C during the time of analysis. 25 µl of this working solution were diluted in 250 µl of blank plasma to make a calibration samples from 20 to 20,000 ng/ml.
- Lumefantrine stock solution for quality control (QC) was dissolved in methanol/HCl (90/10, v/v) and serially diluted with methanol to have a working QC solutions, which were diluted in blank plasma to have a final concentration of 60, 6000 and 15000 ng/ml
- Lumefantrine internal standard TA3039 was dissolved in DMSO and used at the final concentration of 1µg/ml

- DEAQ, pyronaridine, and their internal standard (4-((7-chloro-4-quinolinyl) amino)-1-Pentanol), the final concentration of IS 750 ng/ml, were dissolved in water and kept frozen until need. The standard stock solution of DEAQ and pyronaridine were serially diluted with an appropriate volume of water to have working solutions at a concentration range of 0.2 to 20 µg/ml, and they were kept at 4°C during analysis. These working solutions were diluted in blank plasma to have a calibration samples range from 20 to 2000 ng/ml.
- DEAQ QC stock solution was dissolved in water and serially diluted with water to have the working solution. These working solutions were diluted in blank plasma to have a final QC concentration of 60, 775 and 1600 ng/ml.

2.2.4.5. Quantification

The quantification was done using the internal standard method. Two sets of calibrators were used to measure the level of calibration: one at the beginning and the other at the end. For elimination of probable memory effect after the high calibration level of lumefantrine, two blanks samples were analyzed instantaneously. Seven calibration points were used to construct the calibration curve, with the peak area ratio of the drugs to IS, versus the concentration of the respective drugs in each standard sample.

2.2.4.6.1. Analytical method validation

Methods were validated according to the US Food and Drugs Administration (FDA) recommendations [291] and the European Medicines Agency guidelines.

- **Accuracy, Precision and Lower limit of quantification**

Accuracy and precision were determined using a replicate analysis (6 times) of quality control samples at low, intermediate and high concentration, and at the lower limit of quantification, which was 3 times lower than the concentration of the lower QC sample. This run was repeated on three different days, with the daily calibration curve to determine the concentration in each sample. The coefficient of variations

(CV%) within run (intra-assay), and between different runs (inter-assays) were calculated and reported as precision. The accuracy was described as a bias or the deviation of the true concentration from the measured one.

- **Stability of drugs**

Drugs' stability was evaluated after three freeze-thaw cycles. The three frozen QC samples were thawed at room temperature for 4 hours and froze at -20°C for 24 hours. The concentrations were measured before the first freezing and after the third freezing. For stability issues for each batch the QC samples concentration at the beginning were compared to the one at the end of the batch. The stability was also measured after leaving samples at room temperature for 24h.

- **Extraction recovery**

The recovery of the drugs and the internal standard was measured at different QC concentration. The mean area obtained after extraction (N = 6), was divided by the mean area after direct injection (N = 3), and this result was then multiplied by 100.

- **Selectivity**

The selectivity of the different methods was assessed using plasma from six different sources. The interference of these plasmas was evaluated with the low limit of quantification. Interference with other antimalarial drugs (CQ, sulfadoxine, pyrimethamine, mefloquine) and possible concomitant treatment drugs (paracetamol) was also assessed.

- **Memory effect**

The memory effect or carry-over was assessed after injection of the high concentration of the calibration standard. Blank injection constituted with mobile phase was immediately done after the injection of the high concentration. And if there was a peak at the indicated retention time, the percentage of its area was compared to the area of the high concentration.

2.2.4.6.2. Revalidation of N-desethylamodiaquine method

The method for quantification of AQ and DEAQ was developed and validated by the laboratory of the Department of Clinical Pharmacology and Pharmacoepidemiology, of the University of Heidelberg before the start of this PhD thesis. For utilization of this method to quantify DEAQ concentration, the method has been revalidated. Accuracy and precision were determined using a replicate analysis (6 times) of quality control samples at low, intermediate, high concentration and the lower limit of quantification.

2.2.4.6.3. Validation of the methods during patients' samples analysis

Precision and accuracy of QC samples during patients' samples analysis were evaluated for all of the methods. This precision and accuracy should not exceed 15%

2.2.4.6.4. Clinical sample quantification

Sensitive and specific high-performance liquid chromatography methods were developed and validated according to FDA guidance to quantify DEAQ and lumefantrine in plasma collected from uncomplicated malaria patients treated with ASAQ or AL.

Samples were kept at -80°C until the day of analysis. They were thawed at room temperature, and 250 µl were used for drug quantification. Methods for quantification of the two drugs are described in the upper chapter.

Briefly, after liquid-liquid extraction using borate buffer at PH10 and TBME for DEAQ and phthalate buffer PH3 and ethyl acetate for lumefantrine, the organic phase, which contains the drug were dried under nitrogen gas at 40°C after centrifugation. 200 µl of mobile phase (acetonitrile – TEMED buffer PH3.5, 14 : 86, volume/volume for DEAQ) and (33% of TEMED buffer at PH: 3.5 with Orthophosphoric acid + 300µl of TriEthylAmine (solvent A) and 67% of Acetonitrile (solvent B) v/v for lumefantrine) were added into the dried tube and then mixed, and 50 µl were injected into the column.

For each batch, a new calibration curve was made, and two sets of the three quality control samples were run. Lower QC concentration samples were run just after the calibration curve at the beginning of patients' samples analysis, medium QC

concentration samples were run in the middle of the analysis and high QC concentration samples were run at the end of the analysis.

2.2.5. Data management and analysis

Data were entered and double-checked on access. The data analysis was done using Stata version 12.1. Diagrams were designed using GraphPad Prism version 5.0d.

Chi-square test or Fischer exact test were used as appropriate to compare proportions. Mann-Whitney test or Kruskal-Wallis test was used as appropriate to compare medians of abnormally distributed parameters

2.3. Outcomes and statistical analysis methods

I first computed the proportion of patients without subsequent episode by day 28 and 42 in each treatment arm, defined as the absence of *Pf* parasite by day 28 or 42 of follow-up at each episode in each treatment arm. I also measured these proportions after molecular correction to differentiate true recrudescence from cases of reinfection. Other efficacy outcomes were the post-treatment prophylaxis of each arm during the two years of follow-up measured by the risk of parasite recurrence.

For safety outcomes, the change in QTc (corrected QT interval) from baseline to day 2 (4 to 6 hours after the last dose) was evaluated. Twelve-lead ECG was used to measure the QT-interval, which was corrected for heart rate (using Fridericia formula). A change greater than 30 ms was considered as abnormal [292]. The second safety variable was the change in ALAT between baseline and day 3 with abnormal ALAT at day 3 defined by the laboratory reference ranges of Malaria Research and Training Center (MRTC) in Mali. Was considered abnormal if ALAT level was higher than 61 IU/L in adult patients (age > 14 years), or greater than 53.4 IU/L in 6 to 14 years' age group, or greater than 50 IU/L in less than 6 years' patients.

For pharmacokinetic outcomes, HPLC methods were developed and validated to quantify lumefantrine and DEAQ in plasma. The accumulation of lumefantrine and DEAQ in the study population was evaluated by comparing day 7 concentration of these drugs between the first episode and patients receiving subsequent treatment between 26 and 45 days and patients receiving subsequent treatment after 45 days.

The role of day 7 drug concentration in protection against early reinfection was evaluated by Cox univariate and multivariate modeling of factors associated with risk of parasite recurrence by day 28 and 42.

3 Results:

3.1. Clinical study findings

3.1.1. Study profile and baseline characteristics

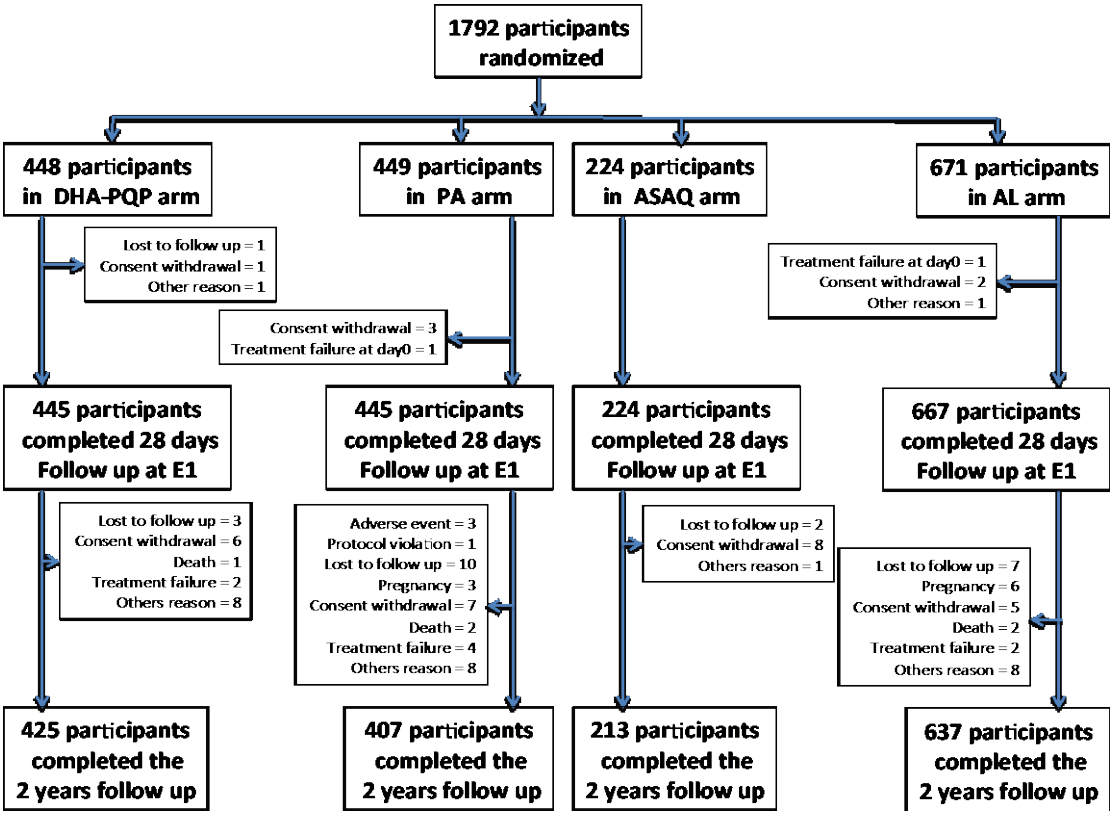
Figure 13 shows the profile of my sub-study. A total 1792 participants were included in this sub-study with 448 in the DHA-PPQ arm, 449 in the PA arm, 671 in the AL arm and 224 in the ASAQ arm. Ninety four point nine percent (94.9%) of participants successfully completed the two-year follow-up in DHA-PPQ arm, 90.6% in the PA arm, 95.1% in the ASAQ arm and 94.9% in the AL arm. Participants were treated with ASAQ in Bougoula-Hameau only.

In the DHA-PPQ arm, 23 participants did not complete the two-year follow-up period. Among these 23 participants, there was one death, a 2 year old girl who died from unknown causes 166 days after her inclusion on study.

In the PA arm, 42 participants did not complete the two-year follow-up period. Of these, there were two deaths. The first was a 4 year-old girl, who died 188 days after her inclusion on study. An 8 year old boy also died 42 days after his inclusion on study after falling from a tree.

For the eleven participants in total who did not complete the two years of follow-up in the ASAQ arm, 8 withdrew consent, 2 were lost to follow-up, and the last was for another undefined reason.

For the 34 participants in the AL arm, who did not complete the two years of follow-up, there were 2 deaths. The first was a 16 year old girl, who died 82 days after her inclusion into the study and the cause of the death was due to another disease not related to malaria or the antimalarial drugs. The second death was of a 7 year old male, who died 28 days after his inclusion into the study after he fell into a well.



<u>Figure 13</u>: Study profile.

(DHA-PPQ) dihydroartemisinin-piperaquine. (PA) pyronaridine-artesunate. (ASAQ) artesunate-amodiaquine. (AL) artemether-lumefantrine.

Baseline characteristics of participants at inclusion appear in Table 7. Participants aged 5 to 9 years old were the most represented in DHA-PPQ, ASAQ and AL treatment arms, with an incidence of 42.19%, 52.23%, and 33.98%, respectively.

During the two years of follow-up, of the 449 participants included in the PA arm, 301 (67.04%) were re-treated with the same drug for 746 episodes with 6 patients re-treated 9 times. Of the 448 participants included in DHA-PPQ arm, 317 (70.76%) were re-treated with the same drug for 824 episodes with 3 patients re-treated 9 times and one patient eleven times. In ASAQ arm, of the 224 participants included, 205 (91.52%) were re-treated during the two-year follow-up period for 837 episodes with 8 patients re-treated 9 times, seven patients 10 times, one patient 11 times and one patient 13 times. Of the 671 participants in AL arm, 453 (67.51%) were re-treated for 1061 episodes with one patient retreated 9 times, two patients 10 times and one patient 11 times (Table 8).

Table 7: Characteristics of participants at inclusion into different treatment arm. Age is expressed in year. *P. falciparum*: median (/µl) (Q1 – Q3) represents median number of asexual forms of parasites per µl with the interquartile range.

Characteristics	PA	DHA-PPQ	ASAQ	AL
Mean age ±SD	13.8 (10.2)	9.1 (5.2)	8.2 (3.3)	12.3 (8.4)
Median age (min, max)	11.1 (1.7 – 62.2)	8.9 (0.6 – 61.6)	7.9 (0.7 – 17.4)	10.1 (1.1 – 69.3)
Age category n (%)				
< 5	58 (12.92)	76 (16.96)	36 (16.07)	99 (14.75)
5 - 9	132 (29.40)	189 (42.19)	117 (52.23)	228 (33.98)
10 -14	101 (22.49)	166 (37.05)	70 (31.25)	160 (23.85)
≥ 15	158 (35.19)	17 (3.79)	1 (0.45)	184 (27.42)
Female n (%)	199 (44.32)	226 (50.45)	105 (46.88)	297 (44.26)
P. falciparum: median (/µl) (Q1 - Q3)	17,980 (3,300 – 44,080)	17,770 (2,380 – 40,010)	14,610 (1,240 – 38,350)	20,840 (4,100 –48,000)

Table 8: Number of episodes per treatment arm. The proportion of patients at each episode (E2, E3, E4 … E13) in each treatment arm was calculated by dividing the number of patients at that episode by the number at the first episode (E1).

	PA	DHA-PQP	ASAQ	AL
E1	449	448	224	671
E2	67.04% (301)	70.76% (317)	91.52% (205)	67.51% (453)
E3	43.21% (194)	47.77% (214)	81.70% (183)	38.60% (259)
E4	22.49% (101)	29.91% (134)	64.73% (145)	24.14% (162)
E5	13.59% (61)	19.20% (86)	52.23% (117)	13.86% (93)
E6	9.58% (43)	9.60% (43)	36.16% (81)	6.86% (46)
E7	6.46% (29)	3.79% (17)	20.98% (47)	3.87% (26)
E8	2.45% (11)	1.56% (7)	12.95% (29)	2.09% (14)
E9	1.34% (6)	0.89% (4)	7.59% (17)	0.60% (4)
E10		0.22% (1)	4.02% (9)	0.45% (3)
E11		0.22% (1)	0.89% (2)	0.15% (1)
E12			0.45% (1)	
E13			0.45% (1)	
TOTAL	1195	1272	1061	1732

3.1.2. Treatment response by episode at day 28 and 42 of follow-up shows the intensity of malaria exposure/recurrence by treatment arm

The proportion of patients without subsequent episodes of malaria or *Plasmodium sp*. asexual parasitaemia relapse by day 28 of follow-up in the PA arm was lower at episode 4 (92.71%) than episode 1, 2, 3 and 5 ($p = 0.023$). In the DHA-PPQ arm there was no difference between episodes. By comparison, in the ASAQ arm the lowest efficacies were recorded at episodes 2 and 3 with 85.93% and 85.29% efficacy, respectively and the highest efficacy at episode 1 with 94.64% ($p = 0.028$). In the AL arm, the lowest efficacy was observed at episode 4 (79.86%), but there was no statistical difference compared to other episodes ($p = 0.051$) (Table 9).

At 42 days of follow-up, the number of patients without subsequent treatment episodes decreased from episode 1 to episode ≥ 6 in the PA arm. This may have occurred as a result of selection of tolerant parasite by this treatment arm or it may be due to the repeated malaria exposure. Participants who are more exposed to malaria may do more episodes. The same trend of decreased efficacy was observed from episode 1 to episode ≥ 6 in the DHA-PPQ arm, but the decrease was not significant in this treatment arm. Contrasting with this scenario, in the ASAQ arm, as for day 28 of follow-up, episode 1 with 77.23% of efficacy had the higher number of patients without subsequent episode at day 42 compared to others episodes ($p < 0.001$). In AL arm there was no decrease in the efficacy from episode 1 to episode 5. The lowest efficacy was recorded at episode ≥ 6 with a proportion of (52.5%) ($p = 0.034$) (Table 10).

Table 9: Proportion of patients without subsequent episode by treatment arm and by episode at day 28 of follow-up. In PA arm episodes 6 – 9 were gathered together as episode ≥ E6. In DHA-PPQ and AL arm episodes 6 – 11 were gathered together as episode ≥ E6. In ASAQ arm, episodes 6 – 13 were gathered together to form episode ≥ E6

	E1 % (N)	E2 % (N)	E3 % (N)	E4 % (N)	E5 % (N)	≥E6 % (N)	p
PA	98.20 (445)	98.10 (263)	96.93 (163)	92.71 (96)	96.30 (54)	93.51 (77)	0.023
DHA-PPQ	99.77 (444)	99.31 (289)	98.84 (172)	100 (105)	100 (66)	98.25 (57)	0.445
ASAQ	94.64 (224)	85.93 (199)	85.29 (170)	88.15 (135)	86.79 (106)	90.57 (159)	0.028
AL	86.06 (667)	87.19 (398)	89.43 (227)	79.86 (139)	85.88 (85)	78.31 (83)	0.051

Table 10: Proportion of patients without subsequent episode by treatment arm and by episode at day 42 of follow-up. In PA arm episodes 6 – 9 were gathered together as episode ≥ E6. In DHA-PPQ and AL arm episodes 6 – 11 were gathered together as episode ≥ E6. In ASAQ arm, episodes 6 – 13 were gathered together to form episode ≥ E6

	E1 % (N)	E2 % (N)	E3 % (N)	E4 % (N)	E5 % (N)	≥E6 % (N)	p
PA	86.85 (441)	86.54 (260)	81.70 (153)	77.42 (93)	70.37 (54)	72.00 (75)	0.001
DHA-PPQ	97.74 (443)	97.52 (282)	94.55 (165)	94.95 (99)	96.88 (64)	94.63 (56)	0.279
ASAQ	77.23 (224)	59.80 (199)	56.55 (168)	64.66 (133)	69.52 (105)	69.68 (155)	0.000
AL	69.82 (666)	71.88 (384)	69.68 (221)	67.67 (133)	69.62 (79)	52.50 (80)	0.034

3.1.3. Treatment response by arm at day 28 and 42 of follow-up shows the intensity of malaria exposure/recurrence by site:

Sotuba, with its lower transmission intensity, had the higher proportion of patients without subsequent episode at day 28 and 42 in all of the treatment arms. DHA-PPQ and PA had an efficacy of more than 96% at day 28 and 42. The proportion of patients without subsequent episode in AL arm in Sotuba was 94.84% at day 28 and 87.76% at day 42 of follow-up. The proportions of patients without subsequent episode in Kollé and Bougoula Hameau were not statistically different except at day 42 in PA and AL arms with the lower proportion in Kollé (Table 11).

In general, a drastic decrease in the proportion of patients without subsequent episodes was observed from day 28 to day 42 of follow-up in each treatment arm and at each study site.

Table 11: Efficacy of treatment arms at day 28 and 42 of follow-up by site (ASAQ was implemented in Bougoula Hameau only)

	Day	Sotuba % (N)	Bougoula hameau % (N)	Kollé % (N)	p
PA	28	99.27 (273)	96.34 (683)	96.48 (142)	0.047
	42	96.86 (255)	80.18 (681)	74.29 (140)	0.000
DHA-PPQ	28	100 (206)	99.45 (723)	99.02 (204)	0.388
	42	99.49 (196)	96.09 (717)	96.41 (195)	0.056
ASAQ	28		88.92 (993)		
	42		66.46 (984)		
AL	28	94.84 (465)	83.17 (719)	80.48 (415)	0.000
	42	87.76 (441)	67.65 (714)	51.96 (408)	0.000

3.1.4. Distribution of the proportion of patients without subsequent episode by treatment arm and by age group at day 28 and 42 of follow-up:

Except for the ASAQ arm and DHA-PPQ arm at day 28 of follow-up, my results showed an increase in the proportion of patients without subsequent episode at day 28 and 42 with age groups in PA, DHA-PPQ and AL arms ($p < 0.005$). In DHA-PPQ arm, at day 28 of follow-up, there was no difference between age groups. Age group ≥ 15 years had a higher proportion of patients without subsequent episode at day 28 of follow-up respectively 100% in PA, DHA-PPQ and ASAQ arms, and 96.35% in AL arm. Patients in the lower age group (< 5 years) had a lower proportion of patients without a subsequent episode. There was no difference between age groups concerning the percentage of patients without subsequent episode by day 28 in ASAQ arm (Table 12).

In ASAQ arm, except for age group ≥ 15, the proportion of patients without subsequent episode by day 42 of follow-up was higher in under 5 years old age group than the others ($p = 0.004$). Age group 5 - < 10 years had a lower proportion of patients without subsequent episode at day 42 of follow-up compared to the others age groups (Table 12).

Table 12: Patients without subsequent episode at day 28 and 42 of follow-up by treatment arm and by age group

	Day	< 5 years % (N)	5 - < 10 years %(N)	10 - <15 years %(N)	≥15 years %(N)	p
PA	28	94.37 (142)	94.97 (378)	97.98 (248)	100 (330)	0.000
	42	68.31 (142)	73.60 (375)	91.77 (243)	95.25 (316)	0.000
DHA-PPQ	28	98.72 (156)	99.60 (501)	99.53 (425)	100 (49)	0.546
	42	92.81 (153)	96.15 (493)	98.80 (416)	97.87 (46)	0.003
ASAQ	28	90.65 (107)	87.88 (553)	89.72 (321)	100 (12)	0.452
	42	76.64 (107)	62.41 (548)	69.09 (317)	91.67 (12)	0.004
AL	28	78.79 (198)	79.94 (633)	88.80 (384)	96.35 (384)	0.000
	42	63.96 (197)	60.10 (619)	70.13 (375)	86.29 (372)	0.000

3.1.5. Safety of the treatment arms

I looked for two major adverse events during this study. The first one was the cardiotoxicity defined by an abnormal QTc at day 2 (4 to 6 hours after the last dose of treatment) as compared to QTc at baseline (day 0). Cases with a QTc higher than 30 milliseconds were considered abnormal. The other adverse event of interest I monitored was the hepatotoxicity defined by the abnormal level of alanine aminotransferase (ALAT) concentration in patients at day 3. As stipulated by laboratory reference ranges of Malaria Research and Training Center (MRTC) in Mali.

Was considered abnormal if ALAT level was higher than:

- 61 IU/L in adult patients (age > 14 years)
- 53.4 IU/L in 6 to 14 years' age group
- 50 IU/L in less than 6 years' patients

Table 13 shows the proportion of patients with abnormal ALAT and QTc in the treatment arms. The higher proportion of abnormal ALAT was in PA arm (6.42%) and the lower in ASAQ arm (2.77%). The higher proportion of patients with abnormal QTc was reported in DHA-PPQ arm (30.37%) and the lower in PA arm (8.60%).

Table 13: Level of abnormal ALAT and QTc in different treatment arms

	PA % (n/N)	DHA-PPQ % (n/N)	ASAQ % (n/N)	AL % (n/N)	value
Abnormal ALAT	6.42 (70/1090)	3.76 (42/1116)	2.77 (27/974)	3.15 (50/1589)	<0.001
Abnormal QTc	8.60 (93/1081)	30.37 (338/1113)	22.05(217/987)	12.74(200/1570)	<0.001

3.2. Genotype results

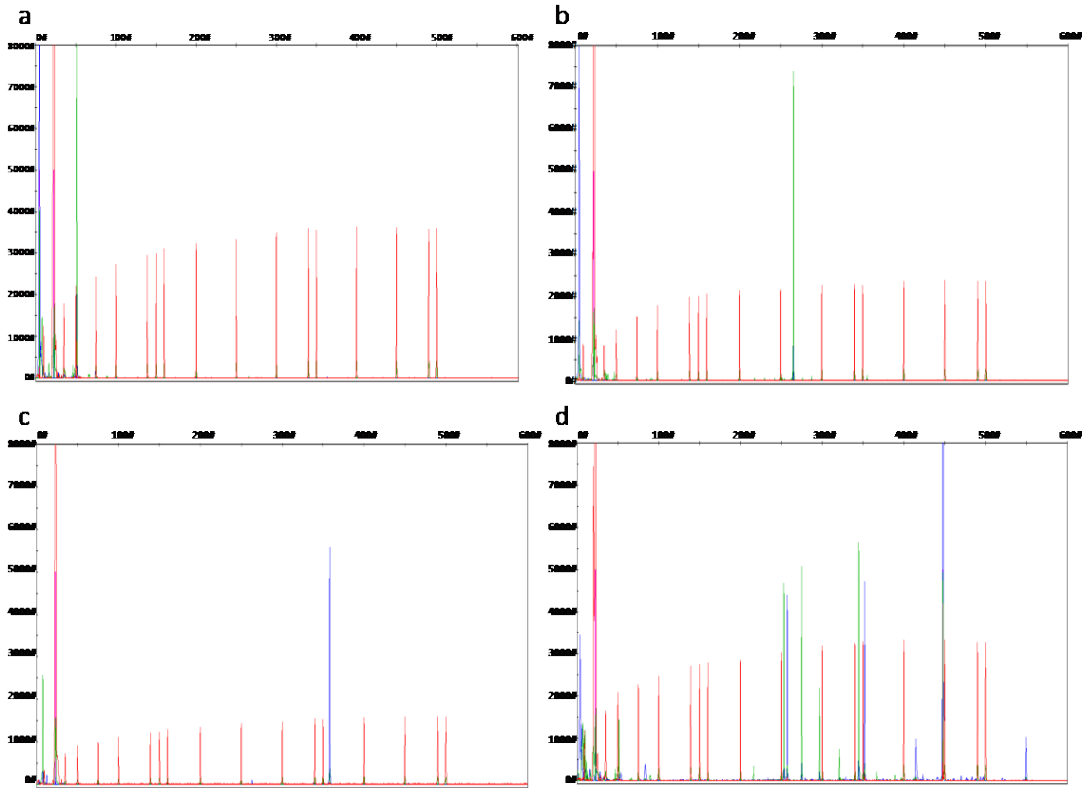
A total of 1027 pairs of recrudescent patient blood samples were analysed at day 42 of follow-up. There were 179 pairs in PA arm, 36 in DHA-PPQ arm, 330 in ASAQ arm and 482 in AL arm. To discriminate cases of reinfection from recrudescent parasites, I used *Pf* size polymorphic genes (*msp2, msp1, and ca1*).

For discriminating parasites with *msp2*, I used a nested PCR for its amplification coupled with capillary electrophoresis for its revelation. This method of evaluation allowed me to better characterize the multiplicity of infection in the study population.

3.2.1. Genetic diversity of parasites in study sites

With *msp2* capillary electrophoresis, I found that the multiplicity of infection was as high as 9, meaning that a patient can harbor up to 9 different clones of parasite at the same time as shown in (Figure 14, d). The mean multiplicity of infection was 4.7 in the study population.

My results showed a high genetic diversity of *Pf* in the study area. The major allele was FC27 (336 bp) with a frequency of 11.7%; followed by FC27 (371 bp and 417 bp) observed respectively in 7.8 and 6.8% of case. The most frequent 3D7 allele was 3D7 (282 bp). The diversity of 3D7 was higher than the one of FC27 in my study area (Figure 15).



<u>Figure 14</u>: Electropherograms of the *msp2* capillary electrophoresis.

(a) negative control of the extraction; (b) 3D7 family's positive control (green color); (c) FC27 family's positive control (blue color); (d) sample from one of the patients included in this study. This patient harbored 4 alleles of the 3D7 family green colored and 5 alleles of the FC27 family blue colored. Red peaks are the size standards (rox); green peaks are 3D7 family alleles; and blue peaks are FC27 family alleles.

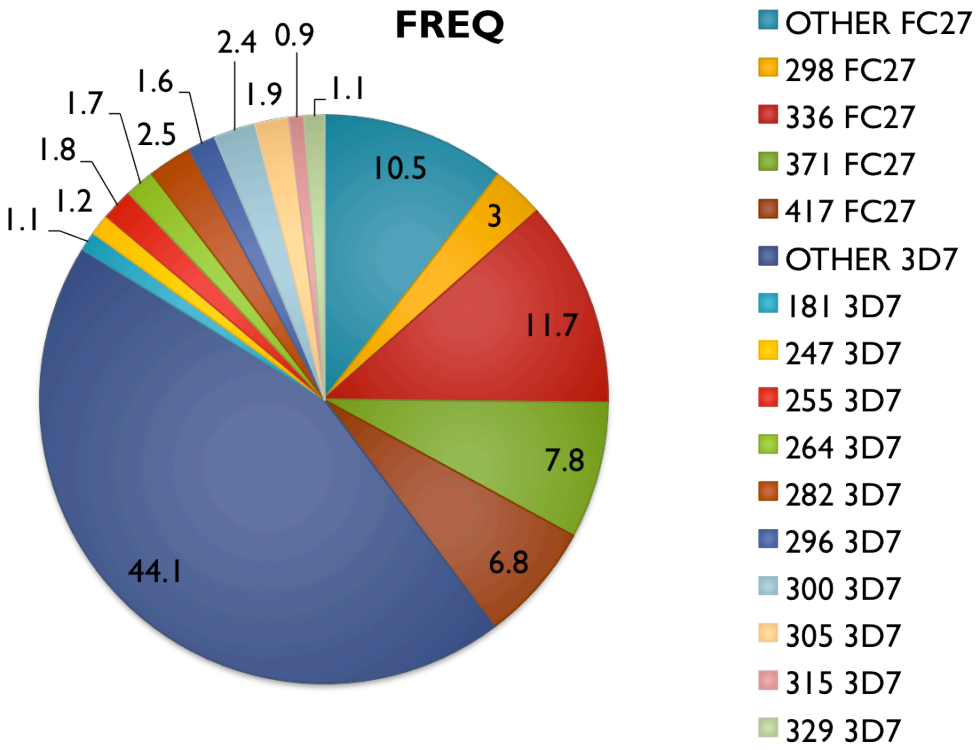


Figure 15: msp2 genetic diversity in my study sites in percent.

FC27 allele 336 is the most frequent with 11.7%. OTHER represents alleles which frequency was less than 0.9%

3.2.2. Treatment response by arms after molecular correction

In antimalarial drug clinical trials, estimation of true drug efficacy is done by identifying recurrent infections detected after drug treatment as recrudescent parasites or as new infection coming from the liver. This categorisation of recurrent infection is done by comparing the size of the length-polymorphic molecular markers before and after treatment. If the sizes of the amplicons at day 0 (before treatment) differ from the one at day of failure (recurrence), the recurrent infection is considered as a new infection, but if the sizes are the same, these are categorized as recrudescent infections and are considered as true treatment failures. Discrimination of recrudescent parasites from reinfection in this work was done sequentially. Firstly, the most length-polymorphic molecular marker *msp2* were used on all of the recurrent infection samples. Secondly, all samples that were identified as recrudescent by *msp2* were

analysed with *msp1*. Lastly, the remaining recrudescent parasites were analysed with *ca1*.

The antimalarial drugs used in this study were highly efficacious, with all demonstrating more than 99% efficacy after molecular correction. DHA-PPQ and ASAQ were 100% efficacious; and all of the recurrent parasites in these treatment arms were cases of reinfection. For PA, there were 3 cases of recrudescence at day 42 of follow-up. In the AL arm, there were 7 cases of recrudescent infections. Table 14 shows the characteristics of patients with the recrudescent parasites in the AL arm. These patients did not share any particular characteristics of note except that the majority were from the Kollé site. Further investigation will determine if drug concentration played a role in the recrudescence of infection in these patients.

Table 14: Characteristics of patients with recrudescent parasites after molecular correction in AL arm. Day 7 lumefantrine concentration was quantified in patients with recrudescence of infection.

SN	01-0126-P	02-0135-P	02-0275-P	03-0019-P	03-0046-D	03-0099-D	03-0214-D
Episode	1	2	1	1	3	2	1
Pf Day 0	6720	2100	87120	8840	107360	11440	105160
Temp Day 0	39.6	36.3	39.2	35.9	36.4	36.9	38.9
Pf Day 1	460	88	840	2680	12060	476	128
Pf Day 2	0	0	0	760	0	0	0
Pf Day 3	0	0		1200	0	0	0
Pf Day 7	0			1680	0	0	0
Pf Day 14	0	0	53800	0	0	0	0
Pf Day 25	13060						
Pf Day 27				2520			
Pf Day 28		0	0		1640	8480	80560
Pf Day 35		740	0	48			
Pf Day 42				0			
Arm	AL	AL	AL	AL	AL	AL	AL
Site	Sotuba	Bougoula	Bougoula	Kollé	Kollé	Kollé	Kollé
Age (years)	21	14	6	10	4	7	4

3.3. Development and validation of HPLC methods for the quantification of lumefantrine, N-desethylamodiaquine, and pyronaridine in plasma.

Methods have been developed and validated for quantification of lumefantrine and pyronaridine in plasma.

For DEAQ, there was already a developed and validated method for its quantification in plasma in the pharmacokinetic lab. For my study, I revalidated the method before quantification of my samples.

The methods for quantification of lumefantrine and DEAQ were sensitive and specifics to be applied for quantification of day 7 concentration of these drugs in malaria patients.

Figure 16 shows electropherograms for lumefantrine, DEAQ, and their ISs. There was a good separation between drugs and IS. The retention times for lumefantrine and its IS were respectively 13.8 minutes and 17.7 minutes. DEAQ had a retention time of 6.3 minutes, and its IS 4.4 minutes. For pyronaridine, it was 7 minutes while its IS come out at 14.4 minutes.

There were no retention time variations with direct injection or spiked samples or drugs obtained from malaria patients.

IS used for the quantification of pyronaridine and DEAQ had structural similarity with these drugs. For lumefantrine, I tested three of its analogs: two were a gift from Novartis (TA3099 and TA 2006-12-14K2) and one was already used as IS for lumefantrine quantification [293]. TA3099 was the one, which didn't interfere with any other drug or endogenous peaks. The other two analogs interfered with endogenous peaks under selected conditions.

All of the methods gave excellent linearity. Calibrations curve's coefficients of correlation were always higher than 0.997 (Figure 17).

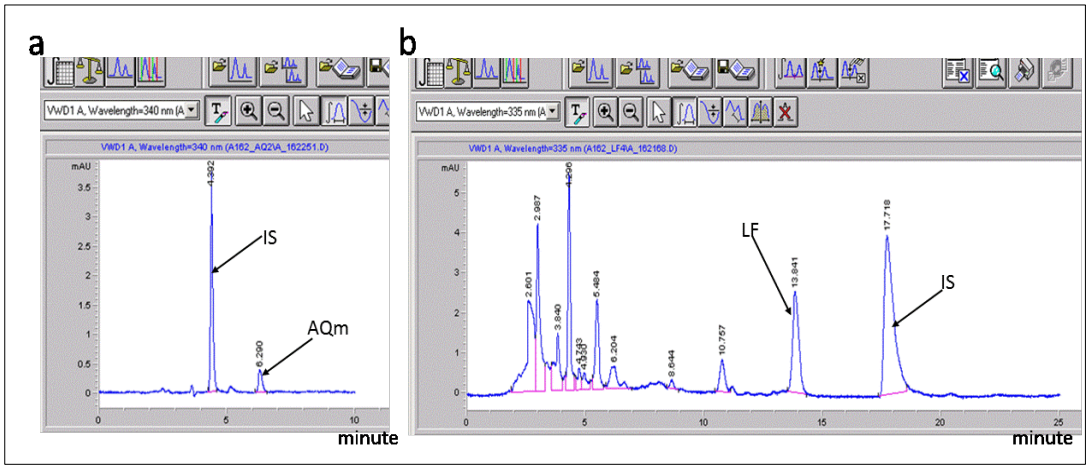


Figure 16: Electropherograms of quantified drugs.

(a) N-desethylamodiaquine, (b) lumefantrine, from my patients and their internal standards. DEAQ has a retention time of 6.3 minutes its IS come at 4.4 minutes. While lumefantrine has a retention time of 13.8 minutes, its IS comes at 17.7 minutes.

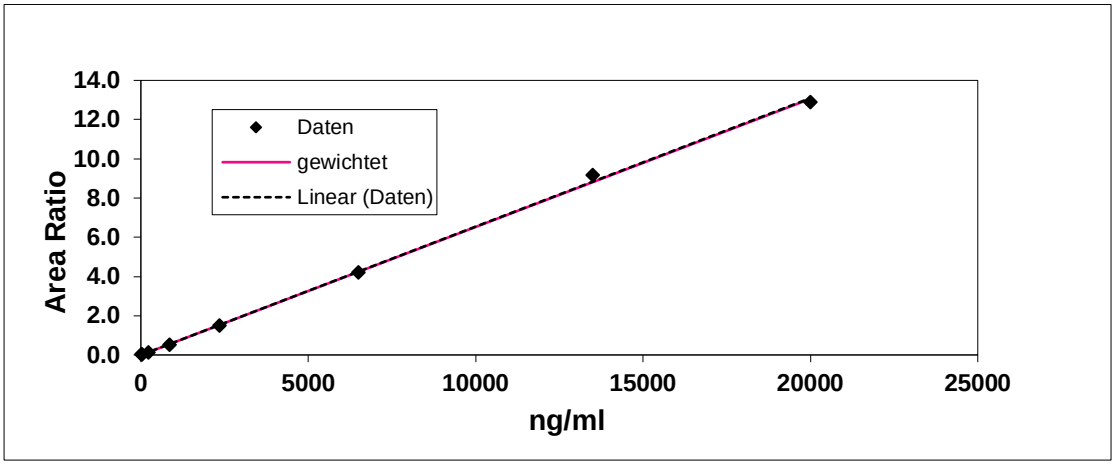


Figure 17: Lumefantrine calibration curve with R-Square = 0.9987

Within-day and between days' coefficients of variation of both methods for pyronaridine and lumefantrine were always less than 12%, showing that the methods have a good precision. The deviations of the true concentration from the measured concentration of the QCs samples were always between 90 and 110% for pyronaridine and lumefantrine confirming the good accuracy of the methods. The

Intra and inter-batch variations of the limits of quantification were less than 10% (Table 15 and 16).

Extraction recovery for pyronaridine, lumefantrine, and their internal standards was excellent. Recoveries from plasma were higher than 75% for all of the drugs and ISs. The recovery of lumefantrine was higher than 80% at differents QC samples. For pyronaridine, the recovery was higher than 90%. The recovery for lumefantrine IS (concentration = 1000ng/ml) was 76.3±3 in percent. Moreover, the recovery of the IS of pyronaridine and DEAQ at the concentration of 750 ng/ml was 91.7 ± 2 in percent.

No other antimalarial drugs interfered with the peaks of my drugs. Neither the main concomitant drug (paracetamol) nor the endogenous substances interfered with the peaks.

Injection of the mobile phase directly after the high drug concentration showed that there was no carry over for the methods.

Table 15: Lumefantrine Intra and inter-day precision, and accuracy

C_{spiked} (ng/ml)	C_{found} mean±S.D (ng/ml)	Withing-asay CV (%)	Between-assay CV (%)	Bias (%)
20	21.8 ± 1.3	6.3	6.1	9.3
60	54.9 ± 3.9	9.2	11.5	-8.4
6000	5973.4 ± 162.3	2.3	2.7	-0.4
15000	14982.6 ± 291	1.3	1.9	-0.1

Table 16: Pyronaridine Intra and inter-day precision, and accuracy

C_{spiked} (ng/ml)	C_{found} mean±S.D (ng/ml)	Withing-assay CV (%)	Between-assay CV (%)	Bias (%)
25	25.7 ± 1.8	2.8	7.0	2.4
80	76.6 ± 2.2	3.2	2.8	-4.3
418	415 ± 18.9	1.1	4.6	-2.3
806	845.6 ± 32.9	3.9	3.9	-0.5

DEAQ method has been revalidated successfully with a withing-assay coefficient of variation of 3% at 60 ng/ml; 2.8% at 775 ng/ml and 2.8% at 1600 ng/ml. The method was accurate and precise at the different quality control concentrations.

Table 17 and 18 showed the precision and accuracy of quality control samples used in the analysis of clinical samples collected from uncomplicated malaria patients at day 7 of follow-up after treatment with AL or ASAQ. A total of 26 batches with two replicates of each QC samples were used to quantify lumefantrine concentrations in patients. From these 52 replicates at each QC concentration only one in high concentration QC was out of the 15% concentration variation recommended (spiked concentration 15000 ng/ml, the concentration found 17255.9 ng/ml).

For DEAQ quantification, I used 11 batches with two replicates of each QC samples. None of the measured QC concentrations varied more than 10% from the spiked concentrations.

The mean variations of precision and accuracy were always less than 10%.

For pyronaridine, after quantification of 40 samples collected from uncomplicated malaria patients at day 7 of follow-up (4 days after the last dose) I found that pyronaridine concentrations were always under the limit of quantification, which was set at 25 ng/ml. The method developed and validated was not able to quantify pyronaridine concentration in malaria patients at day 7 of follow-up.

Table 17: Validation of lumefantrine quantification method during patients' samples analysis

C_{spiked} (ng/ml)	C_{found} mean±S.D (ng/ml) (N)	CV (%)	Accuracy
60	60.8 ± 2.2 (52)	3.7	101.3
6000	6075.1 ± 190.9 (51)	3.1	101.3
15000	15471.4 ± 650.7 (52)	4.2	103.1

Table 18: Validation of N-desethylamodiaquine quantification method during patients' samples analysis

C_{spiked} (ng/ml)	C_{found} mean±S.D (ng/ml) (N)	CV (%)	Accuracy
60	57.6 ± 2.1 (22)	3.6	96.1
775	734.2 ± 33.8 (22)	4.6	94.7
1600	1604.5 ± 46.4 (22)	2.9	100.3

3.4. Pharmacokinetic of lumefantrine and N-desethylamodiaquine at day 7 of follow-up

3.4.1. Number of episodes and baseline characteristics in patients with quantified drug concentrations

Table 19 shows the number of samples analyzed by HPLC in each treatment arm by episode. In total lumefantrine concentration were quantified at day 7 in 1100 samples collected from 659 patients with uncomplicated malaria treated with AL. DEAQ was quantified in 749 samples taken from 217 patients treated with ASAQ.

Episodes' baseline characteristics of patients are summarized in Table 20. There was no difference between mean ages by sex in AL or ASAQ arm. Age group 5 – 9 years contributed to the maximum number of samples. This age group was the most affected by malaria in the two treatments arm.

Sotuba site, where the transmission is low contributed to the maximum number of adult patients 64.7% and Bougoula-Hameau with 29.7%.

Table 19: Number of samples analyzed by episode in different treatment arm to quantify lumefantrine and N-desethylamodiaquine

Episode	Lumefantrine	Desethyl-Amodiaquine
E1	585	150
E2	277	162
E3	139	107
E4	58	124
E5	27	86
E6	8	58
E7	2	30
E8	2	15
E9	1	10
E10	1	5
E11 – E13		2
Total	1100	749

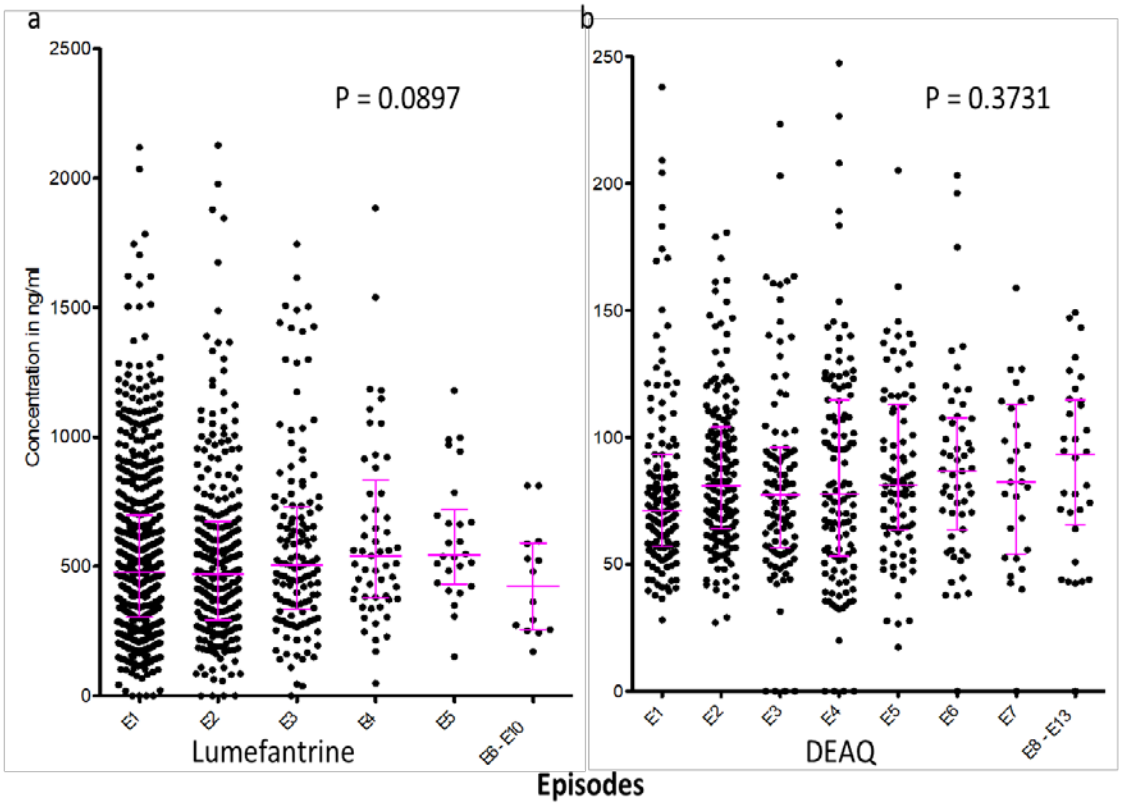
Table 20: Episodes' baseline characteristics of patients with drug concentration

Characteristics	Artemether-lumefantrine		Artesunate-amodiaquine	
	Male	Female	Male	Female
Mean age ±SD	12.2 (8.5)	11.9 (9.3)	8.7 (3.1)	8.5 (2.9)
Age category (n)				
< 5 years	80	60	45	30
5 – 9	201	198	232	181
10 – 14	128	124	143	92
≥ 15	186	99	0	0
Median temperature	37.8	37.6	37.3	37.2
Pf: median (/µl) (Q1 – Q3)	17,980 (3,060 – 46,400)	21,110 (3,380 – 47,920)	19,580 (1,620 – 45,200)	14,400 (1,520 – 34,900)

3.4.2. Accumulation of lumefantrine and N-desethylamodiaquine in study population after repetitive treatment with the same drug: AL or ASAQ

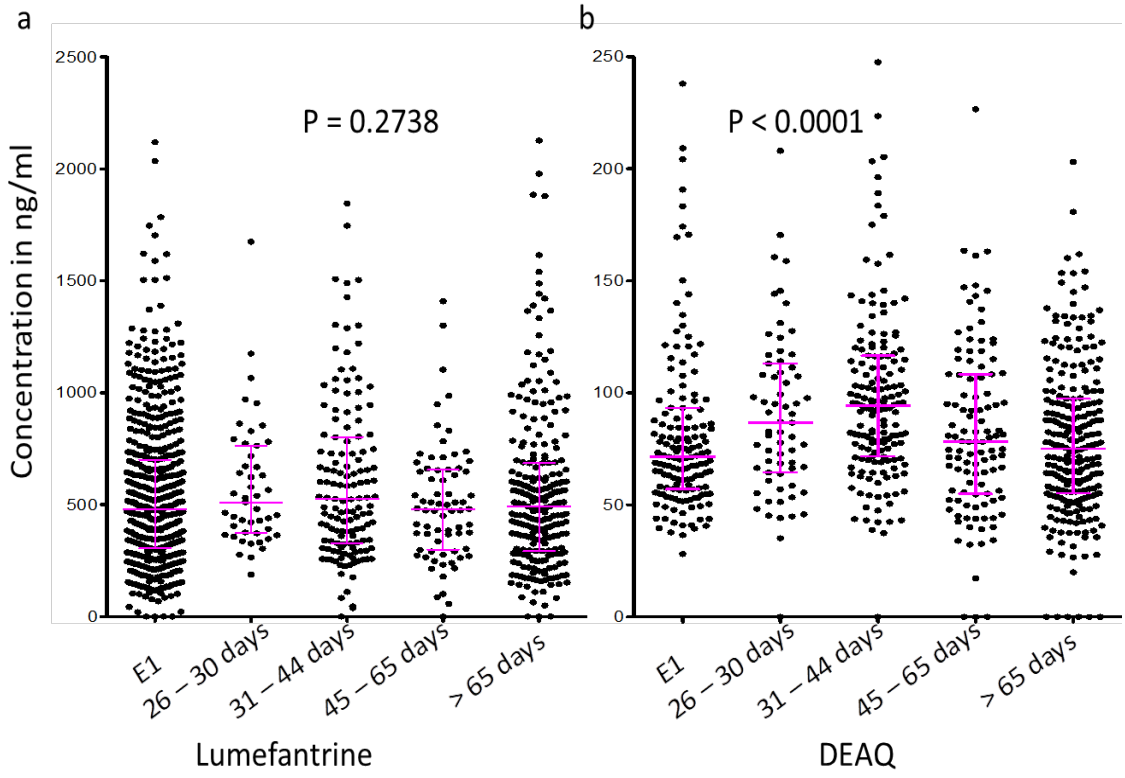
In the AL arm, I did not find any accumulation of lumefantrine in study population after repetitive treatment of patients with the same drug. There was no statistical difference in day 7 concentration of lumefantrine neither between episodes as shown in (Figure 18 a), nor between period at which patients received the subsequent treatment (Figure 19 a). Some patients received 10 times the same treatment during the two years period of follow-up.

Repetitive treatment of uncomplicated malaria patients with ASAQ accumulated DEAQ in study population when patients received subsequent treatment in a short period. In the ASAQ arm, when the patient received a subsequent treatment of ASAQ in a period of 45 days, there was an accumulation of DEAQ in population as shown in (Figure 19 b). Patients, who received subsequent treatment between day 26 and 45 had a high day 7 concentration of DEAQ when compared to the concentration at the first treatment or to the concentration from patients who received a subsequent treatment after 45 days.



<u>Figure 18</u>: Lumefantrine and DEAQ day 7 concentration profile after repetitive treatment of a new episode of uncomplicated malaria with the same drug (AL or ASAQ).

(a) lumefantrine concentration, (b) DEAQ concentration, (E) episode. Number 1, 2, 3…are number of malaria episodes, in individual patients during the two-year follow-up period corresponding also to the number of time an individual patient received the same drug during the two years.



<u>Figure 19</u>: Day 7 concentrations variation according to the time of retreatment.

(a) Lumefantrine did not accumulate in study population after repetitive treatment with AL. (b) DEAQ accumulates in patients receiving a second treatment with ASAQ in a period of 45 days.

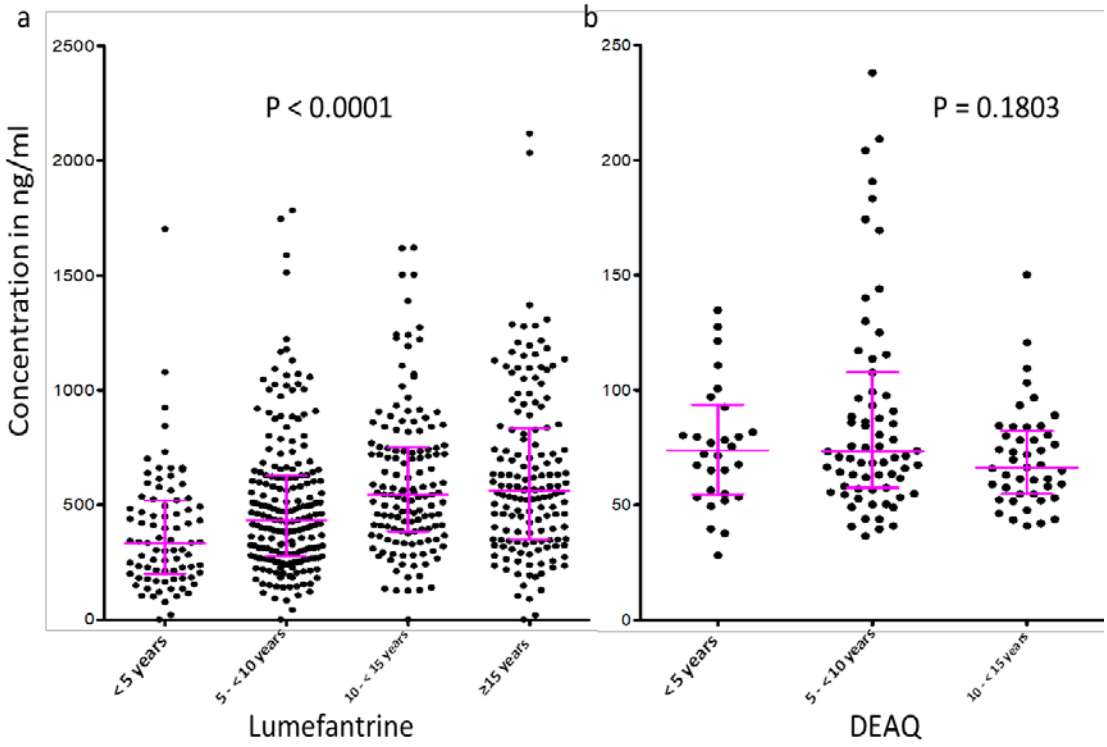
3.4.3. Distribution of lumefantrine and N-desethylamodiaquine day 7 concentration by age group, sex and fever status before treatment

Day 7 concentration of lumefantrine decreases with age. In my study population the lower median day 7 concentration of lumefantrine was measured in under 5 years' patients (305.9 ng/ml (IQR: 207.3 – 491.5)) and the higher in more than 15 years (571.1 ng/ml (IQR: 378.8 – 850.9)). The increase of lumefantrine median day 7 concentration with age group was very highly significant ($p < 0.0001$). This association profile persisted even after normalisation of concentration by body weight and drug dose ($p < 0.0001$) (Figure 20 a).

For DEAQ, there were no differences between medians of day 7 concentration by age group $p = 0.1803$ (Figure 20 b).

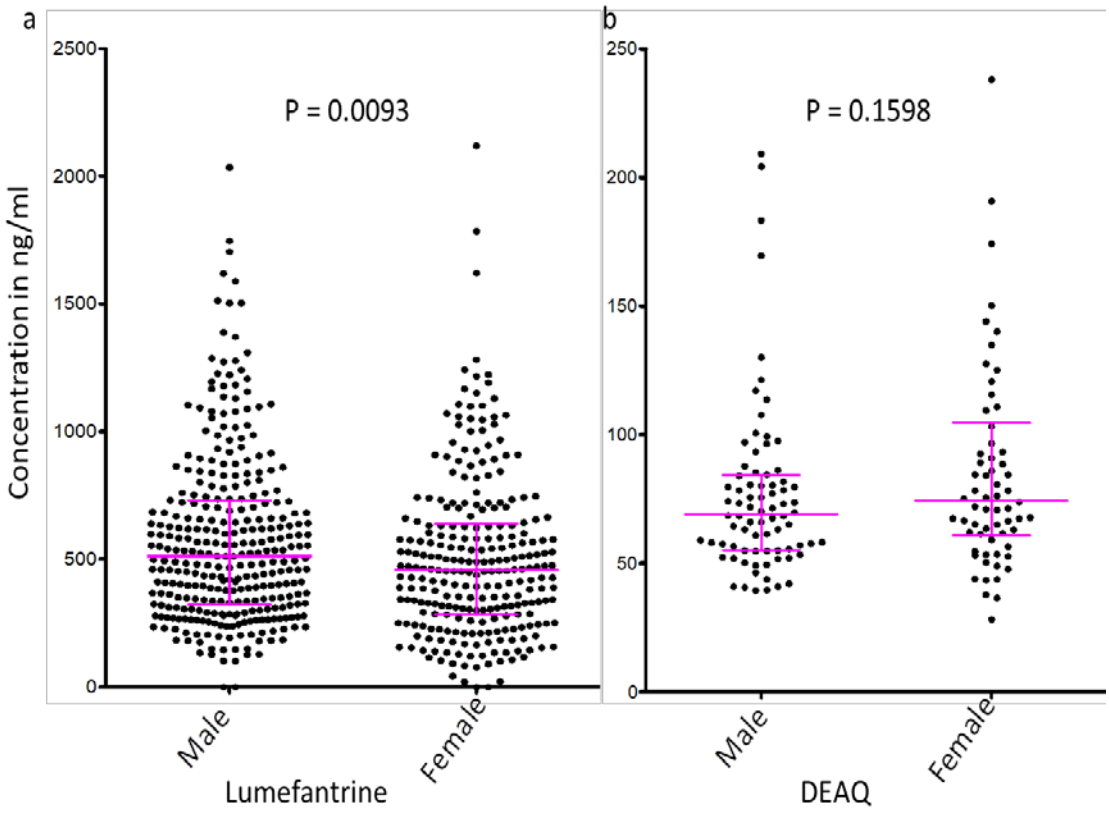
Figure 21 shows the distribution of lumefantrine and DEAQ day 7 concentration by sex. Lumefantrine median concentration was 10.6% lower in female than male (p = 0.0093). Contrasting with this observation, DEAQ median concentrations in both sexes were comparable (p = 0.1598).

Patients with fever at inclusion had lower day 7 median concentration of lumefantrine and DEAQ as compared to patients without fever with highly significant differences (p < 0.001) as shown in Figure 22.



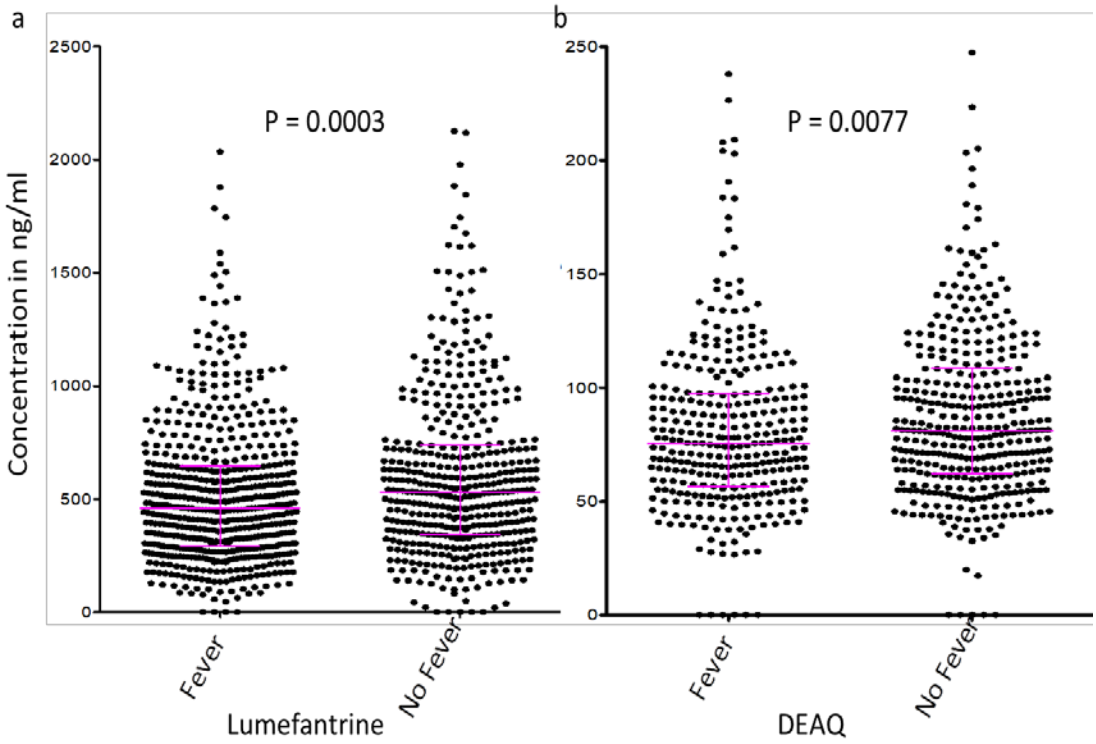
<u>Figure 20</u>: Day 7 concentrations distribution by age group.

All of the patients in ASAQ arm were under 15 years old (b). Lumefantrine day 7 median concentration increased with age (a) but not DEAQ day 7 median concentration after treatment of uncomplicated malaria patients with AL or ASAQ at first episode. The concentrations at E1 were used to avoid the effect of DEAQ accumulation. The lumefantrine concentration difference was statistically significant between age group of patients receiving AL (p < 0.0001).



<u>Figure 21</u>: Day 7 concentration distribution by sex

(a) lumefantrine, (b) DEAQ. Lumefantrine day 7 median concentration was higher in male patients compared to female after treatment of uncomplicated malaria with AL (a). While, there was no impact of sex on desethylamodiaquine day 7 concentration distribution after treatment of uncomplicated malaria patients with ASAQ (b).



<u>Figure 22</u>: Day 7 concentration distribution by fever status.

Baseline fever had an impact on plasma drug concentration. Febrile patients at inclusion had a lower day 7 median concentration compared to non-febrile after treatment of uncomplicated malaria with AL (a). For DEAQ there was no difference of day 7 median concentrations between febrile and non-febrile patients at inclusion after treatment with ASAQ (b).

3.5. Pharmacodynamics of lumefantrine and N-desethylamodiaquine

3.5.1. Relationship of lumefantrine and N-desethylamodiaquine day 7 concentrations with treatment response at day 28 and 42

I looked at the protective effect (post-treatment prophylaxis) of lumefantrine and DEAQ day 7 concentrations at day 28 and 42 of follow-up after treatment of uncomplicated malaria patient with AL or ASAQ.

The median concentration of lumefantrine at day 7 of follow-up was 36.7% higher in patients without subsequent episode before day 28 of follow-up than in patients who had subsequent episode $p < 0.0001$ (Figure 23 a). At day 42 of follow-up patients without subsequent episode had 7.6% higher lumefantrine day 7 median concentration compared to patients with subsequent episode $p = 0.0102$ (Figure 24 a)

For DEAQ, there was no difference between day 7 median concentration of patients with or without subsequent episode at day 28 or 42 of follow-up.

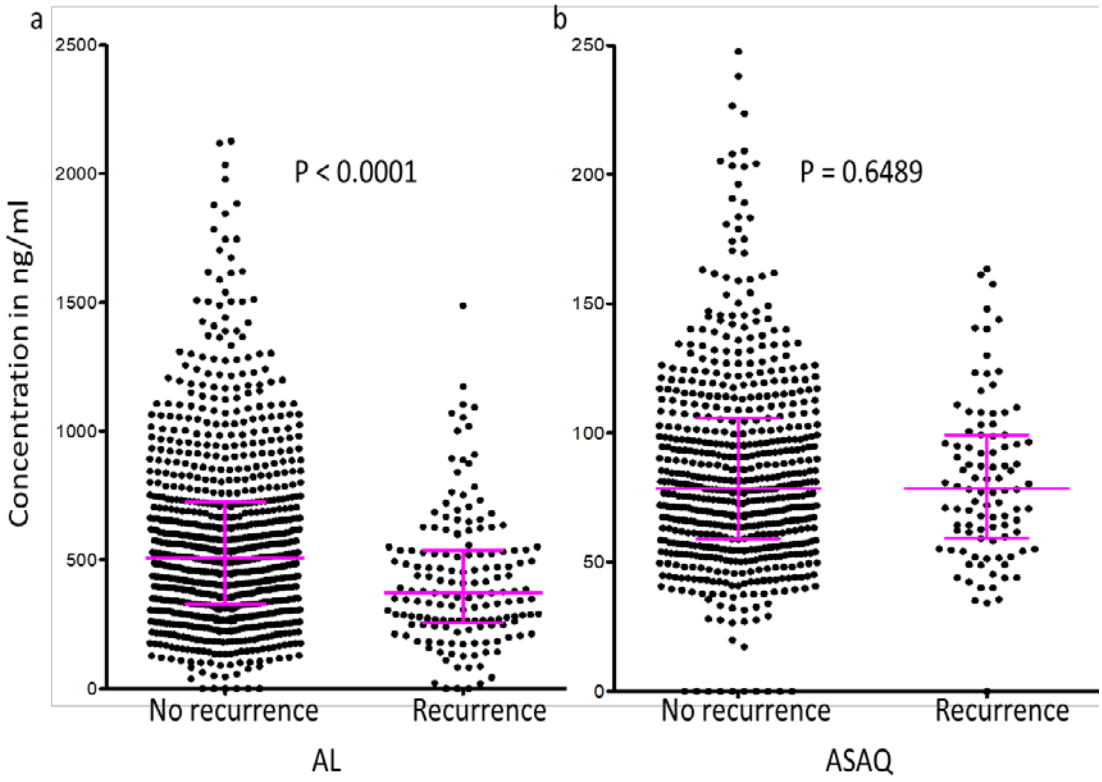


Figure 23: Association of low day 7 concentration with parasite recurrence by day 28 of follow-up.

(a) Lumefantrine day 7 concentration was lower in patients with recurrent parasitaemia before 28 days of follow-up as compared to those without recurrent parasitaemia after treatment with AL. (b) For DEAQ, there was no difference between day 7 concentration in patients with and without recurrent parasitaemia at day 28 of follow-up after treatment with ASAQ.

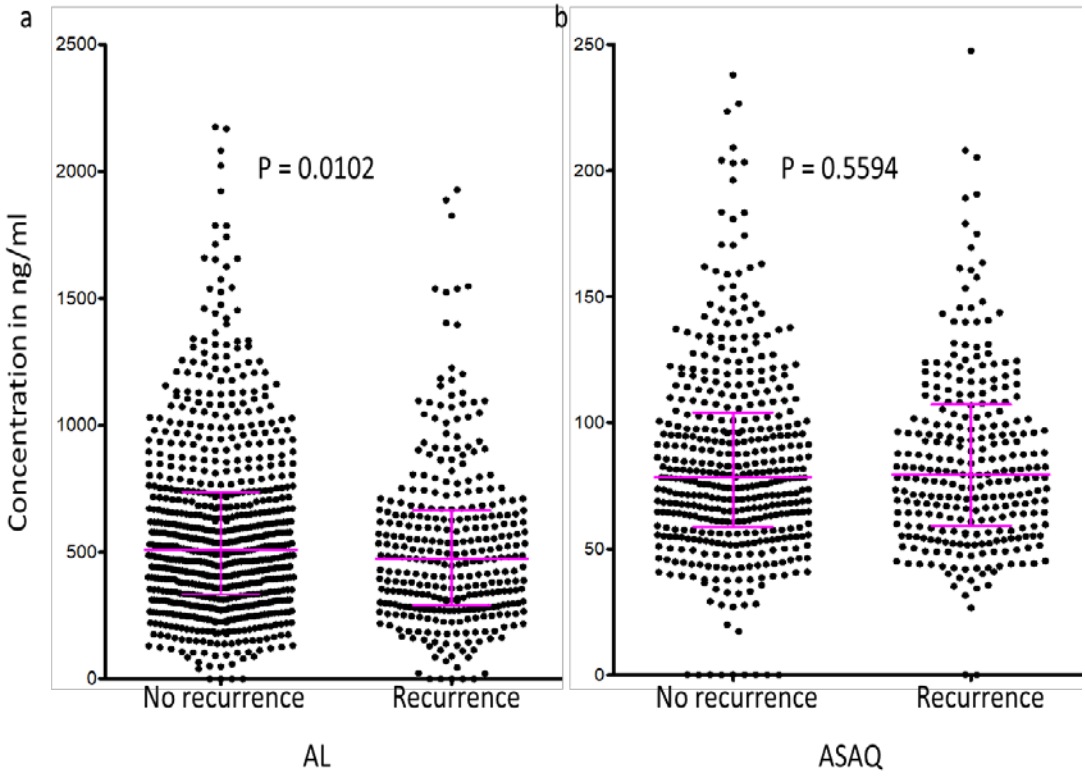


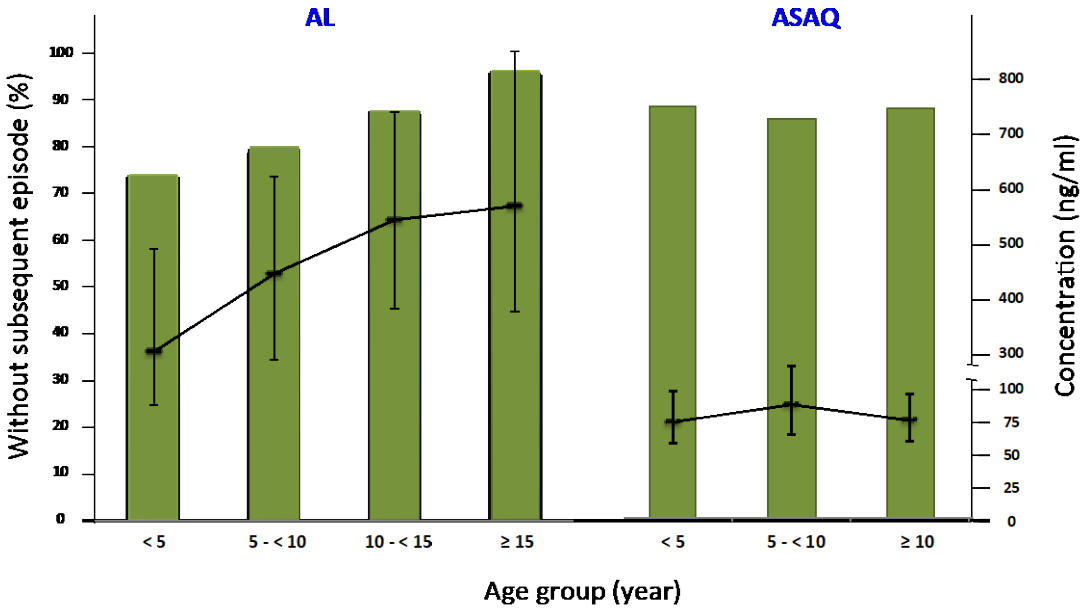
Figure 24: Association of low day 7 concentration with parasite recurrence by day 42.

(a) lumefantrine day 7 concentration is associated with parasite recurrence at day 42 of follow-up after treatment with AL. (b) DEAQ concentration at day 7 is not associated with parasite recurrence at day 42 of follow-up after treatment with ASAQ

3.5.2. Relationship between lumefantrine and N-desethylamodiaquine concentration with treatment response at day 28 by age group

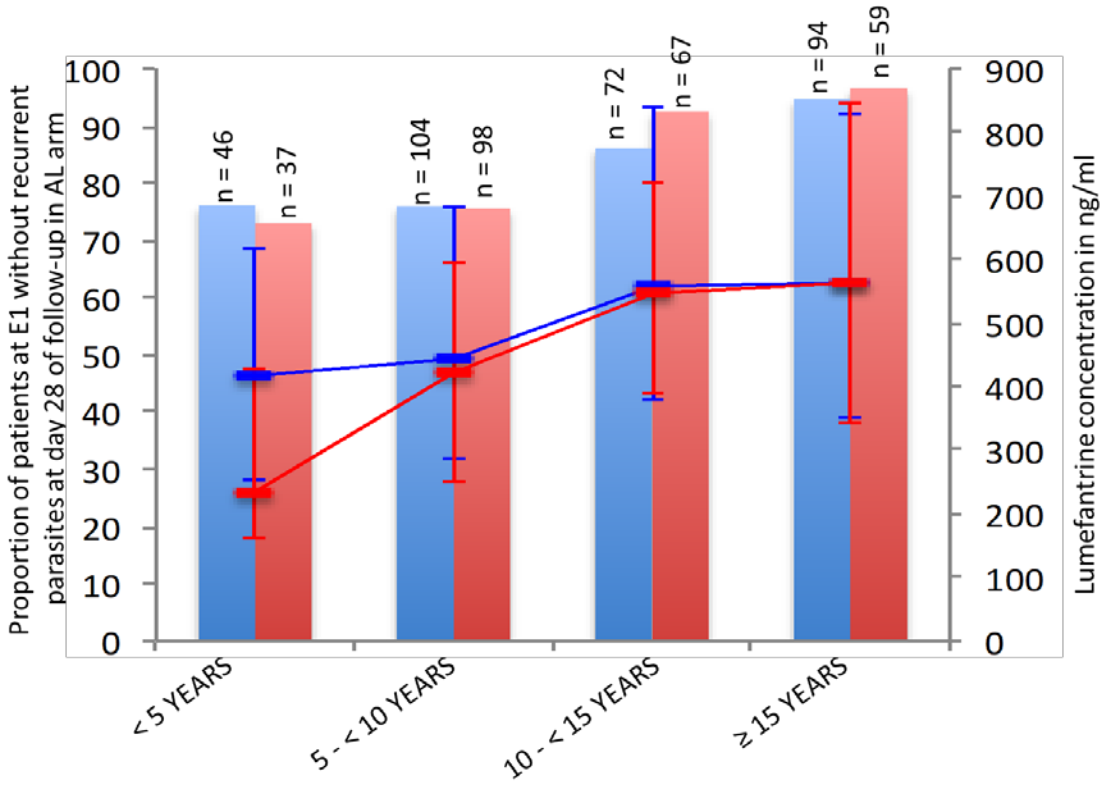
In my study, I found an increase in the proportion of patients without subsequent episode at day 28 of follow-up with age in AL group but not in ASAQ group. I also found an increase in the day 7 concentration of lumefantrine with age. Figure 25 shows collinearity between lumefantrine concentration and the proportion of patients without subsequent episode at day 28 of follow-up, the lower the proportion of patients without subsequent episode at day 28, the lower lumefantrine day 7 concentrations.

When I looked at the distribution of lumefantrine day 7 concentration by sex and age group, I found a lower concentration in under five years old' girls' patients. The median concentration was statistically lower in under five years' girls' patients than boys' p = 0.0018 (Figure 26).



<u>Figure 25</u>: Collinearity between lumefantrine day 7 concentration and proportion of patients without subsequent episode at day 28 of follow-up after treatment with artemether-lumefantrine.

While, there was no relationship between DEAQ day 7 concentration and proportion of patients without subsequent episode at day 28 after treatment with ASAQ. (Black) line with interquartile range represents the drugs concentration (for lumefantrine in AL arm and for DEAQ in ASAQ arm). (Green) filled columns represent the proportion of patients without subsequent episode at day 28 in AL and ASAQ arms respectively.



<u>Figure 26</u>: Low day 7 lumefantrine concentration in girls under five year of age.

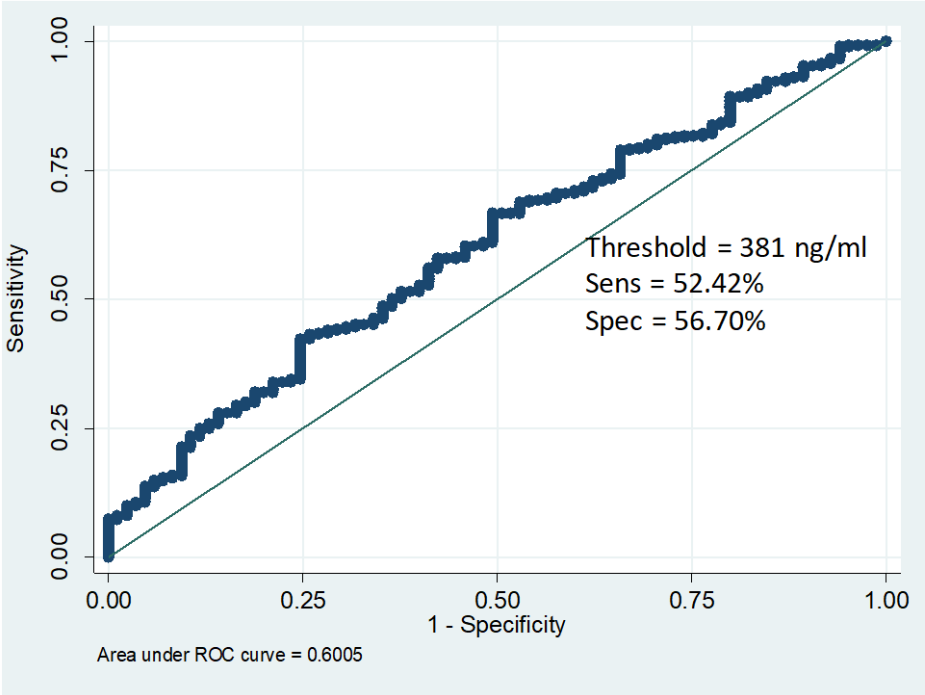
(Blue) color represents male patients. There was no concentration difference by sex in higher age groups. (Red) color is for female. (Lines) represent concentration with interquartile range. (Columns) represent proportion of patient without recurrent parasite at day 28.

3.5.3. Lumefantrine day 7 concentration in under five years' children was too low for post-treatment prophylaxis

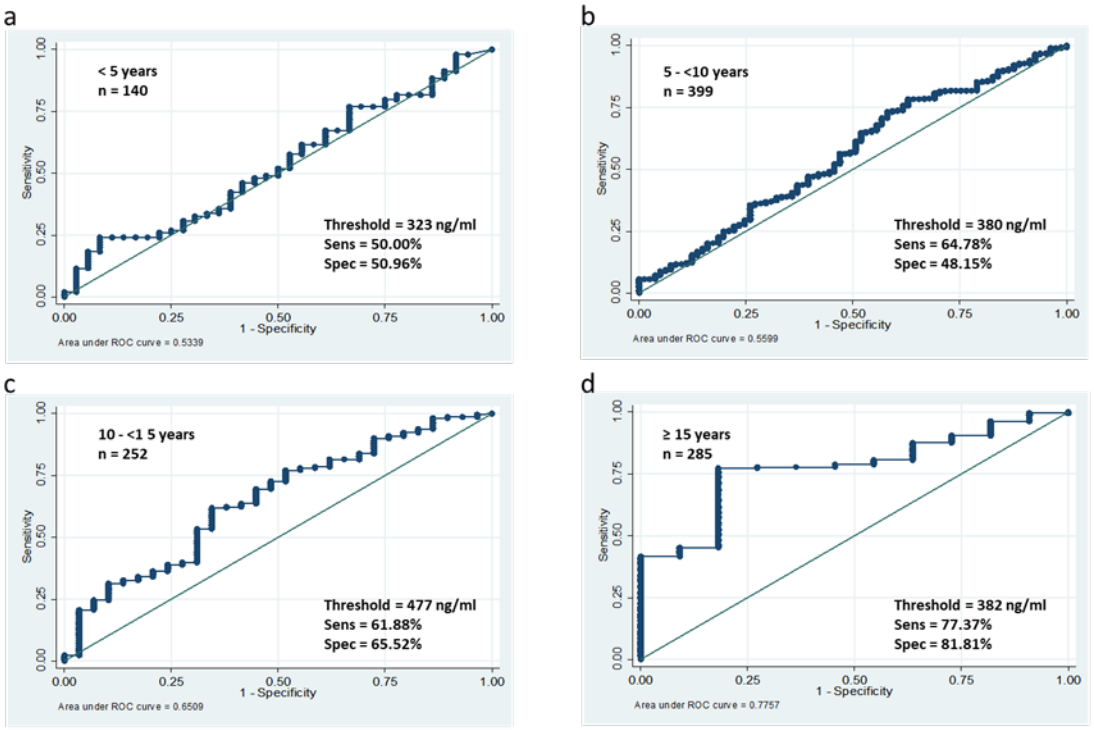
Lumefantrine day 7 concentration in under five years' age group was lower for protection against new infection at day 28 of follow-up. Except, in under five years age group, I found a statistically significant association between lumefantrine day 7 concentration and protection from recurrent parasite by day 28 in the other age groups. These associations were still present after normalization of the concentration per dose and body weight in one hand, and on another hand, they were still significant after adjusting for covariates by multiples episodes and multiples variables, Cox regression model. The association between lumefantrine day 7 concentration and

protection from recurrence by day 28 was stronger as the age increase (Cox regression model, HR = 0.999, 95% CI: [0.997 – 1.000], p = 0.228 for children less than five years old, HR = 0.998, 95% CI: [0.997 – 0.999], p = 0.028 for age group 5 - <10 years, HR = 0.996, 95% CI: [0.993 – 0.998], p = 0.003 for age group 10 - <15 years, and HR = 0.996, 95% CI: [0.994 – 0.998], p = 0.001 for age group higher or equal to 15 years, after adjusting for site, age in each group, transmission season at inclusion and initial parasite count before treatment).

As shown in Figure 27, the best lumefantrine day 7 concentration cut-off for prediction of parasite recurrence before day 28 was 381 ng/mg with a sensitivity and specificity of 52.42% and 56.70% respectively. Because of this low sensitivity and specificity, I look at the cut-off by age group. I found an improvement in the sensitivity and specificity as the age increase (Figure 28), with the best sensitivity and specificity in higher age group (more than 15 years).



<u>Figure 27:</u> Lumefantrine day 7 concentration threshold for prediction of parasite recurrence at 28 days of follow-up



<u>Figure 28</u>: Lumefantrine day 7 concentration threshold for prediction of parasite recurrence at 28 days of follow-up by age group.

Lumefantrine day 7 concentration in under 5 years' patients is too low to protect them against parasite recurrence by day 28 of follow-up. The sensitivity and specificity for prediction of parasite recurrence by day 28 with lumefantrine day 7 concentration threshold improved as the age increase, with the best sensitivity and specificity in age group higher or equal to 15 years.

3.5.4. Multivariable and multivariate analysis of factors associated with parasite recurrence

In the AL arm, lumefantrine day 7 concentration was significantly associated with protection against recurrent parasite by day 28 and 42 after univariate and multivariate Cox modelling analysis and after adjustment for significant cofactors (site, age, season at inclusion and initial parasitaemia). The extent of the implication of lumefantrine day 7 concentrations in protection against recurrence decreased from day 28 to day 42 and disappeared after 42. At day 63 there was no association between lumefantrine day 7 concentrations and protection against recurrence. By day 28 each increase of the natural log of the lumefantrine day 7 concentration had about

40% lower hazard rate to have recurrence while by day 42 each increase of the natural log of the lumefantrine day 7 concentration had about 21% less likely to have a recurrence in Cox multivariable multivariate modelling (Table 21 and 23).

In the ASAQ arm, DEAQ day 7 concentration was not associated with parasite recurrence by day 28 after univariate analysis at episode 1. However, it was associated with parasite recurrence after multivariate analysis. These associations disappeared by day 42 of follow-up (Table 22 and 24). The observed association after multivariate Cox regression analysis may be due to the accumulation of DEAQ in the study population after subsequent treatment during 45 days.

Table 21: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 28 in AL arm (n = number of recurrence; N = total number of episodes)

Variable	Failure by day 28 (n/N)	HR (95% CI)	*p*- value
Univariate model			
For episode 1			
Lumefantrine concentration (natural log)	74/577	0.523 (0.417 – 0.656)	<0.001
Study site			
Sotuba	17/258	(ref)	
Bougoula	26/213	1.855 (1.018 – 3.383)	0.044
Kollé	37/199	2.883 (1.644 – 5.053)	<0.001
Age	80/670	0.908 (0.871 – 0.945)	<0.001
Season at inclusion			<0.001
December - May (low transmission)	0/92	(ref)	
June - November (high transmission)	80/578	$3.36\ 10^{16}$	
Sex			0.916
Male	44/374	(ref)	
Female	36/296	1.023 (0.667 – 1.569)	
Pf day 0 parasitaemia (log10)	80/670	2.020 (1.310 – 3.113)	0.001
Day 0 temperature	80/670	1.037 (0.861 – 1.248)	0.702
For multiple episodes			
Lumefantrine concentration (natural log)	132/1075	0.516 (0.437 – 0.608)	<0.001
Study site			
Sotuba	24/475	(ref)	
Bougoula	104/722	2.86 (1.790 – 4.561)	<0.001
Kollé	75/421	3.634 (2.278 – 5.798)	<0.001
Age	203/1618	0.894 (0.867 – 0.922)	<0.001
Season at inclusion			<0.001
December - May (low transmission)	1/233	(ref)	
June - November (high transmission)	202/1385	35.61 (5.11 – 248.17)	
Sex			0.836
Male	116/914	(ref)	
Female	87/704	0.969 (0.719 – 1.305)	
Pf day 0 parasitaemia (log10)	203/1618	1.593 (1.268 – 2.000)	<0.001
Day 0 temperature	203/1618	1.031 (0.919 – 1.157)	0.597

Table 21 continues on next page

Table 21 continues

Multivariable model			
For episode 1			
Lumefantrine concentration (natural log)	74/577	0.636 (0.493 – 0.820)	<0.001
Study site			
Sotuba	17/220	(ref)	
Bougoula	25/190	1.682 (0.914 – 3.098)	0.095
Kollé	32/167	1.824 (1.008 – 3.299)	0.045
Age	74/577	0.955 (0.920 – 0.991)	0.012
Season at inclusion			<0.001
December - May (low transmission)	0/63	(ref)	
June - November (high transmission)	74/514	$3.12\ 10^{15}$	
Pf day 0 parasitaemia (log10)	74/577	1.458 (0.964 – 2.205)	0.074
For multiple episodes			
Lumefantrine concentration (natural log)	132/1075	0.607 (0.499 – 0.739)	<0.001
Study site			
Sotuba	22/348	(ref)	
Bougoula	47/399	1.691 (1.011 – 2.829)	0.042
Kollé	63/328	2.104 (1.273 – 3.475)	0.004
Age	132/1075	0.946 (0.914 – 0.979)	<0.001
Season at inclusion			<0.001
December - May (low transmission)	0/146	(ref)	
June - November (high transmission)	132/929	$5.20\ 10^{14}$	
Pf day 0 parasitaemia (log10)	132/1075	1.178 (0.912 – 1.521)	0.209

Table 22: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 28 in ASAQ arm (n = number of recurrence; N = total number of episodes)

Variable	Failure by day 28 (n/N)	HR (95% CI)	*P* value
Univariate model			
For episode 1			
DEAQ concentration (ng/ml)	10/151	0.988 (0.967 – 1.009)	0.282
Age	10/224	1.025 (0.873 – 1.202)	0.764
Season at inclusion			0.017
December - May (low transmission)	1/128	(ref)	
June - November (high transmission)	9/96	12.20 (1.561 –95.37)	
Sex			0.280
Male	7/119	(ref)	
Female	3/105	0.479 (0.126 – 1.818)	
Pf day 0 initial parasitaemia (log10)	10/224	1.917 (0.946 – 3.885)	0.071
Day 0 temperature	10/224	0.898 (0.529 – 1.525)	0.691
For multiple episodes			
DEAQ concentration (ng/ml)	64/740	0.992 (0.987 – 0.999)	0.026
Age	77/1002	0.928 (0.861 – 0.999)	0.048
Season at inclusion			<0.001
December - May (low transmission)	6/246	(ref)	
June - November (high transmission)	71/756	3.971 (1.835 – 8.593)	
Sex			0.014
Male	56/567	(ref)	
Female	21/435	0.482 (0.270 – 0.862)	
Pf day 0 initial parasitaemia (log10)	77/1002	0.895 (0.738 – 1.086)	0.262
Day 0 temperature	77/1002	0.788 (0.648 – 0.959)	0.018
Multivariable model			
For multiple episodes			
DEAQ concentration (ng/ml)	64/740	0.992 (0.986 – 0.998)	0.011
Age	64/740	0.918 (0.845 – 0.998)	0.046
Season at inclusion			0.013
December-May (low transmission	5/149	(ref)	
June-November (high transmission)	59/591	3.047 (1.305 – 7.113)	
Sex			0.064
Male	45/432	(ref)	
Female	19/308	0.569 (0.312 – 1.037)	
Pf day 0 parasitaemia (log10)	64/740	0.884 (0.684 – 1.141)	0.343
Day 0 temperature	64/740	0.789 (0.610 – 1.022)	0.070

Table 23: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 42 in AL arm (n = number of recurrence; N = total number of episodes; ref = reference)

Variable	Failure by day 42 (n/N)	HR (95% CI)	*P* value
Univariate model			
For episode 1			
Lumefantrine concentration (natural log)	173/577	0.667 (0.550 – 0.810)	<0.001
Study site			
Sotuba	34/258	(ref)	
Bougoula	69/213	2.681 (1.785 – 4.025)	<0.001
Kollé	84/199	3.715 (2.507 – 5.506)	<0.001
Age	187/670	0.941 (0.915 – 0.967)	<0.001
Season at inclusion			<0.001
December - May (low transmission)	2/92	(ref)	
June - November (high transmission)	185/578	17.05 (4.263 – 68.19)	
Sex			0.892
Male	103/374	(ref)	
Female	84/296	1.019 (0.769 – 1.351)	
Pf day 0 parasitaemia (log10)	187/670	1.432 (1.152 – 1.781)	0.001
Day 0 temperature	187/670	1.007 (0.889 – 1.141)	0.908
For multiple episodes			
Lumefantrine concentration (natural log)	302/1075	0.690 (0.595 – 0.800)	<0.001
Study site			
Sotuba	54/475	(ref)	
Bougoula	222/722	2.937 (2.163 – 3.987)	<0.001
Kollé	182/421	4.435 (3.274 – 6.008)	<0.001
Age	458/1618	0.936 (0.913 – 0.959)	<0.001
Season at inclusion			<0.001
December - May (low transmission)	12/233	(ref)	
June-November (high transmission)	446/1385	7.361 (3.323 – 16.31)	
Sex			0.901
Male	256/914	(ref)	
Female	202/704	1.013 (0.823 – 1.247)	
Pf day 0 parasitaemia (log10)	458/1618	1.336 (1.175 – 1.520)	<0.001
Day 0 temperature	458/1618	1.021 (0.937 – 1.112)	0.633

Table 23 continues on next page

Table 23 continues

Multivariable model			
For episode 1			
Lumefantrine concentration (natural log)	173/577	0.792 (0.643 – 0.976)	0.029
Study site			
Sotuba	33/220	(ref)	
Bougoula	63/190	2.755 (1.815 – 4.183)	<0.001
Kollé	77/167	3.042 (2.021 – 4.579)	<0.001
Age	173/577	0.976 (0953 – 1.000)	0.051
Season at inclusion			0.002
December - May (low transmission)	1/63	(ref)	
June - November (high transmission)	172/514	23.31 (3.269 – 166.2)	
Pf day 0 parasitaemia (log10)	173/577	1.161 (0.935 – 1.441)	0.176
For multiple episodes			
Lumefantrine concentration (natural log)	302/1075	0.797 (0.677 – 0.938)	0.006
Study site			
Sotuba	43/348	(ref)	
Bougoula	107/399	2.575 (1.819 – 3.646)	<0.001
Kollé	152/328	3.761 (2.671 – 5.295)	<0.001
Age	302/1075	0.974 (0.948 – 1.000)	0.058
Season at inclusion			<0.001
December-May (low transmission)	5/146	(ref)	
June-November (high transmission)	297/929	51.17 (7.097 – 368.9)	
Pf day 0 parasitaemia (log10)	302/1075	1.090 (0.944 – 1.259)	0.238

Table 24: Cox univariate and multivariate modelling of factors associated with risk of parasite recurrence by day 42 in ASAQ arm (n = number of recurrence; N = total number of episodes)

Variable	Failure by day 42 (n/N)	HR (95% CI)	*P* value
Univariate model			
For episodes 1			
DEAQ concentration (ng/ml)	42/151	0.995 (0.987 – 1.004)	0.295
Age	44/224	1.006 (0.933 – 1.083)	0.881
Season at inclusion			<0.001
December - May (low transmission)	14/128	(ref)	
June - November (high transmission)	30/96	3.326 (1.805 – 6.130)	
Sex			0.748
Male	24/119	(ref)	
Female	20/105	0.909 (0.511 – 1.619)	
Pf day 0 initial parasitaemia (log10)	44/224	1.259 (0.932 – 1.702)	0.134
Day 0 temperature	44/224	1.014 (0.793 – 1.296)	0.919
For multiple episodes			
DEAQ concentration (ng/ml)	235/741	1.000 (0.998 – 1.002)	0.773
Age	293/1002	0.971 (0.933 – 1.012)	0.169
Season at inclusion			<0.001
December - May (low transmission)	22/246	(ref)	
June - November (high transmission)	271/756	4.754 (3.114 – 7.256)	
Sex			0.009
Male	187/567	(ref)	
Female	106/435	0.690 (0.523 – 0.910)	
Pf day 0 initial parasitaemia (log10)	293/1002	0.994 (0.888 – 1.114)	0.926
Day 0 temperature	293/1002	0.868 (0.784 – 0.962)	0.007
Multivariable model			
For multiple episodes			
Season at inclusion			<0.001
December - May (low transmission)	22/246	(ref)	
June-November (high transmission)	271/756	4.744 (3.094 – 7.275)	
Sex			0.005
Male	187/567	(ref)	
Female	106/435	0.677 (0.517 – 0.887)	
Day 0 temperature	293/1002	0.868 (0.786 – 0.959)	0.005

3.5.5. N-desethylamodiaquine day 7 concentration was positively correlated with adverse events

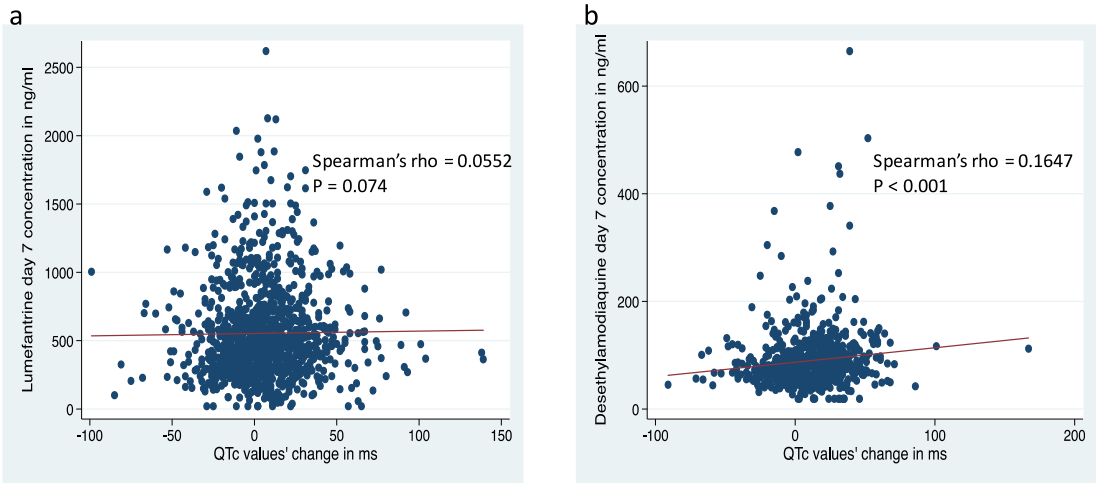
The effects of day 7 concentration of lumefantrine and DEAQ on measures of hepatotoxicity and cardiotoxicity was assessed.

The change in Fridericia-corrected QTc from day 0 (before treatment) to day 2 (4 to 6 hours after the last dose) was positively correlated with DEAQ concentration. As this change increased, the concentration of DEAQ increased too as shown on (Figure 29. b). The correlation was statistically significant $p < 0.001$.

For lumefantrine, the correlation was not statistically significant even if there is a trend (Figure 29. a).

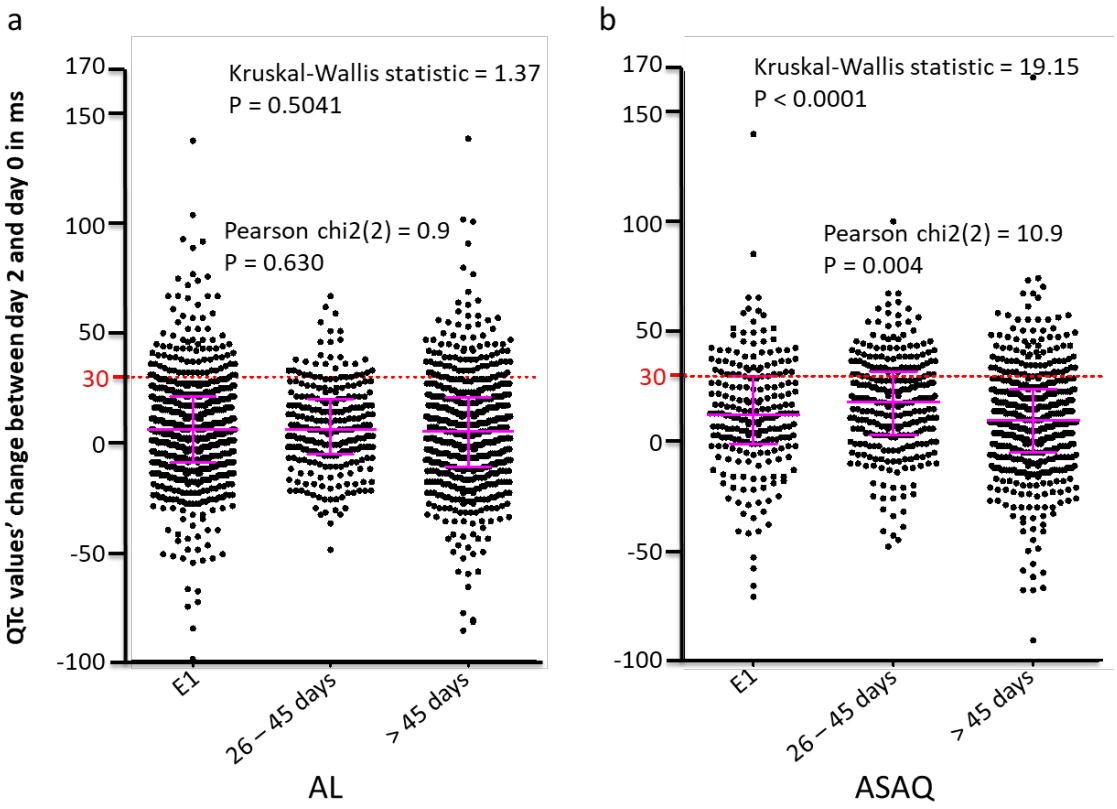
To consolidate this implication of DEAQ day 7 concentration in QTc prolongation, I also looked at the median change in different categories of patients according to the time of retreatment. As DEAQ accumulates in the study population when patients received a subsequent treatment between 26 – 45 days, the median change of QTc was higher in patients receiving a subsequent treatment between 26 – 45 days (median (IQR) 18 (3 – 32.25) and mean (95% CI) 17.12 (14.51 – 19.74)) compared to patients at first episode (median (IQR) 12.5 (-1 – 30) mean (95% CI) 12.52 (9.08 – 16.07)) or patients receiving subsequent treatment after 45 days (median (IQR) 10 (-5 – 24) and mean (95% CI) 9.62 (7.35 – 11.89)) (Kruskal-Wallis' $P < 0.0001$). Following the same line, when I considered QTc change higher than 30 milliseconds as abnormal, I found a higher proportion of abnormal QTc in patients receiving subsequent treatment of ASAQ between 26 – 45 days (Pearson's $p = 0.004$) (Figure 30. b).

In the AL arm, there was no correlation between median QTc change and lumefantrine day 7 concentration. There was also no association between median QTc change and time frame of AL ingestion. The median change of QTc in patients receiving a subsequent treatment between 26 – 45 days was (median (IQR) 6 (-5.25 – 20.00) and mean (95% CI) 6.97 (4.59 – 9.34)) compared to patients at first episode (median (IQR) 6 (-9 – 21) mean (95% CI) 6.4 (4.44 – 8.36)) or patients receiving subsequent treatment after 45 days (median (IQR) 5 (-11 – 20.75) and mean (95% CI) 5.24 (3.23 – 7.25)) (Kruskal-Wallis' $P = 0.5041$). There was also no difference in abnormal QTc proportion among patients receiving subsequent dose of AL between 26 -45 days compared to the others (Pearson's $P = 0.630$) (Figure 30 a)



<u>Figure 29</u>: Positive correlation between drugs concentration QTc change.

No correlation with lumefantrine day 7 concentration (a), positive correlation with DEAQ day 7 concentration (b). QTc change represents change in QTc value from day 0 (before treatment) to day 2 (4 to 6 hours after the last dose), at each episode

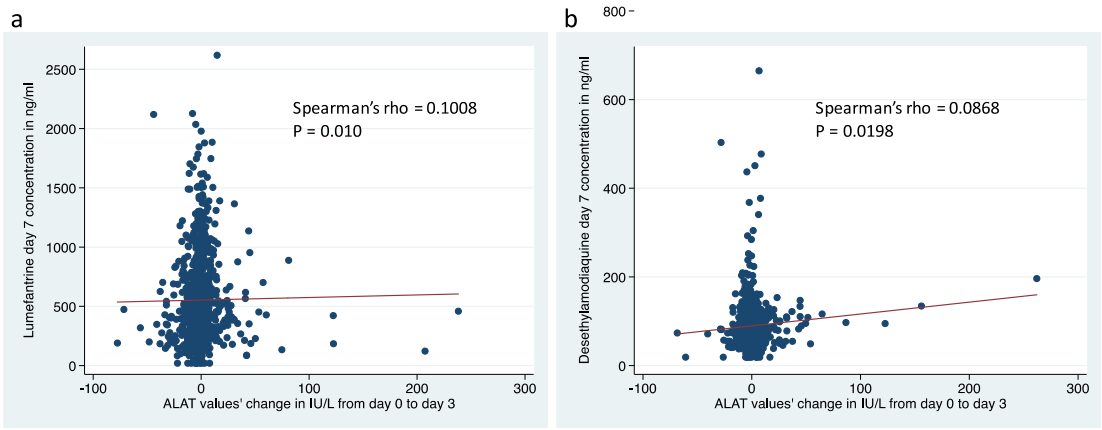


<u>Figure 30</u>: Median QTc change and proportion of QTc change higher than 30 ms in each treatment arm.

Median QTc change was higher in patients receiving subsequent treatment of ASAQ between 26 and 45 days compared to QTc change at first episode or QTc change in patients receiving subsequent treatment after 45 days (Kruskal-Wallis' $P < 0.0001$). In AL arm there was no difference (Kruskal-Wallis' $P = 0.5041$). The proportion of patients with QTc change > 30 ms was higher in patients receiving subsequent ASAQ treatment between 26 – 45 days compared to patients at first episode or patients receiving subsequent treatment after 45 days (Pearson's $P = 0.004$). In AL arm there was no difference (Pearson's $P = 0.630$). QTc change represents change in QTc value from day 0 (before treatment) to day 2 (4 to 6 hours after the last dose) at each episode

The second adverse event of interest I looked at was day 7 concentration of lumefantrine and DEAQ on alanine aminotransferase change. This change was defined as the ALAT change from day 0 before treatment to day 3. There was a statistically significant positive correlation between this change and day 7 concentration of lumefantrine (P = 0.010) and DEAQ (P = 0.0198) (Figure 31).

These implications were reinforced when I considered outputs in Table 25 for DEAQ but not for lumefantrine. Patients, who received the subsequent treatment of ASAQ between 26 and 45 days and having abnormal ALAT at day 3 had a higher day 7 concentration of DEAQ compared to others categories (Chi2 = 37.831, P = 0.0001). In AL arm, lumefantrine day 7 concentration didn't vary significantly between different categories.



<u>Figure 31</u>: Positive correlation between day 7 drugs concentration and ALAT change.

(a) lumefantrine, (b) DEAQ. ALAT change represents change in ALAT value from day 0 (before treatment) to day 3 (one day after the last dose)

Table 25: Effect of day 7 concentration on ALAT. Patients, who received a subsequent treatment of ASAQ between 26 – 45 days and had elevated ALAT had a higher day 7 concentration of DEAQ when compared to other categories. There was no difference between lumefantrine day 7 concentrations by ALAT and treatment period categories. E1 represents initial treatment at first episode, (26 – 45 days) represents patients who received a subsequent treatment between 26 and 45 days, and (>45 days) represents patients who received subsequent treatment after 45 days.

Category	Artemether-lumefantrine		Artesunate-amodiaquine	
ALAT status - subsequent treatment period	N	Day 7 lumefantrine median (IQR) in ng/ml	n	Day 7 DEAQ median (IQR) in ng/ml
Normal ALAT – E1	534	487.1 (309.8 – 704,8)	141	71.5 (57.1 – 93.3)
Normal ALAT – (26 – 45 days)	181	524.3 (346.5 – 781.5)	220	91.9 (70.1 – 115. 6)
Normal ALAT – (>45 days)	293	483.6 (295.8 – 685.7)	323	74.5 (54.2 – 98.9)
Abnormal ALAT – E1	16	423.6 (198.4 – 788.1)	0	-
Abnormal ALAT – (26 – 45 days)	2	810.7 (668.2 – 953.3)	6	112.5 (95.2 – 121.8)
Abnormal ALAT – (>45 days)	5	459.0 (86.5 – 566.3)	16	96.1 (79.0 – 117.8)
Total	1031	Chi2 = 9.065 P = 0.1065	706	Chi2 = 37.831 p = 0.0001

4 Discussion

My study shows the implication of day 7 concentrations of lumefantrine, and desethylamodiaquine, in parasite recurrence and drug-related safety after repeated treatment with the same ACT during a period of two years of follow-up

The major finding of this study was the accumulation of DEAQ after retreating with ASAQ over a short period (26 to 45 days) in the study population. The study population was consisted of all of the population of the study sites with microscopically confirmed acute, uncomplicated *Plasmodium sp* malaria. Accumulation of DEAQ had safety implications in that it was associated with an increase in the incidence of adverse events. A second major finding was that day 7 DEAQ concentration was not associated with treatment outcome, which compared to findings with lumefantrine. A third major finding was confirmation, in a large sample size spanning all of the age groups (< 5 and ≥ 5 years), of the strong and independent effect of lumefantrine day 7 concentrations on protection from new malaria infection by day 28. This independent effect of lumefantrine day 7 concentration on parasite recurrence was still present at day 42 but was less pronounced when compared to the protective effect observed at day 28. The effect was not detectable at day 63. The protection observed was lumefantrine concentration dependent; with concentrations below 381 ng/ml proving not effective against subsequent infection by day 28. The majority of lumefantrine concentrations in children aged <5 years (60%) was below this 381 ng/ml protective threshold, explaining at least in part why lumefantrine day 7 concentrations in patients in this age group did not reflect the findings of a protective effect observed at day 28 in older patients.

Clinical study findings

Patients aged 5 to 9 years old were the most represented age group in the DHA-PPQ, ASAQ and AL groups, comprising 42.19%, 52.23%, and 33.98% of each treatment arm, respectively.

In the PA arm, 35.19% of the participants were ≥14 years; due to the fact that randomization of this treatment arm was initiated in adult patients. Children were included in this treatment arm after an interim analysis. The proportion of adult patients was also high in the AL arm (27.42%), which served as the comparison group for the PA arm. Except for the DHA-PPQ arm, where the sex ratio was around one, a

lower proportion of female compared to male participants were included in the PA, ASAQ and AL arms.

The results of this study showed that after approximately 10 years of use in Mali, ACTs are still almost 100% efficacious based on molecular correction against reinfection in this test study population. This finding is in agreement with previous studies, reporting an efficacy ≥95% at day 28 of follow-up [104-106, 294]. The focus of this study when compared to others was the repeated treatment for subsequent episodes of malaria with the same drug over a two-year period without age restriction. The ACTs used (DHA-PPQ, PA, ASAQ and AL) conserved their efficacy by day 28 after molecular correction against reinfection and after treatment of subsequent episodes with the same drug over the two year study period. In contrast to South-Asian countries where resistance to artemisinin and its derivatives and in some cases to their partner drugs is increasing, in Africa, particularly in West Africa there is no resistance to ACTs. It is already known that resistance to both CQ and sulfadoxine-pyrimethamine spread from Southeast Asia to Africa, occurring first in East Africa before reaching West Africa. There is a concern that ACT resistance will also display the same pattern of spread. Fortunately, to date there is no clear sign of ACT resistance in East Africa. Reassuring data from Uganda demonstrated that repeat treatment with the same drug in young children [295] was associated with a high efficacy of AL and DHA-PPQ with low numbers of recrudescence cases by day 28. In the study reported in this thesis, the number of recrudescence cases was also very low with only 3 and 7 cases reported in the PA and AL arms, respectively.

Exploring the function of the partner drugs of artemisinin and its derivatives in protecting against early reinfection or recurrence, I studied the parasite recurrence by day 28 or 42. I found that recurrence rate was higher in AL and ASAQ arm than DHA-PPQ and PA. Of note, the DHA-PPQ arm was the only arm that exhibited a lower rate of recurrent parasites by day 28 or 42. This finding was in agreement with previous studies showing a lower proportion of recurrence with DHA-PPQ compared to AL or ASAQ. This lower proportion of recurrence by day 28 or 42 with DHA-PPQ may be explained by the long half-life of piperaquine, which is around 4 weeks. After complete elimination of piperaquine from the blood or a reduction in concentration below its minimal inhibitory concentration, the recurrence of parasites in DHA-PPQ increased and reached the same levels as in the other treatment arms. It seems that

piperaquine, during its post-treatment prophylactic period, does not eliminate parasites so much as delay their rapid reappearance.

The high recurrence level observed by day 28 or 42 in each treatment arm may also be explained by the endemicity of malaria in study sites. Regardless of treatment arm, Sotuba, which is the peri-urban village of Bamako the capital city of Mali, with its low endemicity (malaria exposure), had the lower proportion of parasite recurrence by day 28 or 42 as compared to Bougoula-Hameau and Kollé. Other factors which played a role in parasite recurrence were the season at time of study inclusion and the patient's age. The level of recurrence by day 28 or 42 was lower during the dry season (December – May) in each treatment arm regardless of the site when compared to the high transmission season (June – November). During the dry season malaria exposure is very low. Similar observations were reported by Sagara *et al*. in Bougoula Hameau between 2005 to 2007 [104]. These authors found a seasonal variation in the parasite recurrence after treatment with ACTs with lower recurrence by day 28 recorded during the dry season (January – March).

The safety profile of different treatment arms was evaluated by examining the drug's effects on the liver function and the heart. For the liver function test, it was found that the level of abnormal ALAT was two times higher in PA arm as compared to the other treatment arm. This observation was previously described in another report, where they found a four-fold rise in the occurrence of ALAT and ASAT grade 3 or 4 toxicity in PA arm as compared to other ACTs[296]. In my study, PA was re-administered to more than 67% of the patients, among which some received it 9 times during the two-year follow-up period. However, an interim analysis on PA re-treatment did not find any difference in the level of hepatotoxicity between initial treatment and re-treatment group [105]. These ALAT elevations were transient, without clinical manifestations and became normal before the end of the 28 days' follow-up.

In the ASAQ arm, I found the lowest rate of abnormal ALAT. I can explain this by the age of the participants in this treatment arm. Hepatotoxicity due to AQ has generally been described in adult's patients and after its use in prophylaxis [140, 297, 298]. In children, most of the studies did not find any hepatotoxicity after treatment with ASAQ [299, 300].

The second adverse event analysed during this sub-study was the cardiotoxicity through the measurement of QTc. Almost one-third of patients in DHA-PPQ arm experienced some QTc lengthening defined as a QTc's change from day 0 to day 2 above than 30 ms. In ASAQ arm the proportion of patient with QTc prolongation was 22.05%. AL and PA had lower proportions with respectively 12.74% and 8.60%. These finding corroborate with published data. These QTc prolongations were transient without clinical signs. Quinoline antimalarial drugs are known to have an impact on QT interval; some prolong the QRS wave while others prolong JT or both. Quinidine, the diastereoisomer of quinine is an example of drugs prolonging the QT interval [301]. The second antimalarial drug inducing a QT prolongation associated sometimes with torsades de pointes is Halofantrine [302, 303]. Both contain the quinoline moiety like piperaquine, which is a bisquinoline and AQ. The high proportion of QTc prolongation in DHA-PPQ arm compared to ASAQ arm may be due to the presence of two quinoline moieties in piperaquine. Other similar studies showed a significant lengthening of the mean QTc in DHA-PPQ arm but without significant cardiac effects [304, 305]

Many factors can affect QT interval, among which malaria status, age, gender, electrolytes concentrations and some classes of drugs[306]. The contribution of these factors is minimised by randomization of treatment arms.

Pharmacokinetic and pharmacodynamics

To study the relationship between *in vivo* results (efficacy and safety) and drug concentration, we developed and validated or revalidated high performance liquid chromatographic methods to quantify lumefantrine and DEAQ in plasma collected from uncomplicated malaria patients treated with AL or ASAQ. The analytical methods developed were able to quantify day 7 concentration of lumefantrine and DEAQ in plasma accurately.

For pyronaridine, the quantification method developed and validated had a low limit of quantification set at 25 ng/ml and a limit of detection of 7 ng/ml, and was not able to quantify drug concentration in malaria patients' plasma sample, 4 days after the last dose. In the literature, pyronaridine day 7 concentration in whole blood was estimated to be around 37 ng/ml[307, 308]. Moreover, some studies, most often in rabbits showed that pyronaridine concentrates in blood cells, with blood: plasma ratio varying from 4.9 to 17.8 in rabbits [161, 164].

For lumefantrine quantification, the LLOQ (20 ng/ml) was far lower than the mean concentration of this drug in plasma at day 7 [207, 309].

The methods for lumefantrine and DEAQ quantification were sensitive, specific, reproducible, and accurate. They have been validated according to FDA guidance. Moreover, the accuracies and coefficients of variation obtained with the QCs samples during patients' samples analysis (Table 17) showed that it is a good method for clinical studies.

The DEAQ method of detection was fully revalidated. It was a sensitive, specific, reproducible, and accurate method for quantification of DEAQ in plasma. The LLOQ set at 24 ng/ml showed that the method was sensitive to quantify DEAQ in patients' samples at day 7 of follow-up (4 days after the last dose). The median day 7 plasma concentration of DEAQ in African children with malaria treated with ASAQ is estimated to be around 91.5 ng/ml [144], which is far higher than the LLOQ of my method. As shown by the accuracy and coefficient of variation of QCs samples during patients' samples analysis (Table 18), it is a good method for clinical studies.

The methods I developed and validated to quantify lumefantrine, DEAQ and pyronaridine in human plasma gave excellent linearity. Calibrations curve's coefficients of correlation were always higher than 0.997 (Figure 17).

The internal standards used (TA3099 for lumefantrine and (4-((7-chloro-4-quinolinyl) amino)-1-Pentanol) for DEAQ and pyronaridine) are structurally related to the drugs and are not usually found in patients' blood. Because they are not drugs to treat any human diseases, this makes them appropriate for quantification. They also did not interfere with any other peaks coming from endogenous or concomitant treatment or any other antimalarial drugs. They had a good extraction recovery more than 75% and stable in the analytical conditions.

I found a high inter-individual variability in day 7 plasma concentration of lumefantrine and DEAQ. The median lumefantrine day 7 concentration with interquartile range at first episode was 477 (304.7 to 701) ng/ml (mean (SD) = 543.1(329.1)), for DEAQ it was 71.3 (57.1 to 93.3) ng/ml (mean (SD) = 88.8 (65.2) ng/ml). These concentrations were comparable to data published elsewhere [310]. During the two years period of follow-up of this study, some patients suffered more than 10 episodes of uncomplicated malaria, and they always received the same treatment if the time period between the following episodes was equal or superior to

28 days. When I compared day 7 drug concentrations between episodes, I found similar concentration between the different episodes. I did not find any increase in lumefantrine or DEAQ day 7 concentration episode by episode. However, when I compared the concentration of the drug at episode 1 to its concentration in patients who received a subsequent treatment, I found a high concentration of DEAQ in patients who received a subsequent treatment between 26 and 44 days ($p < 0.0001$). This finding suggests that, when patients received a second treatment with ASAQ in a period between 26 to 44 days, there will be an accumulation of DEAQ in those patients' blood (Figure 19 b). I did not find any accumulation of lumefantrine in my study population regardless of the time of the subsequent treatment after 26 days of the precedent treatment. This accumulation of DEAQ in study population has to be considered in places where AQ is use in IPTi and SMC[8]. If DEAQ is involved in any of the adverse events (cytotoxicity, hepatotoxicity or cardiotoxicity)[311, 312], this accumulation may exacerbate it.

Lumefantrine day 7 concentration increases with age in my study population. I found a high day 7 concentration of lumefantrine in above 15 years' age group. The concentration I found in this age group was comparable to the concentration found in 10 - <15 years age group but higher than the concentration in 5 - <10 and under five years' age group ($p < 0.0001$). The lower lumefantrine day 7 concentration was recorded in under five year's age group. This profile of lumefantrine day 7 concentration may be explained by the activity of the CYP3A4, which is the primary enzyme implicated in lumefantrine metabolism. This CYP3A4 is more active in 1 to 12 years old children than adult [184, 313, 314]. My findings is in agreement with other studies indicating an increase of lumefantrine disposition with age [207]. Unlike lumefantrine, DEAQ concentration didn't exhibit any correlation with age group ($p = 0.1803$).

Lumefantrine day 7 concentration varied also significantly between sexes. It was lower in female patients than male ($p = 0.0093$). This difference was statistically significant between under 5 years male and female. It may also be explained by the expression profile of the CYP3A4, which is higher in female than in male [185]. As for age groups, DEAQ day 7 concentration didn't vary significantly with sex ($p = 0.1598$).

Both lumefantrine and DEAQ day 7 concentrations were lower in patients with fever (axillary temperature equal or higher than 37.5°C) at the start of the treatment compared to patients without fever.

Regarding the pharmacokinetic pharmacodynamics relationship of lumefantrine and DEAQ on recurrent parasite or post-treatment prophylaxis after repetitive treatment with AL or ASAQ, I found that lumefantrine day 7 concentration was lower in patients with a subsequent episode of malaria by day 28 ($p < 0.0001$) and day 42 ($p = 0.0102$) of follow-up when compared to patients without a subsequent episode. The median concentration was 66 % higher ($p <0.0001$) in patients without recurrent parasite by day 28 as compared to those with recurrent parasitaemia. For DEAQ, there was no difference in its day 7 concentration between patients with or without recurrent parasite by day 28 and day 42 of follow-up. This implication of lumefantrine concentration in post-treatment prophylaxis is strenghten by its high association at day 28 of follow-up ($p < 0.0001$), which was diminished by day 42 of follow-up ($p = 0.0102$) and was no longer detectable at day 63. This association is confirmed with Cox proportional hazard multivariable and multiple episodes modelling, which showed good correlation between lumefantrine day 7 concentrations and patients protection against new episode of malaria before day 28 of follow-up. Why lumefantrine day 7 concentration impacts on parasite recurrence by day 28 and 42, but not DEAQ day 7 concentration? There is no clear answer to this question. However, a study showed that CQ (same family like AQ and DEAQ) efficacy is time dependent (time above minimum inhibitory concentration) [315]. If that apply to DEAQ, which minimum inhibitory concentration did not reach 28 days post treatment, may explain why its concentration at day 7 did not impact on parasite recurrence by 28 days of follow-up. Lumefantrine and quinine belong to the same family. A study showed that quinine efficacy is concentration and time dependent [316] and if that apply to lumefantrine, it may explain its day 7 concentrations effect on parasite recurrence. Low day 7 lumefantrine concentration has been involved in parasite recrudescence to AL [202, 203]. In my study, parasites recrudescent level was very low. There was no case of recrudescence in the ASAQ arm. In the AL arm, we found 7 cases of recrudescence. In these 7 cases, day 7 lumefantrine concentrations were available for 6 cases and unavailable for one case. Within the six available cases, a 10 year old patient did not eliminate his parasite burden. He came

with parasites at day 1, 2, 3, 7 and 27 of follow-up and there was no detectable lumefantrine in his blood at day 7 of follow-up. After investigation of this last patient, it was found that he was keeping drugs under his tongue and spat them out when far from the study team. This particular patient showed the importance of pharmacokinetic in monitoring drug efficacy. All patients, except one, who had parasite recrudescence had day 7 concentrations of lumefantrine lower than the low interquartile concentration. This is in agreement with previous reported data linking parasite recrudescence to low lumefantrine day 7 concentration [207].

I found collinearity between lumefantrine day 7 concentration and AL 28 days uncorrected efficacy by age group. The lower lumefantrine day 7 concentrations and the lower 28 days uncorrected efficacy rates were observed in under five years' age group, and those higher were found in 15 years old and above age group.

The lowest lumefantrine day 7 concentrations were observed in under five years' female children as compared to under five years' male children ($p < 0.01$). This difference may be explained by the high activity of the metabolism enzyme CYP3A4 in female than male [185].

Lumefantrine day 7 concentration of 175 ng/ml and 280 ng/ml were used as cut-off values for prediction of parasite recrudescence to AL [202, 203]. In my study, the level of recrudescence was very low. We tried to look at these cut-off values of lumefantrine day 7 concentrations, which could predict parasite recurrence at day 28 of follow-up using the receiver operating characteristic (ROC curve) statistics. Lumefantrine day 7 concentration threshold, which can predict parasite recurrence at day 28 of follow-up, was estimated to 381ng/ml with a sensitivity of 52.4% and specificity of 56.7%. Because of these low sensitivity and specificity, we looked at the threshold in age group. Sensitivity and specificity increase as age increase with the best sensitivity and specificity in the highest age group. This finding was surprising because higher age groups had high lumefantrine concentration and the malaria premunition should also play a role in the post-treatment prophylaxis in this age group in malaria endemic areas. Participants in the lower age groups should be those that give the best sensitivity and specificity because of their lower drug concentration and their lower premunition. However, it is already known that drug efficacy depends on the proportion of the free drug in the blood (unbound to plasma

proteins). It is unknown if there is a difference in the percentage of free lumefantrine concentration between these age groups to explain this phenomenon.

DEAQ concentration has been shown to be associated with parasite recurrence. The strong association was described with day 3 concentration of DEAQ after treatment with AQ in monotherapy [317]. At day 3 the inter-individual variability was high compared to day 7, and concentration at day 3 did not correlate well with AUC, which is the best pharmacokinetic parameter for the determination of treatment outcome in uncomplicated malaria. Another study showed a weak association of day 7 concentration of DEAQ with parasite recurrence [318], however this study used a loose combination of AQ plus AS, and the majority of patients with recurrent parasites did not have detectable DEAQ at day 7. The authors thought that patients with undetectable DEAQ used AS only as monotherapy [318]. In the current sub-study analysis, I did not find any association between DEAQ day 7 concentration and parasite recurrence by day 28 or 42. This implies that, unlike for lumefantrine, DEAQ day 7 concentration cannot be used to predict parasite recurrence by day 28.

Repeat treatment with ASAQ for a short period of time leads to accumulation of DEAQ in the study population investigated. If DEAQ accumulation is associated with adverse events this may ultimately affect treatment compliance and efficacy therapy adherence. I found a good correlation between DEAQ day 7 concentrations and QTc prolongation. The effect of DEAQ day 7 concentration on QTc was mainly observed when I considered the QTc change from day 0 to day 2. When I used the absolute QTc at day2 and monitored the effect of day 7 concentration on it, I found no correlation between the two parameters. However, when I considered absolute QTc at day 2 higher than 450 ms as abnormal, the concentration of DEAQ was higher in patients with abnormal than normal QTc ($p = 0.0182$). In another hand, the change in QTc from day 0 to day 2 was correlated with DEAQ day 7 concentrations with a spearman's correlation coefficient of 0.1647 and $p < 0.001$. To emphasize this correlation I looked at the QTc change between episode 1 treated patients, 26 to 45 days subsequent treated patients and above 45 days subsequent treated patients. That was based on knowing that DEAQ concentrates in study population when patients receive the subsequent treatment between 26 and 45 days. I found a higher median change of QTc ($p < 0.0001$), and a higher proportion ($p = 0.004$) of patients with QTc change higher than 30 ms, in patients receiving subsequent treatment after a short

period as compared to episode 1 or to patients who received subsequent treatment after 45 days.

AQ, like other aminoquinolines, has been shown to affect cardiac electrophysiology, but very few studies have investigated the concentration-dependent QTc prolongation of AQ or its metabolite DEAQ. Halofantrine is the only quinolone antimalarial for which the effects on QTc prolongation have been extensively studied [302, 303, 319, 320]. Other quinoline antimalarial drugs have been implicated in QTc prolongation, but their concentration-dependent QTc prolongation has not been fully characterised.

QTc has been evaluated in many AL studies. AL was associated with a small increase in mean QTc (around 7.45 ms) compared to the placebo. In my study, the mean increase was less than 5 ms at episode 1 or at any period of subsequent treatment. I did not find any correlation between lumefantrine day 7 concentration and change in QTc from day 0 to day 2 (Spearman's coefficient of correlation of 0.0552 and $p = 0.074$). AL does not prolong the QTc.

Another major adverse event finding was the correlation between day 7 concentration and hepatotoxicity as measured through ALAT levels. I found a positive correlation between the change in ALAT value from day 0 to day 3 and day 7 concentration of lumefantrine (Spearman's coefficient of correlation of 0.1008 and $p = 0.010$) and DEAQ (Spearman's coefficient of correlation of 0.0868 and $p = 0.0198$). This concentration-dependent action on ALAT change was strengthen in ASAQ arm by the elevated concentration of DEAQ in patients who received a subsequent treatment between 26 and 45 days and had abnormal ALAT at day 3 as compared to the others categories ($p = 0.0001$) (Table 25). AQ has been implicated in hepatotoxicity, which was linked to its metabolite quinone-immune in the absence of glutathione; the authors have suggested an immune-mediated idiosyncratic hepatotoxicity [321]. This idiosyncratic hepatotoxicity was supposed to be multifactorial with formation of reactive metabolite of the drug, genetic and environmental factors as well as drug exposure [322]. It is also known that glutathione decreases with ageing [323]. In my study, all of the participants in ASAQ arm were young children under 15 years meaning with a high glutathione concentration that could explain at least partly the low proportion of abnormal ALAT in ASAQ arm.

All the cases of abnormal cardiac and liver functions were resolved by day 28 of follow-up.

This work was part of one of the largest clinical trial on malaria, which was a phase IIIb/IV comparative, randomised, multi-centre, open label, parallel 3-arm clinical study to assess the safety and efficacy of repeated administration of pyronaridine-artesunate, dihydroartemisinin-piperaquine or artemether-lumefantrine or artesunate-amodiaquine over a two-year period in children and adult patients with acute uncomplicated *Plasmodium* sp. malaria. Data from repeat treatment episodes over a short period of time demonstrated the accumulation of DEAQ in this study population. Repeat treatment with a long follow-up period was necessary to define the risk of malaria exposure and parasite recurrence at an individual level based on patient data from a single study site in Mali.

5 Conclusion

Repetitive treatment with ASAQ over a short period of time (between 26 and 45 days) leads to accumulation of DEAQ, the main and active metabolite of AQ in this study population. The accumulation of DEAQ corresponds to an increase in the occurrence of adverse events like cardiotoxicity (prolongation of QTc interval) and hepatotoxicity (elevation of alanine aminotransferase enzyme). Contrary to lumefantrine concentration on day 7, DEAQ day 7 concentrations were not associated with treatment outcome.

Lumefantrine day 7 concentration was strongly associated with attainment of protection against new *Pf* reinfection by day 28. However, the association decreased by day 42 and was no longer detectable at day 63. The effect was still present after normalization of lumefantrine dose and patient weight and also after adjusting for other significant cofactors (study site, age, season at inclusion, and initial parasitaemia).

Lumefantrine day 7 concentrations lower than 381 ng/ml were not protective against new infection by day 28. Children aged <5 years had a low day 7 concentration of lumefantrine compared to other age groups. AL dosage should be adjusted in this age group, particularly in girls who have lower lumefantrine day 7 concentrations than boys of the same age.

6 References:

1. WHO: **World malaria report 2017. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO.** 2017.
2. Doumbo O, Traore SF, Sow Y, Dembele M, Soula G, Coulibaly A, Dolo A, Sangare O, Koita O, Pichard E, et al.: **[Impact of curtains and blankets impregnated with permethrin on the malarial indicators and the number of malarial attacks per child in a village in an area hyperendemic for malaria on the Malian savannah (preliminary results of the first year study)].** *Bull Soc Pathol Exot* 1991, **84:**761-774.
3. Koita OA, Sangare L, Sango HA, Dao S, Keita N, Maiga M, Mounkoro M, Fane Z, Maiga AS, Traore K, et al: **Effect of seasonality and ecological factors on the prevalence of the four malaria parasite species in northern mali.** *J Trop Med* 2012, **2012:**367160.
4. Bernabeu M, Gomez-Perez GP, Sissoko S, Niambele MB, Haibala AA, Sanz A, Thera MA, Fernandez-Becerra C, Traore K, Alonso PL, et al: **Plasmodium vivax malaria in Mali: a study from three different regions.** *Malar J* 2012, **11:**405.
5. Niangaly A, Karthigayan G, Amed O, Coulibaly D, Sa JM, Adams M, Travassos MA, Ferrero J, Laurens MB, Kone AK, et al: **Plasmodium vivax Infections over 3 Years in Duffy Blood Group Negative Malians in Bandiagara, Mali.** *Am J Trop Med Hyg* 2017, **97:**744-752.
6. Toure M, Sanogo D, Dembele S, Diawara SI, Oppfeldt K, Schioler KL, Haidara DB, Traore SF, Alifrangis M, Konradsen F, Doumbia S: **Seasonality and shift in age-specific malaria prevalence and incidence in Binko and Carriere villages close to the lake in Selingue, Mali.** *Malar J* 2016, **15:**219.
7. Coulibaly D, Travassos MA, Tolo Y, Laurens MB, Kone AK, Traore K, Sissoko M, Niangaly A, Diarra I, Daou M, et al: **Spatio-Temporal Dynamics of Asymptomatic Malaria: Bridging the Gap Between Annual Malaria Resurgences in a Sahelian Environment.** *Am J Trop Med Hyg* 2017, **97:**1761-1769.
8. WHO: **World malaria report.** Geneva, Switzerland2015.
9. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, Battle KE, Moyes CL, Henry A, Eckhoff PA, et al: **The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015.** *Nature* 2015, **526:**207-211.
10. Gething PW, Casey DC, Weiss DJ, Bisanzio D, Bhatt S, Cameron E, Battle KE, Dalrymple U, Rozier J, Rao PC, et al: **Mapping Plasmodium falciparum Mortality in Africa between 1990 and 2015.** *N Engl J Med* 2016.
11. Woodring JV, Ogutu B, Schnabel D, Waitumbi JN, Olsen CH, Walsh DS, Heppner DG, Jr., Polhemus ME: **Evaluation of recurrent parasitemia after artemether-lumefantrine treatment for uncomplicated malaria in children in western Kenya.** *Am J Trop Med Hyg* 2010, **83:**458-464.
12. Perignon JL, Druilhe P: **Immune mechanisms underlying the premunition against Plasmodium falciparum malaria.** *Mem Inst Oswaldo Cruz* 1994, **89 Suppl 2:**51-53.

13. Tran TM, Li S, Doumbo S, Doumtabe D, Huang CY, Dia S, Bathily A, Sangala J, Kone Y, Traore A, et al: **An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to Plasmodium falciparum infection.** *Clin Infect Dis* 2013, **57:**40-47.
14. Goncalves BP, Sagara I, Coulibaly M, Wu Y, Assadou MH, Guindo A, Ellis RD, Diakite M, Gabriel E, Prevots DR, et al: **Hemoglobin variants shape the distribution of malaria parasites in human populations and their transmission potential.** *Sci Rep* 2017, **7:**14267.
15. Cyrklaff M, Sanchez CP, Frischknecht F, Lanzer M: **Host actin remodeling and protection from malaria by hemoglobinopathies.** *Trends Parasitol* 2012, **28:**479-485.
16. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Williams TN, Weatherall DJ, Hay SI: **Global distribution of the sickle cell gene and geographical confirmation of the malaria hypothesis.** *Nat Commun* 2010, **1:**104.
17. Flint J, Harding RM, Boyce AJ, Clegg JB: **The population genetics of the haemoglobinopathies.** *Baillieres Clin Haematol* 1998, **11:**1-51.
18. Kwiatkowski DP: **How malaria has affected the human genome and what human genetics can teach us about malaria.** *Am J Hum Genet* 2005, **77:**171-192.
19. Allison AC: **Protection afforded by sickle-cell trait against subtertian malareal infection.** *Br Med J* 1954, **1:**290-294.
20. Ackerman H, Usen S, Jallow M, Sisay-Joof F, Pinder M, Kwiatkowski DP: **A comparison of case-control and family-based association methods: the example of sickle-cell and malaria.** *Ann Hum Genet* 2005, **69:**559-565.
21. Agarwal A, Guindo A, Cissoko Y, Taylor JG, Coulibaly D, Kone A, Kayentao K, Djimde A, Plowe CV, Doumbo O, et al: **Hemoglobin C associated with protection from severe malaria in the Dogon of Mali, a West African population with a low prevalence of hemoglobin S.** *Blood* 2000, **96:**2358-2363.
22. Modiano D, Luoni G, Sirima BS, Simpore J, Verra F, Konate A, Rastrelli E, Olivieri A, Calissano C, Paganotti GM, et al: **Haemoglobin C protects against clinical Plasmodium falciparum malaria.** *Nature* 2001, **414:**305-308.
23. Chotivanich K, Udomsangpetch R, Pattanapanyasat K, Chierakul W, Simpson J, Looareesuwan S, White N: **Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria.** *Blood* 2002, **100:**1172-1176.
24. Luzzatto L, Usanga FA, Reddy S: **Glucose-6-phosphate dehydrogenase deficient red cells: resistance to infection by malarial parasites.** *Science* 1969, **164:**839-842.
25. Gilles HM, Fletcher KA, Hendrickse RG, Lindner R, Reddy S, Allan N: **Glucose-6-phosphate-dehydrogenase deficiency, sickling, and malaria in African children in South Western Nigeria.** *Lancet* 1967, **1:**138-140.
26. Ganczakowski M, Town M, Bowden DK, Vulliamy TJ, Kaneko A, Clegg JB, Weatherall DJ, Luzzatto L: **Multiple glucose 6-phosphate**

dehydrogenase-deficient variants correlate with malaria endemicity in the Vanuatu archipelago (southwestern Pacific). *Am J Hum Genet* 1995, **56:**294-301.
27. Modiano G, Morpurgo G, Terrenato L, Novelletto A, Di Rienzo A, Colombo B, Purpura M, Mariani M, Santachiara-Benerecetti S, Brega A, et al.: **Protection against malaria morbidity: near-fixation of the alpha-thalassemia gene in a Nepalese population.** *Am J Hum Genet* 1991, **48:**390-397.
28. Mockenhaupt FP, Ehrhardt S, Gellert S, Otchwemah RN, Dietz E, Anemana SD, Bienzle U: **Alpha(+)-thalassemia protects African children from severe malaria.** *Blood* 2004, **104:**2003-2006.
29. Cattani JA, Gibson FD, Alpers MP, Crane GG: **Hereditary ovalocytosis and reduced susceptibility to malaria in Papua New Guinea.** *Trans R Soc Trop Med Hyg* 1987, **81:**705-709.
30. Genton B, al-Yaman F, Mgone CS, Alexander N, Paniu MM, Alpers MP, Mokela D: **Ovalocytosis and cerebral malaria.** *Nature* 1995, **378:**564-565.
31. Miller LH, Mason SJ, Clyde DF, McGinniss MH: **The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy.** *N Engl J Med* 1976, **295:**302-304.
32. Malaria Genomic Epidemiology N, Band G, Rockett KA, Spencer CC, Kwiatkowski DP: **A novel locus of resistance to severe malaria in a region of ancient balancing selection.** *Nature* 2015, **526:**253-257.
33. Ndila CM, Uyoga S, Macharia AW, Nyutu G, Peshu N, Ojal J, Shebe M, Awuondo KO, Mturi N, Tsofa B, et al: **Human candidate gene polymorphisms and risk of severe malaria in children in Kilifi, Kenya: a case-control association study.** *Lancet Haematol* 2018, **5:**e333-e345.
34. Karunaweera ND, Grau GE, Gamage P, Carter R, Mendis KN: **Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in Plasmodium vivax malaria.** *Proc Natl Acad Sci U S A* 1992, **89:**3200-3203.
35. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA, Jr., O'Connor JV: **Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1.** *J Exp Med* 1986, **163:**1433-1450.
36. Grau GE, Taylor TE, Molyneux ME, Wirima JJ, Vassalli P, Hommel M, Lambert PH: **Tumor necrosis factor and disease severity in children with falciparum malaria.** *N Engl J Med* 1989, **320:**1586-1591.
37. Richards AL: **Tumour necrosis factor and associated cytokines in the host's response to malaria.** *Int J Parasitol* 1997, **27:**1251-1263.
38. WHO: **WHO malaria terminology.** 2016.
39. WHO: **Guidelines for the treatment of malaria - 3rd edition.** 2015.
40. Marsh K, Forster D, Waruiru C, Mwangi I, Winstanley M, Marsh V, Newton C, Winstanley P, Warn P, Peshu N, et al.: **Indicators of life-threatening malaria in African children.** *N Engl J Med* 1995, **332:**1399-1404.
41. Naing C, Whittaker MA, Nyunt Wai V, Mak JW: **Is Plasmodium vivax malaria a severe malaria?: a systematic review and meta-analysis.** *PLoS Negl Trop Dis* 2014, **8:**e3071.

42. Kute VB, Trivedi HL, Vanikar AV, Shah PR, Gumber MR, Patel HV, Goswami JG, Kanodia KV: **Plasmodium vivax malaria-associated acute kidney injury, India, 2010-2011.** *Emerg Infect Dis* 2012, **18:**842-845.
43. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, Menard R: **Quantitative imaging of Plasmodium transmission from mosquito to mammal.** *Nat Med* 2006, **12:**220-224.
44. De Niz M, Burda PC, Kaiser G, Del Portillo HA, Spielmann T, Frischknecht F, Heussler VT: **Progress in imaging methods: insights gained into Plasmodium biology.** *Nat Rev Microbiol* 2017, **15:**37-54.
45. Frevert U, Engelmann S, Zougbede S, Stange J, Ng B, Matuschewski K, Liebes L, Yee H: **Intravital observation of Plasmodium berghei sporozoite infection of the liver.** *PLoS Biol* 2005, **3:**e192.
46. Sultan AA, Thathy V, Frevert U, Robson KJ, Crisanti A, Nussenzweig V, Nussenzweig RS, Menard R: **TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites.** *Cell* 1997, **90:**511-522.
47. Baldacci P, Menard R: **The elusive malaria sporozoite in the mammalian host.** *Mol Microbiol* 2004, **54:**298-306.
48. Krotoski WA, Collins WE, Bray RS, Garnham PC, Cogswell FB, Gwadz RW, Killick-Kendrick R, Wolf R, Sinden R, Koontz LC, Stanfill PS: **Demonstration of hypnozoites in sporozoite-transmitted Plasmodium vivax infection.** *Am J Trop Med Hyg* 1982, **31:**1291-1293.
49. Graewe S, Rankin KE, Lehmann C, Deschermeier C, Hecht L, Froehlke U, Stanway RR, Heussler V: **Hostile takeover by Plasmodium: reorganization of parasite and host cell membranes during liver stage egress.** *PLoS Pathog* 2011, **7:**e1002224.
50. Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, Krueger A, Pollok JM, Menard R, Heussler VT: **Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids.** *Science* 2006, **313:**1287-1290.
51. John GK, Douglas NM, von Seidlein L, Nosten F, Baird JK, White NJ, Price RN: **Primaquine radical cure of Plasmodium vivax: a critical review of the literature.** *Malar J* 2012, **11:**280.
52. Rajapakse S, Rodrigo C, Fernando SD: **Tafenoquine for preventing relapse in people with Plasmodium vivax malaria.** *Cochrane Database Syst Rev* 2015**:**CD010458.
53. Ashley EA, Phyo AP: **Drugs in Development for Malaria.** *Drugs* 2018, **78:**861-879.
54. Boyle MJ, Richards JS, Gilson PR, Chai W, Beeson JG: **Interactions with heparin-like molecules during erythrocyte invasion by Plasmodium falciparum merozoites.** *Blood* 2010, **115:**4559-4568.
55. Beeson JG, Drew DR, Boyle MJ, Feng G, Fowkes FJ, Richards JS: **Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria.** *FEMS Microbiol Rev* 2016, **40:**343-372.
56. Gilson PR, Crabb BS: **Morphology and kinetics of the three distinct phases of red blood cell invasion by Plasmodium falciparum merozoites.** *Int J Parasitol* 2009, **39:**91-96.
57. Riglar DT, Richard D, Wilson DW, Boyle MJ, Dekiwadia C, Turnbull L, Angrisano F, Marapana DS, Rogers KL, Whitchurch CB, et al: **Super-**

resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. *Cell Host Microbe* 2011, **9:**9-20.
58. Besteiro S, Dubremetz JF, Lebrun M: **The moving junction of apicomplexan parasites: a key structure for invasion.** *Cell Microbiol* 2011, **13:**797-805.
59. Lamarque M, Besteiro S, Papoin J, Roques M, Vulliez-Le Normand B, Morlon-Guyot J, Dubremetz JF, Fauquenoy S, Tomavo S, Faber BW, et al: **The RON2-AMA1 interaction is a critical step in moving junction-dependent invasion by apicomplexan parasites.** *PLoS Pathog* 2011, **7:**e1001276.
60. Cowman AF, Berry D, Baum J: **The cellular and molecular basis for malaria parasite invasion of the human red blood cell.** *J Cell Biol* 2012, **198:**961-971.
61. Marti M, Good RT, Rug M, Knuepfer E, Cowman AF: **Targeting malaria virulence and remodeling proteins to the host erythrocyte.** *Science* 2004, **306:**1930-1933.
62. Gruring C, Heiber A, Kruse F, Ungefehr J, Gilberger TW, Spielmann T: **Development and host cell modifications of Plasmodium falciparum blood stages in four dimensions.** *Nat Commun* 2011, **2:**165.
63. White NJ: **Assessment of the pharmacodynamic properties of antimalarial drugs in vivo.** *Antimicrob Agents Chemother* 1997, **41:**1413-1422.
64. Miller LH, Baruch DI, Marsh K, Doumbo OK: **The pathogenic basis of malaria.** *Nature* 2002, **415:**673-679.
65. Baruch DI, Pasloske BL, Singh HB, Bi X, Ma XC, Feldman M, Taraschi TF, Howard RJ: **Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes.** *Cell* 1995, **82:**77-87.
66. Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Peterson DS, Pinches R, Newbold CI, Miller LH: **Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes.** *Cell* 1995, **82:**101-110.
67. Su XZ, Heatwole VM, Wertheimer SP, Guinet F, Herrfeldt JA, Peterson DS, Ravetch JA, Wellems TE: **The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes.** *Cell* 1995, **82:**89-100.
68. Wickham ME, Rug M, Ralph SA, Klonis N, McFadden GI, Tilley L, Cowman AF: **Trafficking and assembly of the cytoadherence complex in Plasmodium falciparum-infected human erythrocytes.** *EMBO J* 2001, **20:**5636-5649.
69. Spillman NJ, Beck JR, Goldberg DE: **Protein export into malaria parasite-infected erythrocytes: mechanisms and functional consequences.** *Annu Rev Biochem* 2015, **84:**813-841.
70. Smith TG, Walliker D, Ranford-Cartwright LC: **Sexual differentiation and sex determination in the Apicomplexa.** *Trends Parasitol* 2002, **18:**315-323.
71. Joice R, Nilsson SK, Montgomery J, Dankwa S, Egan E, Morahan B, Seydel KB, Bertuccini L, Alano P, Williamson KC, et al: **Plasmodium falciparum**

transmission stages accumulate in the human bone marrow. *Sci Transl Med* 2014, **6:**244re245.
72. Jeffery GM, Young MD, Eyles DE: **The treatment of Plasmodium falciparum infection with chloroquine, with a note on infectivity to mosquitoes of primaquine- and pyrimethamine-treated cases.** *Am J Hyg* 1956, **64:**1-11.
73. Mikolajczak SA, Vaughan AM, Kangwanrangsan N, Roobsoong W, Fishbaugher M, Yimamnuaychok N, Rezakhani N, Lakshmanan V, Singh N, Kaushansky A, et al: **Plasmodium vivax liver stage development and hypnozoite persistence in human liver-chimeric mice.** *Cell Host Microbe* 2015, **17:**526-535.
74. Greenwood BM, Fidock DA, Kyle DE, Kappe SH, Alonso PL, Collins FH, Duffy PE: **Malaria: progress, perils, and prospects for eradication.** *J Clin Invest* 2008, **118:**1266-1276.
75. Aravind L, Iyer LM, Wellems TE, Miller LH: **Plasmodium biology: genomic gleanings.** *Cell* 2003, **115:**771-785.
76. Coatney GR, National Institute of Allergy and Infectious Diseases (U.S.): *The primate malarias.* Bethesda, Md.,: U.S. National Institute of Allergy and Infectious Diseases; for sale by the Supt. of Docs., U.S. Govt. Print. Off., Washington; 1971.
77. Janse CJ, Van der Klooster PF, Van der Kaay HJ, Van der Ploeg M, Overdulve JP: **Rapid repeated DNA replication during microgametogenesis and DNA synthesis in young zygotes of Plasmodium berghei.** *Trans R Soc Trop Med Hyg* 1986, **80:**154-157.
78. Baton LA, Ranford-Cartwright LC: **Spreading the seeds of million-murdering death: metamorphoses of malaria in the mosquito.** *Trends Parasitol* 2005, **21:**573-580.
79. Zieler H, Dvorak JA: **Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells.** *Proc Natl Acad Sci U S A* 2000, **97:**11516-11521.
80. Vlachou D, Zimmermann T, Cantera R, Janse CJ, Waters AP, Kafatos FC: **Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion.** *Cell Microbiol* 2004, **6:**671-685.
81. Meis JF, Pool G, van Gemert GJ, Lensen AH, Ponnudurai T, Meuwissen JH: **Plasmodium falciparum ookinetes migrate intercellularly through Anopheles stephensi midgut epithelium.** *Parasitol Res* 1989, **76:**13-19.
82. Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, Florens L, Janssen CS, Pain A, Christophides GK, et al: **A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses.** *Science* 2005, **307:**82-86.
83. Golenda CF, Starkweather WH, Wirtz RA: **The distribution of circumsporozoite protein (CS) in Anopheles stephensi mosquitoes infected with Plasmodium falciparum malaria.** *J Histochem Cytochem* 1990, **38:**475-481.
84. Kappe SH, Kaiser K, Matuschewski K: **The Plasmodium sporozoite journey: a rite of passage.** *Trends Parasitol* 2003, **19:**135-143.
85. Frischknecht F, Baldacci P, Martin B, Zimmer C, Thiberge S, Olivo-Marin JC, Shorte SL, Menard R: **Imaging movement of malaria parasites**

during transmission by Anopheles mosquitoes. *Cell Microbiol* 2004, **6:**687-694.
86. Beier JC: **Malaria parasite development in mosquitoes.** *Annu Rev Entomol* 1998, **43:**519-543.
87. Billingsley PF, Rudin W: **The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of Plasmodium species.** *J Parasitol* 1992, **78:**430-440.
88. Smith RC, King JG, Tao D, Zeleznik OA, Brando C, Thallinger GG, Dinglasan RR: **Molecular Profiling of Phagocytic Immune Cells in Anopheles gambiae Reveals Integral Roles for Hemocytes in Mosquito Innate Immunity.** *Mol Cell Proteomics* 2016, **15:**3373-3387.
89. Clayton AM, Dong Y, Dimopoulos G: **The Anopheles innate immune system in the defense against malaria infection.** *J Innate Immun* 2014, **6:**169-181.
90. Osta MA, Christophides GK, Vlachou D, Kafatos FC: **Innate immunity in the malaria vector Anopheles gambiae: comparative and functional genomics.** *J Exp Biol* 2004, **207:**2551-2563.
91. Moreno-Garcia M, Recio-Totoro B, Claudio-Piedras F, Lanz-Mendoza H: **Injury and immune response: applying the danger theory to mosquitoes.** *Front Plant Sci* 2014, **5:**451.
92. Riehle MM, Markianos K, Niare O, Xu J, Li J, Toure AM, Podiougou B, Oduol F, Diawara S, Diallo M, et al: **Natural malaria infection in Anopheles gambiae is regulated by a single genomic control region.** *Science* 2006, **312:**577-579.
93. Belachew EB: **Immune Response and Evasion Mechanisms of Plasmodium falciparum Parasites.** *J Immunol Res* 2018, **2018:**6529681.
94. Blasco B, Leroy D, Fidock DA: **Antimalarial drug resistance: linking Plasmodium falciparum parasite biology to the clinic.** *Nat Med* 2017, **23:**917-928.
95. WHO: **Antimalarial drug combination therapy, report of a technical consultation.** 4-5 April 2001.
96. WHO: **Q&A on artemisinin resistance.** 2018.
97. WHO: **Artemisinin resistance and artemisinin-based combination therapy efficacy.** WHO/CDC/GMP/2018.18; 2018.
98. WHO: **World malaria report. Geneva.** ((WHO/HTM/GMP/2010.1).2010 ed.2010.
99. Sirima SB, Ogutu B, Lusingu JP, Mtoro A, Mrango Z, Ouedraogo A, Yaro JB, Onyango KO, Gesase S, Mnkande E, et al: **Comparison of artesunate-mefloquine and artemether-lumefantrine fixed-dose combinations for treatment of uncomplicated Plasmodium falciparum malaria in children younger than 5 years in sub-Saharan Africa: a randomised, multicentre, phase 4 trial.** *Lancet Infect Dis* 2016.
100. Faye B, Offianan AT, Ndiaye JL, Tine RC, Toure W, Djoman K, Sylla K, Ndiaye PS, Penali L, Gaye O: **Efficacy and tolerability of artesunate-amodiaquine (Camoquin plus) versus artemether-lumefantrine (Coartem) against uncomplicated Plasmodium falciparum malaria: multisite trial in Senegal and Ivory Coast.** *Trop Med Int Health* 2010, **15:**608-613.

101. Zongo I, Some FA, Somda SA, Parikh S, Rouamba N, Rosenthal PJ, Tarning J, Lindegardh N, Nosten F, Ouedraogo JB: **Efficacy and day 7 plasma piperaquine concentrations in African children treated for uncomplicated malaria with dihydroartemisinin-piperaquine.** *PLoS One* 2014, **9:**e103200.
102. Niare K, Dara A, Sagara I, Sissoko MS, Guindo CO, Cisse NH, Coulibaly CO, Ringwald P, Benoit-Vical F, Berry A, et al: **In Vivo Efficacy and Parasite Clearance of Artesunate + Sulfadoxine-Pyrimethamine Versus Artemether-Lumefantrine in Mali.** *Am J Trop Med Hyg* 2016, **94:**634-639.
103. Kayentao K, Doumbo OK, Penali LK, Offianan AT, Bhatt KM, Kimani J, Tshefu AK, Kokolomami JH, Ramharter M, de Salazar PM, et al: **Pyronaridine-artesunate granules versus artemether-lumefantrine crushed tablets in children with Plasmodium falciparum malaria: a randomized controlled trial.** *Malar J* 2012, **11:**364.
104. Sagara I, Fofana B, Gaudart J, Sidibe B, Togo A, Toure S, Sanogo K, Dembele D, Dicko A, Giorgi R, et al: **Repeated artemisinin-based combination therapies in a malaria hyperendemic area of Mali: efficacy, safety, and public health impact.** *Am J Trop Med Hyg* 2012, **87:**50-56.
105. Sagara I, Beavogui AH, Zongo I, Soulama I, Borghini-Fuhrer I, Fofana B, Camara D, Some AF, Coulibaly AS, Traore OB, et al: **Safety and efficacy of re-treatments with pyronaridine-artesunate in African patients with malaria: a substudy of the WANECAM randomised trial.** *Lancet Infect Dis* 2015.
106. Sagara I, Diallo A, Kone M, Coulibaly M, Diawara SI, Guindo O, Maiga H, Niambele MB, Sissoko M, Dicko A, et al: **A randomized trial of artesunate-mefloquine versus artemether-lumefantrine for treatment of uncomplicated Plasmodium falciparum malaria in Mali.** *Am J Trop Med Hyg* 2008, **79:**655-661.
107. Kayentao K, Maiga H, Newman RD, McMorrow ML, Hoppe A, Yattara O, Traore H, Kone Y, Guirou EA, Saye R, et al: **Artemisinin-based combinations versus amodiaquine plus sulphadoxine-pyrimethamine for the treatment of uncomplicated malaria in Faladje, Mali.** *Malar J* 2009, **8:**5.
108. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM: **Evidence of artemisinin-resistant malaria in western Cambodia.** *N Engl J Med* 2008, **359:**2619-2620.
109. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, et al: **Artemisinin resistance in Plasmodium falciparum malaria.** *N Engl J Med* 2009, **361:**455-467.
110. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, et al: **A molecular marker of artemisinin-resistant Plasmodium falciparum malaria.** *Nature* 2014, **505:**50-55.
111. Zeng W, Bai Y, Wang M, Wang Z, Deng S, Ruan Y, Feng S, Yang Z, Cui L: **Significant Divergence in Sensitivity to Antimalarial Drugs between Neighboring Plasmodium falciparum Populations along the Eastern Border of Myanmar.** *Antimicrob Agents Chemother* 2017, **61**.
112. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, et al: **Mefloquine resistance**

in Plasmodium falciparum and increased pfmdr1 gene copy number. *Lancet* 2004, **364:**438-447.
113. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, Sam B, Dek D, Try V, Amato R, et al: **Dihydroartemisinin-piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study.** *Lancet Infect Dis* 2016.
114. Borrmann S, Sasi P, Mwai L, Bashraheil M, Abdallah A, Muriithi S, Fruhauf H, Schaub B, Pfeil J, Peshu J, et al: **Declining responsiveness of Plasmodium falciparum infections to artemisinin-based combination treatments on the Kenyan coast.** *PLoS One* 2011, **6:**e26005.
115. Chang HH, Meibalan E, Zelin J, Daniels R, Eziefula AC, Meyer EC, Tadesse F, Grignard L, Joice RC, Drakeley C, et al: **Persistence of Plasmodium falciparum parasitemia after artemisinin combination therapy: evidence from a randomized trial in Uganda.** *Sci Rep* 2016, **6:**26330.
116. Staimer J: **Studies on evolution of drug resistance in *P. falciparum* malaria *in vivo* and *in vitro*.** *Dissertation.* Ruperto-Carola University of Heidelberg, Germany, Combined Faculties for the Natural Sciences and for Mathematics; 2010.
117. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, Nussenzweig V: **The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites.** *Nature* 2006, **444:**937-940.
118. Herrington DA, Clyde DF, Losonsky G, Cortesia M, Murphy JR, Davis J, Baqar S, Felix AM, Heimer EP, Gillessen D, et al.: **Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites.** *Nature* 1987, **328:**257-259.
119. Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, Heppner DG, Yuan G, Hollingdale M, Slaoui M, et al.: **Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine.** *J Infect Dis* 1995, **171:**1576-1585.
120. Rts SCTP, Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, Kabwende AL, Adegnika AA, Mordmuller B, Issifou S, et al: **A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants.** *N Engl J Med* 2012, **367:**2284-2295.
121. Rts SCTP, Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, Methogo BG, Doucka Y, Flamen A, et al: **First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children.** *N Engl J Med* 2011, **365:**1863-1875.
122. Rieckmann KH, Carson PE, Beaudoin RL, Cassells JS, Sell KW: **Letter: Sporozoite induced immunity in man against an Ethiopian strain of Plasmodium falciparum.** *Trans R Soc Trop Med Hyg* 1974, **68:**258-259.
123. Clyde DF, McCarthy VC, Miller RM, Hornick RB: **Specificity of protection of man immunized against sporozoite-induced falciparum malaria.** *Am J Med Sci* 1973, **266:**398-403.
124. Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, Arens T, Beckers P, van Gemert G, van de Vegte-Bolmer M, et

al: **Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study.** *Lancet* 2011, **377:**1770-1776.
125. Bijker EM, Bastiaens GJ, Teirlinck AC, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, Siebelink-Stoter R, Arens T, Teelen K, Nahrendorf W, et al: **Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity.** *Proc Natl Acad Sci U S A* 2013, **110:**7862-7867.
126. Bijker EM, Schats R, Obiero JM, Behet MC, van Gemert GJ, van de Vegte-Bolmer M, Graumans W, van Lieshout L, Bastiaens GJ, Teelen K, et al: **Sporozoite immunization of human volunteers under mefloquine prophylaxis is safe, immunogenic and protective: a double-blind randomized controlled clinical trial.** *PLoS One* 2014, **9:**e112910.
127. VanBuskirk KM, O'Neill MT, De La Vega P, Maier AG, Krzych U, Williams J, Dowler MG, Sacci JB, Jr., Kangwanrangsan N, Tsuboi T, et al: **Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design.** *Proc Natl Acad Sci U S A* 2009, **106:**13004-13009.
128. Spring M, Murphy J, Nielsen R, Dowler M, Bennett JW, Zarling S, Williams J, de la Vega P, Ware L, Komisar J, et al: **First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers.** *Vaccine* 2013, **31:**4975-4983.
129. Mikolajczak SA, Lakshmanan V, Fishbaugher M, Camargo N, Harupa A, Kaushansky A, Douglass AN, Baldwin M, Healer J, O'Neill M, et al: **A next-generation genetically attenuated Plasmodium falciparum parasite created by triple gene deletion.** *Mol Ther* 2014, **22:**1707-1715.
130. van Schaijk BC, Ploemen IH, Annoura T, Vos MW, Foquet L, van Gemert GJ, Chevalley-Maurel S, van de Vegte-Bolmer M, Sajid M, Franetich JF, et al: **A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites.** *Elife* 2014, **3**.
131. Mordmuller B, Surat G, Lagler H, Chakravarty S, Ishizuka AS, Lalremruata A, Gmeiner M, Campo JJ, Esen M, Ruben AJ, et al: **Sterile protection against human malaria by chemoattenuated PfSPZ vaccine.** *Nature* 2017, **542:**445-449.
132. Sissoko MS, Healy SA, Katile A, Omaswa F, Zaidi I, Gabriel EE, Kamate B, Samake Y, Guindo MA, Dolo A, et al: **Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial.** *Lancet Infect Dis* 2017, **17:**498-509.
133. Yayon A, Cabantchik ZI, Ginsburg H: **Identification of the acidic compartment of Plasmodium falciparum-infected human erythrocytes as the target of the antimalarial drug chloroquine.** *EMBO J* 1984, **3:**2695-2700.
134. Krogstad DJ, Schlesinger PH, Gluzman IY: **Antimalarials increase vesicle pH in Plasmodium falciparum.** *J Cell Biol* 1985, **101:**2302-2309.
135. Dupont AG, Vanderniepen P, Lefebvre RA, Bogaert MG: **Pharmacological characterization of neuronal dopamine receptors in the rat hindquarters, renal and superior mesenteric vascular beds.** *J Auton Pharmacol* 1986, **6:**305-309.

136. Fitch CD, Chevli R, Banyal HS, Phillips G, Pfaller MA, Krogstad DJ: **Lysis of Plasmodium falciparum by ferriprotoporphyrin IX and a chloroquine-ferriprotoporphyrin IX complex.** *Antimicrob Agents Chemother* 1982, **21:**819-822.
137. Slater AF, Cerami A: **Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites.** *Nature* 1992, **355:**167-169.
138. **ASAQ - Fixed-Dose Artesunate/Amodiaquine** [www.dndi.org/diseases-projects/portfolio/asaq.html]
139. WHO: **Guidelines for the treatment of malaria Second edition.** 2010.
140. Neftel KA, Woodtly W, Schmid M, Frick PG, Fehr J: **Amodiaquine induced agranulocytosis and liver damage.** *Br Med J (Clin Res Ed)* 1986, **292:**721-723.
141. Winstanley P, Edwards G, Orme M, Breckenridge A: **The disposition of amodiaquine in man after oral administration.** *Br J Clin Pharmacol* 1987, **23:**1-7.
142. Winstanley PA, Edwards G, Orme ML, Breckenridge AM: **Effect of dose size on amodiaquine pharmacokinetics after oral administration.** *Eur J Clin Pharmacol* 1987, **33:**331-333.
143. Krishna S, White NJ: **Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications.** *Clin Pharmacokinet* 1996, **30:**263-299.
144. Stepniewska K, Taylor W, Sirima SB, Ouedraogo EB, Ouedraogo A, Gansane A, Simpson JA, Morgan CC, White NJ, Kiechel JR: **Population pharmacokinetics of artesunate and amodiaquine in African children.** *Malar J* 2009, **8:**200.
145. Li XQ, Bjorkman A, Andersson TB, Ridderstrom M, Masimirembwa CM: **Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate.** *J Pharmacol Exp Ther* 2002, **300:**399-407.
146. Parikh S, Ouedraogo JB, Goldstein JA, Rosenthal PJ, Kroetz DL: **Amodiaquine metabolism is impaired by common polymorphisms in CYP2C8: implications for malaria treatment in Africa.** *Clin Pharmacol Ther* 2007, **82:**197-203.
147. Naraharisetti SB, Lin YS, Rieder MJ, Marciante KD, Psaty BM, Thummel KE, Totah RA: **Human liver expression of CYP2C8: gender, age, and genotype effects.** *Drug Metab Dispos* 2010, **38:**889-893.
148. Tingle MD, Jewell H, Maggs JL, O'Neill PM, Park BK: **The bioactivation of amodiaquine by human polymorphonuclear leucocytes in vitro: chemical mechanisms and the effects of fluorine substitution.** *Biochem Pharmacol* 1995, **50:**1113-1119.
149. Li XQ, Bjorkman A, Andersson TB, Gustafsson LL, Masimirembwa CM: **Identification of human cytochrome P(450)s that metabolise anti-parasitic drugs and predictions of in vivo drug hepatic clearance from in vitro data.** *Eur J Clin Pharmacol* 2003, **59:**429-442.
150. Johansson T, Jurva U, Gronberg G, Weidolf L, Masimirembwa C: **Novel metabolites of amodiaquine formed by CYP1A1 and CYP1B1:**

structure elucidation using electrochemistry, mass spectrometry, and NMR. *Drug Metab Dispos* 2009, **37:**571-579.
151. Furukawa M, Nishimura M, Ogino D, Chiba R, Ikai I, Ueda N, Naito S, Kuribayashi S, Moustafa MA, Uchida T, et al: **Cytochrome p450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver.** *Cancer Sci* 2004, **95:**520-529.
152. Croft SL, Duparc S, Arbe-Barnes SJ, Craft JC, Shin CS, Fleckenstein L, Borghini-Fuhrer I, Rim HJ: **Review of pyronaridine anti-malarial properties and product characteristics.** *Malar J* 2012, **11:**270.
153. Childs GE, Hausler B, Milhous W, Chen C, Wimonwattrawatee T, Pooyindee N, Boudreau EF: **In vitro activity of pyronaridine against field isolates and reference clones of Plasmodium falciparum.** *Am J Trop Med Hyg* 1988, **38:**24-29.
154. Ringwald P, Bickii J, Basco LK: **In vitro activity of antimalarials against clinical isolates of Plasmodium falciparum in Yaounde, Cameroon.** *Am J Trop Med Hyg* 1996, **55:**254-258.
155. Ringwald P, Bickii J, Basco L: **Randomised trial of pyronaridine versus chloroquine for acute uncomplicated falciparum malaria in Africa.** *Lancet* 1996, **347:**24-28.
156. Schildbach S, Wernsdorfer WH, Suebsaeng L, Rooney W: **In vitro sensitivity of multiresistant Plasmodium falciparum to new candidate antimalarial drugs in western Thailand.** *Southeast Asian J Trop Med Public Health* 1990, **21:**29-38.
157. Auparakkitanon S, Chapoomram S, Kuaha K, Chirachariyavej T, Wilairat P: **Targeting of hematin by the antimalarial pyronaridine.** *Antimicrob Agents Chemother* 2006, **50:**2197-2200.
158. Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG: **An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials.** *Biochem Pharmacol* 1998, **55:**727-736.
159. Famin O, Krugliak M, Ginsburg H: **Kinetics of inhibition of glutathione-mediated degradation of ferriprotoporphyrin IX by antimalarial drugs.** *Biochem Pharmacol* 1999, **58:**59-68.
160. Hodel EM, Zanolari B, Mercier T, Biollaz J, Keiser J, Olliaro P, Genton B, Decosterd LA: **A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2009, **877:**867-886.
161. Chen YC, Fleckenstein L: **Improved assay method for the determination of pyronaridine in plasma and whole blood by high-performance liquid chromatography for application to clinical pharmacokinetic studies.** *J Chromatogr B Biomed Sci Appl* 2001, **752:**39-46.
162. Jayaraman SD, Ismail S, Nair NK, Navaratnam V: **Determination of pyronaridine in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies.** *J Chromatogr B Biomed Sci Appl* 1997, **690:**253-257.
163. Naik H, Imming P, Schmidt MS, Murry DJ, Fleckenstein L: **Development and validation of a liquid chromatography-mass spectrometry assay**

for the determination of pyronaridine in human blood for application to clinical pharmacokinetic studies. *J Pharm Biomed Anal* 2007, **45:**112-119.
164. Feng Z, Jiang NX, Wang CY, Zhang W: **[Pharmacokinetics of pyronaridine, an antimalarial in rabbits].** *Yao Xue Xue Bao* 1986, **21:**801-805.
165. Ni YC, Zhan CQ, Ha SH, Shao BR: **[The embryotoxicity of a new antimalarial pyronaridine in rats].** *Yao Xue Xue Bao* 1982, **17:**401-406.
166. Ramanathan S, Karupiah S, Nair NK, Olliaro PL, Navaratnam V, Wernsdorfer WH, Mansor SM: **A new and simple solid-phase extraction method for LC determination of pyronaridine in human plasma.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2005, **824:**45-50.
167. Ayyoub A, Methaneethorn J, Ramharter M, Djimde AA, Tekete M, Duparc S, Borghini-Fuhrer I, Shin JS, Fleckenstein L: **Population Pharmacokinetics of Pyronaridine in Pediatric Malaria Patients.** *Antimicrob Agents Chemother* 2015, **60:**1450-1458.
168. Morris CA, Dueker SR, Lohstroh PN, Wang LQ, Fang XP, Jung D, Lopez-Lazaro L, Baker M, Duparc S, Borghini-Fuhrer I, et al: **Mass balance and metabolism of the antimalarial pyronaridine in healthy volunteers.** *Eur J Drug Metab Pharmacokinet* 2015, **40:**75-86.
169. WHO: **Advances in antimalarial chemotherapy.** In *Technical report series no 711* (World Health Organization G, Switzerland ed.1984.
170. Chen L, Qu FY, Zhou YC: **Field observations on the antimalarial piperaquine.** *Chin Med J (Engl)* 1982, **95:**281-286.
171. Thompson PE, Werbel LM: *Antimalarial agents: chemistry and pharmacology.* New York,: Academic Press; 1972.
172. Tarning J, Zongo I, Some FA, Rouamba N, Parikh S, Rosenthal PJ, Hanpithakpong W, Jongrak N, Day NP, White NJ, et al: **Population pharmacokinetics and pharmacodynamics of piperaquine in children with uncomplicated falciparum malaria.** *Clin Pharmacol Ther* 2012, **91:**497-505.
173. Hung TY, Davis TM, Ilett KF, Karunajeewa H, Hewitt S, Denis MB, Lim C, Socheat D: **Population pharmacokinetics of piperaquine in adults and children with uncomplicated falciparum or vivax malaria.** *Br J Clin Pharmacol* 2004, **57:**253-262.
174. Lee TM, Huang L, Johnson MK, Lizak P, Kroetz D, Aweeka F, Parikh S: **In vitro metabolism of piperaquine is primarily mediated by CYP3A4.** *Xenobiotica* 2012, **42:**1088-1095.
175. Hung TY, Davis TM, Ilett KF: **Measurement of piperaquine in plasma by liquid chromatography with ultraviolet absorbance detection.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2003, **791:**93-101.
176. Lindegardh N, Ashton M, Bergqvist Y: **Automated solid-phase extraction method for the determination of piperaquine in whole blood by rapid liquid chromatography.** *Ther Drug Monit* 2003, **25:**544-551.
177. Lindegardh N, White NJ, Day NP: **High throughput assay for the determination of piperaquine in plasma.** *J Pharm Biomed Anal* 2005, **39:**601-605.

178. Tarning J, Singtoroj T, Annerberg A, Ashton M, Bergqvist Y, White NJ, Day NP, Lindegardh N: **Development and validation of an automated solid phase extraction and liquid chromatographic method for the determination of piperaquine in urine.** *J Pharm Biomed Anal* 2006, **41:**213-218.
179. Lindegardh N, Annerberg A, White NJ, Day NP: **Development and validation of a liquid chromatographic-tandem mass spectrometric method for determination of piperaquine in plasma stable isotope labeled internal standard does not always compensate for matrix effects.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **862:**227-236.
180. Tarning J, Lindegardh N: **Quantification of the antimalarial piperaquine in plasma.** *Trans R Soc Trop Med Hyg* 2008, **102:**409-411.
181. Combrinck JM, Mabotha TE, Ncokazi KK, Ambele MA, Taylor D, Smith PJ, Hoppe HC, Egan TJ: **Insights into the role of heme in the mechanism of action of antimalarials.** *ACS Chem Biol* 2013, **8:**133-137.
182. Colussi D, Parisot C, Legay F, Lefevre G: **Binding of artemether and lumefantrine to plasma proteins and erythrocytes.** *Eur J Pharm Sci* 1999, **9:**9-16.
183. Novartis PC: **Coartem (artemether/lumefantrine) tablets prescrinbing information.** *East Hanover* 2013 Apr, **Nj**.
184. de Wildt SN, Kearns GL, Leeder JS, van den Anker JN: **Cytochrome P450 3A: ontogeny and drug disposition.** *Clin Pharmacokinet* 1999, **37:**485-505.
185. Wolbold R, Klein K, Burk O, Nussler AK, Neuhaus P, Eichelbaum M, Schwab M, Zanger UM: **Sex is a major determinant of CYP3A4 expression in human liver.** *Hepatology* 2003, **38:**978-988.
186. Lindegardh N, Annerberg A, Blessborn D, Bergqvist Y, Day N, White NJ: **Development and validation of a bioanalytical method using automated solid-phase extraction and LC-UV for the simultaneous determination of lumefantrine and its desbutyl metabolite in plasma.** *J Pharm Biomed Anal* 2005, **37:**1081-1088.
187. Huang L, Li X, Marzan F, Lizak PS, Aweeka FT: **Determination of lumefantrine in small-volume human plasma by LC-MS/MS: using a deuterated lumefantrine to overcome matrix effect and ionization saturation.** *Bioanalysis* 2012, **4:**157-166.
188. Blessborn D, Romsing S, Annerberg A, Sundquist D, Bjorkman A, Lindegardh N, Bergqvist Y: **Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of lumefantrine in capillary blood on sampling paper.** *J Pharm Biomed Anal* 2007, **45:**282-287.
189. Ntale M, Ogwal-Okeng JW, Mahindi M, Gustafsson LL, Beck O: **A field-adapted sampling and HPLC quantification method for lumefantrine and its desbutyl metabolite in whole blood spotted on filter paper.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **876:**261-265.
190. Wang J, Zhang CJ, Chia WN, Loh CC, Li Z, Lee YM, He Y, Yuan LX, Lim TK, Liu M, et al: **Haem-activated promiscuous targeting of artemisinin in Plasmodium falciparum.** *Nat Commun* 2015, **6:**10111.

191. White NJ, van Vugt M, Ezzet F: **Clinical pharmacokinetics and pharmacodynamics and pharmacodynamics of artemether-lumefantrine.** *Clin Pharmacokinet* 1999, **37:**105-125.
192. Hanpithakpong W, Kamanikom B, Dondorp AM, Singhasivanon P, White NJ, Day NP, Lindegardh N: **A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **876:**61-68.
193. Muhia DK, Mberu EK, Watkins WM: **Differential extraction of artemether and its metabolite dihydroartemisinin from plasma and determination by high-performance liquid chromatography.** *J Chromatogr B Biomed Appl* 1994, **660:**196-199.
194. Hilhorst MJ, Hendriks G, de Vries R, Hillewaert V, Verhaege T, van de Merbel NC: **A high-performance liquid chromatography-tandem mass spectrometry method for the determination of artemether and dihydroartemisinin in human plasma.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2014, **965:**45-53.
195. mal ERACGoD: **A research agenda for malaria eradication: drugs.** *PLoS Med* 2011, **8:**e1000402.
196. Baragana B, Hallyburton I, Lee MC, Norcross NR, Grimaldi R, Otto TD, Proto WR, Blagborough AM, Meister S, Wirjanata G, et al: **A novel multiple-stage antimalarial agent that inhibits protein synthesis.** *Nature* 2015, **522:**315-320.
197. Kato N, Comer E, Sakata-Kato T, Sharma A, Sharma M, Maetani M, Bastien J, Brancucci NM, Bittker JA, Corey V, et al: **Diversity-oriented synthesis yields novel multistage antimalarial inhibitors.** *Nature* 2016.
198. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell B, Seitz P, Plouffe DM, Dharia NV, Tan J, et al: **Spiroindolones, a potent compound class for the treatment of malaria.** *Science* 2010, **329:**1175-1180.
199. Simpson JA, Price R, ter Kuile F, Teja-Isavatharm P, Nosten F, Chongsuphajaisiddhi T, Looareesuwan S, Aarons L, White NJ: **Population pharmacokinetics of mefloquine in patients with acute falciparum malaria.** *Clin Pharmacol Ther* 1999, **66:**472-484.
200. Barnes KI, Little F, Smith PJ, Evans A, Watkins WM, White NJ: **Sulfadoxine-pyrimethamine pharmacokinetics in malaria: pediatric dosing implications.** *Clin Pharmacol Ther* 2006, **80:**582-596.
201. White NJ, Stepniewska K, Barnes K, Price RN, Simpson J: **Simplified antimalarial therapeutic monitoring: using the day-7 drug level?** *Trends Parasitol* 2008, **24:**159-163.
202. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S, et al: **Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria.** *Clin Infect Dis* 2006, **42:**1570-1577.
203. Ezzet F, Mull R, Karbwang J: **Population pharmacokinetics and therapeutic response of CGP 56697 (artemether + benflumetol) in malaria patients.** *Br J Clin Pharmacol* 1998, **46:**553-561.
204. Smithuis F, Kyaw MK, Phe O, Aye KZ, Htet L, Barends M, Lindegardh N, Singtoroj T, Ashley E, Lwin S, et al: **Efficacy and effectiveness of**

dihydroartemisinin-piperaquine versus artesunate-mefloquine in falciparum malaria: an open-label randomised comparison. *Lancet* 2006, **367:**2075-2085.

205. Slutsker LM, Khoromana CO, Payne D, Allen CR, Wirima JJ, Heymann DL, Patchen L, Steketee RW: **Mefloquine therapy for Plasmodium falciparum malaria in children under 5 years of age in Malawi: in vivo/in vitro efficacy and correlation of drug concentration with parasitological outcome.** *Bull World Health Organ* 1990, **68:**53-59.
206. Borrmann S, Sallas WM, Machevo S, Gonzalez R, Bjorkman A, Martensson A, Hamel M, Juma E, Peshu J, Ogutu B, et al: **The effect of food consumption on lumefantrine bioavailability in African children receiving artemether-lumefantrine crushed or dispersible tablets (Coartem) for acute uncomplicated Plasmodium falciparum malaria.** *Trop Med Int Health* 2010, **15:**434-441.
207. WorldWide Antimalarial Resistance Network Lumefantrine PKPDSG: **Artemether-lumefantrine treatment of uncomplicated Plasmodium falciparum malaria: a systematic review and meta-analysis of day 7 lumefantrine concentrations and therapeutic response using individual patient data.** *BMC Med* 2015, **13:**227.
208. German P, Parikh S, Lawrence J, Dorsey G, Rosenthal PJ, Havlir D, Charlebois E, Hanpithakpong W, Lindegardh N, Aweeka FT: **Lopinavir/ritonavir affects pharmacokinetic exposure of artemether/lumefantrine in HIV-uninfected healthy volunteers.** *J Acquir Immune Defic Syndr* 2009, **51:**424-429.
209. Gitau EN, Muchohi SN, Ogutu BR, Githiga IM, Kokwaro GO: **Selective and sensitive liquid chromatographic assay of amodiaquine and desethylamodiaquine in whole blood spotted on filter paper.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2004, **799:**173-177.
210. Minzi OM, Rais M, Svensson JO, Gustafsson LL, Ericsson O: **High-performance liquid chromatographic method for determination of amodiaquine, chloroquine and their monodesethyl metabolites in biological samples.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2003, **783:**473-480.
211. **Methods and techniques for assessing exposure to antimalarial drugs in clinical field studies** [http://www.who.int/iris/handle/10665/44653]
212. WHO: **Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations. Geneva.** 2009.
213. WHO: **Methods for surveillance of antimalarial drug efficacy. Geneva.** 2009.
214. Stepniewska K, Taylor WR, Mayxay M, Price R, Smithuis F, Guthmann JP, Barnes K, Myint HY, Adjuik M, Olliaro P, et al: **In vivo assessment of drug efficacy against Plasmodium falciparum malaria: duration of follow-up.** *Antimicrob Agents Chemother* 2004, **48:**4271-4280.
215. White NJ: **The assessment of antimalarial drug efficacy.** *Trends Parasitol* 2002, **18:**458-464.
216. Borrmann S, Peto T, Snow RW, Gutteridge W, White NJ: **Revisiting the design of phase III clinical trials of antimalarial drugs for**

uncomplicated Plasmodium falciparum malaria. *PLoS Med* 2008, **5:**e227.
217. White NJ: **Malaria parasite clearance.** *Malar J* 2017, **16:**88.
218. Rieckmann KH, McNamara JV, Frischer H, Stockert TA, Carson PE, Powell RD: **Effects of chloroquine, quinine, and cycloguanil upon the maturation of asexual erythrocytic forms of two strains of Plasmodium falciparum in vitro.** *Am J Trop Med Hyg* 1968, **17:**661-671.
219. Rieckmann KH, Campbell GH, Sax LJ, Mrema JE: **Drug sensitivity of plasmodium falciparum. An in-vitro microtechnique.** *Lancet* 1978, **1:**22-23.
220. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD: **Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique.** *Antimicrob Agents Chemother* 1979, **16:**710-718.
221. Chulay JD, Haynes JD, Diggs CL: **Plasmodium falciparum: assessment of in vitro growth by [3H]hypoxanthine incorporation.** *Exp Parasitol* 1983, **55:**138-146.
222. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M: **Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening.** *Antimicrob Agents Chemother* 2004, **48:**1803-1806.
223. Elabbadi N, Ancelin ML, Vial HJ: **Use of radioactive ethanolamine incorporation into phospholipids to assess in vitro antimalarial activity by the semiautomated microdilution technique.** *Antimicrob Agents Chemother* 1992, **36:**50-55.
224. Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ: **Parasite lactate dehydrogenase as an assay for Plasmodium falciparum drug sensitivity.** *Am J Trop Med Hyg* 1993, **48:**739-741.
225. Brasseur P, Agnamey P, Moreno A, Druilhe P: **[Evaluation of in vitro drug sensitivity of antimalarials for Plasmodium falciparum using a colorimetric assay (DELI-microtest)].** *Med Trop (Mars)* 2001, **61:**545-547.
226. Druilhe P, Moreno A, Blanc C, Brasseur PH, Jacquier P: **A colorimetric in vitro drug sensitivity assay for Plasmodium falciparum based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay.** *Am J Trop Med Hyg* 2001, **64:**233-241.
227. Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C: **Histidine-rich protein II: a novel approach to malaria drug sensitivity testing.** *Antimicrob Agents Chemother* 2002, **46:**1658-1664.
228. van Vianen PH, Thaithong S, Reinders PP, van Engen A, van der Keur M, Tanke HJ, van der Kaay HJ, Mons B: **Automated flow cytometric analysis of drug susceptibility of malaria parasites.** *Am J Trop Med Hyg* 1990, **43:**602-607.
229. Waki S, Tamura J, Jingu M, Adachi M, Suzuki M: **A new technique for drug susceptibility tests for Plasmodium falciparum by ethidium bromide fluoroassay.** *Trans R Soc Trop Med Hyg* 1986, **80:**47-49.

230. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, et al: **Novel phenotypic assays for the detection of artemisinin-resistant Plasmodium falciparum malaria in Cambodia: in-vitro and ex-vivo drug-response studies.** *Lancet Infect Dis* 2013, **13:**1043-1049.
231. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193:**673-675.
232. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Coulibaly D, Dicko A, Su XZ, Nomura T, et al: **A molecular marker for chloroquine-resistant falciparum malaria.** *N Engl J Med* 2001, **344:**257-263.
233. Djimde A, Doumbo OK, Steketee RW, Plowe CV: **Application of a molecular marker for surveillance of chloroquine-resistant falciparum malaria.** *Lancet* 2001, **358:**890-891.
234. Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, Mukadam RA, Rogerson SJ, Lescano AG, Molyneux ME, et al: **Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of Plasmodium falciparum malaria.** *J Infect Dis* 2002, **185:**380-388.
235. Kyabayinze D, Cattamanchi A, Kamya MR, Rosenthal PJ, Dorsey G: **Validation of a simplified method for using molecular markers to predict sulfadoxine-pyrimethamine treatment failure in African children with falciparum malaria.** *Am J Trop Med Hyg* 2003, **69:**247-252.
236. Sa JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, Wellems TE: **Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine.** *Proc Natl Acad Sci U S A* 2009, **106:**18883-18889.
237. Wellems TE, Hayton K, Fairhurst RM: **The impact of malaria parasitism: from corpuscles to communities.** *J Clin Invest* 2009, **119:**2496-2505.
238. Chabasse D, De Gentile L, Ligny C, Le Bras J, Rialland X, Bouchara JP: **Chloroquine-resistant Plasmodium falciparum in Mali revealed by congenital malaria.** *Trans R Soc Trop Med Hyg* 1988, **82:**547.
239. Hurwitz ES, Johnson D, Campbell CC: **Resistance of Plasmodium falciparum malaria to sulfadoxine-pyrimethamine ('Fansidar') in a refugee camp in Thailand.** *Lancet* 1981, **1:**1068-1070.
240. Vinayak S, Alam MT, Mixson-Hayden T, McCollum AM, Sem R, Shah NK, Lim P, Muth S, Rogers WO, Fandeur T, et al: **Origin and evolution of sulfadoxine resistant Plasmodium falciparum.** *PLoS Pathog* 2010, **6:**e1000830.
241. Basco LK, Tahar R, Ringwald P: **Molecular basis of in vivo resistance to sulfadoxine-pyrimethamine in African adult patients infected with Plasmodium falciparum malaria parasites.** *Antimicrob Agents Chemother* 1998, **42:**1811-1814.
242. Plowe CV, Djimde A, Wellems TE, Diop S, Kouriba B, Doumbo OK: **Community pyrimethamine-sulfadoxine use and prevalence of resistant Plasmodium falciparum genotypes in Mali: a model for deterring resistance.** *Am J Trop Med Hyg* 1996, **55:**467-471.

243. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, et al: **Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance.** *Mol Cell* 2000, **6:**861-871.
244. Menard D, Yapou F, Manirakiza A, Djalle D, Matsika-Claquin MD, Talarmin A: **Polymorphisms in pfcrt, pfmdr1, dhfr genes and in vitro responses to antimalarials in Plasmodium falciparum isolates from Bangui, Central African Republic.** *Am J Trop Med Hyg* 2006, **75:**381-387.
245. Muangnoicharoen S, Johnson DJ, Looareesuwan S, Krudsood S, Ward SA: **Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin in vitro.** *Antimicrob Agents Chemother* 2009, **53:**1362-1366.
246. Sisowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, Fidock DA, Gil JP: **In vivo selection of Plasmodium falciparum parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with artemether-lumefantrine in Africa.** *J Infect Dis* 2009, **199:**750-757.
247. Tekete M, Djimde AA, Beavogui AH, Maiga H, Sagara I, Fofana B, Ouologuem D, Dama S, Kone A, Dembele D, et al: **Efficacy of chloroquine, amodiaquine and sulphadoxine-pyrimethamine for the treatment of uncomplicated falciparum malaria: revisiting molecular markers in an area of emerging AQ and SP resistance in Mali.** *Malar J* 2009, **8:**34.
248. Cowman AF, Karcz S, Galatis D, Culvenor JG: **A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole.** *J Cell Biol* 1991, **113:**1033-1042.
249. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF: **Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum.** *Nature* 2000, **403:**906-909.
250. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnadig N, Uhlemann AC, Martin RE, Lehane AM, Fidock DA: **Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies.** *Nat Commun* 2016, **7:**11553.
251. Phyo AP, Ashley EA, Anderson TJ, Bozdech Z, Carrara VI, Sriprawat K, Nair S, White MM, Dziekan J, Ling C, et al: **Declining Efficacy of Artemisinin Combination Therapy Against P. Falciparum Malaria on the Thai-Myanmar Border (2003-2013): The Role of Parasite Genetic Factors.** *Clin Infect Dis* 2016.
252. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA: **Decreasing pfmdr1 copy number in plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin.** *J Infect Dis* 2006, **194:**528-535.
253. Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE: **Dissecting the loci of low-level quinine resistance in malaria parasites.** *Mol Microbiol* 2004, **52:**985-997.
254. Kone A, Mu J, Maiga H, Beavogui AH, Yattara O, Sagara I, Tekete MM, Traore OB, Dara A, Dama S, et al: **Quinine treatment selects the pfnhe-1**

ms4760-1 polymorphism in Malian patients with Falciparum malaria. *J Infect Dis* 2013, **207:**520-527.
255. Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, Estrada-Franco JG, Mollinedo RE, Avila JC, Cespedes JL, et al: **Mutations in Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance.** *J Infect Dis* 1997, **176:**1590-1596.
256. Hyde JE: **The dihydrofolate reductase-thymidylate synthetase gene in the drug resistance of malaria parasites.** *Pharmacol Ther* 1990, **48:**45-59.
257. Wu Y, Kirkman LA, Wellems TE: **Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine.** *Proc Natl Acad Sci U S A* 1996, **93:**1130-1134.
258. Wang P, Read M, Sims PF, Hyde JE: **Sulfadoxine resistance in the human malaria parasite Plasmodium falciparum is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization.** *Mol Microbiol* 1997, **23:**979-986.
259. Reynolds MG, Roos DS: **A biochemical and genetic model for parasite resistance to antifolates. Toxoplasma gondii provides insights into pyrimethamine and cycloguanil resistance in Plasmodium falciparum.** *J Biol Chem* 1998, **273:**3461-3469.
260. Gil JP, Nogueira F, Stromberg-Norklit J, Lindberg J, Carrolo M, Casimiro C, Lopes D, Arez AP, Cravo PV, Rosario VE: **Detection of atovaquone and Malarone resistance conferring mutations in Plasmodium falciparum cytochrome b gene (cytb).** *Mol Cell Probes* 2003, **17:**85-89.
261. Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q: **Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site.** *Antimicrob Agents Chemother* 2000, **44:**2100-2108.
262. Hall N, Pain A, Berriman M, Churcher C, Harris B, Harris D, Mungall K, Bowman S, Atkin R, Baker S, et al: **Sequence of Plasmodium falciparum chromosomes 1, 3-9 and 13.** *Nature* 2002, **419:**527-531.
263. Adams J, Kelso R, Cooley L: **The kelch repeat superfamily of proteins: propellers of cell function.** *Trends Cell Biol* 2000, **10:**17-24.
264. Chu CS, White NJ: **Management of relapsing Plasmodium vivax malaria.** *Expert Rev Anti Infect Ther* 2016, **14:**885-900.
265. Rieckmann KH, Davis DR, Hutton DC: **Plasmodium vivax resistance to chloroquine?** *Lancet* 1989, **2:**1183-1184.
266. Baird JK, Basri H, Purnomo, Bangs MJ, Subianto B, Patchen LC, Hoffman SL: **Resistance to chloroquine by Plasmodium vivax in Irian Jaya, Indonesia.** *Am J Trop Med Hyg* 1991, **44:**547-552.
267. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, Prasetyorini B, Piera KA, Barends M, Brockman A, et al: **Chloroquine resistant Plasmodium vivax: in vitro characterisation and association with molecular polymorphisms.** *PLoS One* 2007, **2:**e1089.

268. Price RN, Auburn S, Marfurt J, Cheng Q: **Phenotypic and genotypic characterisation of drug-resistant Plasmodium vivax.** *Trends Parasitol* 2012, **28:**522-529.
269. Suwanarusk R, Chavchich M, Russell B, Jaidee A, Chalfein F, Barends M, Prasetyorini B, Kenangalem E, Piera KA, Lek-Uthai U, et al: **Amplification of pvmdr1 associated with multidrug-resistant Plasmodium vivax.** *J Infect Dis* 2008, **198:**1558-1564.
270. Korsinczky M, Fischer K, Chen N, Baker J, Rieckmann K, Cheng Q: **Sulfadoxine resistance in Plasmodium vivax is associated with a specific amino acid in dihydropteroate synthase at the putative sulfadoxine-binding site.** *Antimicrob Agents Chemother* 2004, **48:**2214-2222.
271. Leartsakulpanich U, Imwong M, Pukrittayakamee S, White NJ, Snounou G, Sirawaraporn W, Yuthavong Y: **Molecular characterization of dihydrofolate reductase in relation to antifolate resistance in Plasmodium vivax.** *Mol Biochem Parasitol* 2002, **119:**63-73.
272. Imwong M, Pukrittayakamee S, Cheng Q, Moore C, Looareesuwan S, Snounou G, White NJ, Day NP: **Limited polymorphism in the dihydropteroate synthetase gene (dhps) of Plasmodium vivax isolates from Thailand.** *Antimicrob Agents Chemother* 2005, **49:**4393-4395.
273. Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS, Lee HW, Chen JH, Wang Y, Sattabongkot J, Han ET: **Mutations in the antifolate-resistance-associated genes dihydrofolate reductase and dihydropteroate synthase in Plasmodium vivax isolates from malaria-endemic countries.** *Am J Trop Med Hyg* 2010, **83:**474-479.
274. Farnert A, Arez AP, Babiker HA, Beck HP, Benito A, Bjorkman A, Bruce MC, Conway DJ, Day KP, Henning L, et al: **Genotyping of Plasmodium falciparum infections by PCR: a comparative multicentre study.** *Trans R Soc Trop Med Hyg* 2001, **95:**225-232.
275. Smythe JA, Peterson MG, Coppel RL, Saul AJ, Kemp DJ, Anders RF: **Structural diversity in the 45-kilodalton merozoite surface antigen of Plasmodium falciparum.** *Mol Biochem Parasitol* 1990, **39:**227-234.
276. Kiwanuka GN: **Genetic diversity in Plasmodium falciparum merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997-2007.** *J Vector Borne Dis* 2009, **46:**1-12.
277. Smythe JA, Coppel RL, Brown GV, Ramasamy R, Kemp DJ, Anders RF: **Identification of two integral membrane proteins of Plasmodium falciparum.** *Proc Natl Acad Sci U S A* 1988, **85:**5195-5199.
278. Tanabe K, Mackay M, Goman M, Scaife JG: **Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum.** *J Mol Biol* 1987, **195:**273-287.
279. Certa U, Rotmann D, Matile H, Reber-Liske R: **A naturally occurring gene encoding the major surface antigen precursor p190 of Plasmodium falciparum lacks tripeptide repeats.** *EMBO J* 1987, **6:**4137-4142.
280. Ferreira MU, Ribeiro WL, Tonon AP, Kawamoto F, Rich SM: **Sequence diversity and evolution of the malaria vaccine candidate merozoite**

surface protein-1 (MSP-1) of Plasmodium falciparum. *Gene* 2003, **304:**65-75.
281. Tanabe K, Sakihama N, Walliker D, Babiker H, Abdel-Muhsin AM, Bakote'e B, Ohmae H, Arisue N, Horii T, Rooth I, et al: **Allelic dimorphism-associated restriction of recombination in Plasmodium falciparum msp1.** *Gene* 2007, **397:**153-160.
282. Miller LH, Roberts T, Shahabuddin M, McCutchan TF: **Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1).** *Mol Biochem Parasitol* 1993, **59:**1-14.
283. Robson KJ: **Sequence diversity in the intron of the calmodulin gene from Plasmodium falciparum.** *Mol Biochem Parasitol* 1993, **60:**1-8.
284. Su X, Wellems TE: **Toward a high-resolution Plasmodium falciparum linkage map: polymorphic markers from hundreds of simple sequence repeats.** *Genomics* 1996, **33:**430-444.
285. Adjei GO, Kurtzhals JA, Rodrigues OP, Alifrangis M, Hoegberg LC, Kitcher ED, Badoe EV, Lamptey R, Goka BQ: **Amodiaquine-artesunate vs artemether-lumefantrine for uncomplicated malaria in Ghanaian children: a randomized efficacy and safety trial with one year follow-up.** *Malar J* 2008, **7:**127.
286. Sagara I, Sangare D, Dolo G, Guindo A, Sissoko M, Sogoba M, Niambele MB, Yalcoue D, Kaslow DC, Dicko A, et al: **A high malaria reinfection rate in children and young adults living under a low entomological inoculation rate in a periurban area of Bamako, Mali.** *Am J Trop Med Hyg* 2002, **66:**310-313.
287. West African Network for Clinical Trials of Antimalarial D: **Pyronaridine-artesunate or dihydroartemisinin-piperaquine versus current first-line therapies for repeated treatment of uncomplicated malaria: a randomised, multicentre, open-label, longitudinal, controlled, phase 3b/4 trial.** *Lancet* 2018, **391:**1378-1390.
288. Liljander A, Wiklund L, Falk N, Kweku M, Martensson A, Felger I, Farnert A: **Optimization and validation of multi-coloured capillary electrophoresis for genotyping of Plasmodium falciparum merozoite surface proteins (msp1 and 2).** *Malar J* 2009, **8:**78.
289. Brownstein MJ, Carpten JD, Smith JR: **Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping.** *Biotechniques* 1996, **20:**1004-1006, 1008-1010.
290. Methaneethorn J: **Population pharmacokinetics and pharmacodynamics of pyronaridine.** *PhD dissertations.* University of Iowa, Graduate College, The university of Iowa, Iowa City, Iowa; 2013.
291. U.S. Department of Health and Human Services FaDA, Center for Drug Evaluation and Research, Center for Veterinary Medecine. : **Guidance for Industry: Bioanalytical Method Validation.** May 2001.
292. U.S. Department of Health and Human Services FaDA, Center for Drug Evaluation and Research, Center for Veterinary Medecine:: **E14 Clinical Evaluation of QT/QTc. Interval Prolongation and. Proarrhythmic Potential for Non-Antiarrhythmic Drugs.** 2005.
293. Annerberg A, Singtoroj T, Tipmanee P, White NJ, Day NP, Lindegardh N: **High throughput assay for the determination of lumefantrine in**

plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005, **822:**330-333.
294. Maiga AW, Fofana B, Sagara I, Dembele D, Dara A, Traore OB, Toure S, Sanogo K, Dama S, Sidibe B, et al: **No evidence of delayed parasite clearance after oral artesunate treatment of uncomplicated falciparum malaria in Mali.** *Am J Trop Med Hyg* 2012, **87:**23-28.
295. Arinaitwe E, Sandison TG, Wanzira H, Kakuru A, Homsy J, Kalamya J, Kamya MR, Vora N, Greenhouse B, Rosenthal PJ, et al: **Artemether-lumefantrine versus dihydroartemisinin-piperaquine for falciparum malaria: a longitudinal, randomized trial in young Ugandan children.** *Clin Infect Dis* 2009, **49:**1629-1637.
296. Bukirwa H, Unnikrishnan B, Kramer CV, Sinclair D, Nair S, Tharyan P: **Artesunate plus pyronaridine for treating uncomplicated Plasmodium falciparum malaria.** *Cochrane Database Syst Rev* 2014**:**CD006404.
297. Guevart E, Aguemon A: **[Two cases of fulminant hepatitis during a curative treatment with an artesunate-amodiaquine combination].** *Med Mal Infect* 2009, **39:**57-60.
298. Larrey D, Castot A, Pessayre D, Merigot P, Machayekhy JP, Feldmann G, Lenoir A, Rueff B, Benhamou JP: **Amodiaquine-induced hepatitis. A report of seven cases.** *Ann Intern Med* 1986, **104:**801-803.
299. Coulibaly B, Pritsch M, Bountogo M, Meissner PE, Nebie E, Klose C, Kieser M, Berens-Riha N, Wieser A, Sirima SB, et al: **Efficacy and safety of triple combination therapy with artesunate-amodiaquine-methylene blue for falciparum malaria in children: a randomized controlled trial in Burkina Faso.** *J Infect Dis* 2015, **211:**689-697.
300. Yeka A, Kigozi R, Conrad MD, Lugemwa M, Okui P, Katureebe C, Belay K, Kapella BK, Chang MA, Kamya MR, et al: **Artesunate/Amodiaquine Versus Artemether/Lumefantrine for the Treatment of Uncomplicated Malaria in Uganda: A Randomized Trial.** *J Infect Dis* 2016, **213:**1134-1142.
301. White NJ, Looareesuwan S, Warrell DA: **Quinine and quinidine: a comparison of EKG effects during the treatment of malaria.** *J Cardiovasc Pharmacol* 1983, **5:**173-175.
302. Nosten F, ter Kuile FO, Luxemburger C, Woodrow C, Kyle DE, Chongsuphajaisiddhi T, White NJ: **Cardiac effects of antimalarial treatment with halofantrine.** *Lancet* 1993, **341:**1054-1056.
303. Wesche DL, Schuster BG, Wang WX, Woosley RL: **Mechanism of cardiotoxicity of halofantrine.** *Clin Pharmacol Ther* 2000, **67:**521-529.
304. Mytton OT, Ashley EA, Peto L, Price RN, La Y, Hae R, Singhasivanon P, White NJ, Nosten F: **Electrocardiographic safety evaluation of dihydroartemisinin piperaquine in the treatment of uncomplicated falciparum malaria.** *Am J Trop Med Hyg* 2007, **77:**447-450.
305. Karunajeewa H, Lim C, Hung TY, Ilett KF, Denis MB, Socheat D, Davis TM: **Safety evaluation of fixed combination piperaquine plus dihydroartemisinin (Artekin) in Cambodian children and adults with malaria.** *Br J Clin Pharmacol* 2004, **57:**93-99.
306. White NJ: **Cardiotoxicity of antimalarial drugs.** *Lancet Infect Dis* 2007, **7:**549-558.

307. Methaneethorn J: **Population pharmacokinetics and pharmacodynamics of pyronaridine.** University of Iowa, 2013.
308. Jittamala P, Pukrittayakamee S, Ashley EA, Nosten F, Hanboonkunupakarn B, Lee SJ, Thana P, Chairat K, Blessborn D, Panapipat S, et al: **Pharmacokinetic interactions between primaquine and pyronaridine-artesunate in healthy adult Thai subjects.** *Antimicrob Agents Chemother* 2015, **59:**505-513.
309. Djimde AA, Tekete M, Abdulla S, Lyimo J, Bassat Q, Mandomando I, Lefevre G, Borrmann S: **Pharmacokinetic and pharmacodynamic characteristics of a new pediatric formulation of artemether-lumefantrine in African children with uncomplicated Plasmodium falciparum malaria.** *Antimicrob Agents Chemother* 2011, **55:**3994-3999.
310. Winstanley PA, Simooya O, Kofi-Ekue JM, Walker O, Salako LA, Edwards G, Orme ML, Breckenridge AM: **The disposition of amodiaquine in Zambians and Nigerians with malaria.** *Br J Clin Pharmacol* 1990, **29:**695-701.
311. Winstanley PA, Coleman JW, Maggs JL, Breckenridge AM, Park BK: **The toxicity of amodiaquine and its principal metabolites towards mononuclear leucocytes and granulocyte/monocyte colony forming units.** *Br J Clin Pharmacol* 1990, **29:**479-485.
312. German P, Greenhouse B, Coates C, Dorsey G, Rosenthal PJ, Charlebois E, Lindegardh N, Havlir D, Aweeka FT: **Hepatotoxicity due to a drug interaction between amodiaquine plus artesunate and efavirenz.** *Clin Infect Dis* 2007, **44:**889-891.
313. Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, Kauffman RE: **Developmental pharmacology--drug disposition, action, and therapy in infants and children.** *N Engl J Med* 2003, **349:**1157-1167.
314. Lacroix D, Sonnier M, Moncion A, Cheron G, Cresteil T: **Expression of CYP3A in the human liver--evidence that the shift between CYP3A7 and CYP3A4 occurs immediately after birth.** *Eur J Biochem* 1997, **247:**625-634.
315. Bakshi RP, Nenortas E, Tripathi AK, Sullivan DJ, Shapiro TA: **Model system to define pharmacokinetic requirements for antimalarial drug efficacy.** *Sci Transl Med* 2013, **5:**205ra135.
316. Mapaba E, Hellgren U, Landberg-Lindgren A, Rombo L: **Susceptibility of Plasmodium falciparum to quinine in vitro: effects of drug concentrations and time of exposure.** *Trans R Soc Trop Med Hyg* 1995, **89:**85-89.
317. Aubouy A, Bakary M, Keundjian A, Mbomat B, Makita JR, Migot-Nabias F, Cot M, Le Bras J, Deloron P: **Combination of drug level measurement and parasite genotyping data for improved assessment of amodiaquine and sulfadoxine-pyrimethamine efficacies in treating Plasmodium falciparum malaria in Gabonese children.** *Antimicrob Agents Chemother* 2003, **47:**231-237.
318. Hietala SF, Bhattarai A, Msellem M, Roshammar D, Ali AS, Stromberg J, Hombhanje FW, Kaneko A, Bjorkman A, Ashton M: **Population pharmacokinetics of amodiaquine and desethylamodiaquine in**

pediatric patients with uncomplicated falciparum malaria. *J Pharmacokinet Pharmacodyn* 2007, **34:**669-686.
319. Abernethy DR, Wesche DL, Barbey JT, Ohrt C, Mohanty S, Pezzullo JC, Schuster BG: **Stereoselective halofantrine disposition and effect: concentration-related QTc prolongation.** *Br J Clin Pharmacol* 2001, **51:**231-237.
320. Milton KA, Edwards G, Ward SA, Orme ML, Breckenridge AM: **Pharmacokinetics of halofantrine in man: effects of food and dose size.** *Br J Clin Pharmacol* 1989, **28:**71-77.
321. Shimizu S, Atsumi R, Itokawa K, Iwasaki M, Aoki T, Ono C, Izumi T, Sudo K, Okazaki O: **Metabolism-dependent hepatotoxicity of amodiaquine in glutathione-depleted mice.** *Arch Toxicol* 2009, **83:**701-707.
322. Li AP: **A review of the common properties of drugs with idiosyncratic hepatotoxicity and the "multiple determinant hypothesis" for the manifestation of idiosyncratic drug toxicity.** *Chem Biol Interact* 2002, **142:**7-23.
323. Hernanz A, Fernandez-Vivancos E, Montiel C, Vazquez JJ, Arnalich F: **Changes in the intracellular homocysteine and glutathione content associated with aging.** *Life Sci* 2000, **67:**1317-1324.