Modeling response and delayance to parasite clearance time to artemisinin combination therapies(ACT)

Akindeh Mbuh Nji

Dissertation
zum Erwerb des Doctor of Philosophy (Ph.D.)
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Doctoral Thesis for the awarding of a Doctor of Philosophy (Ph.D.)
at the Medical Faculty of
Ludwig-Maximilians-Universität, Munich

vorgelegt von
submitted by

Akindeh Mbuh Nji

aus (Geburtsort)
born in (place of birth)

Wum- Cameroon

am (Tag an dem die Dissertation abgeschlossen wurde)
submitted on (day of finalization of the thesis)

30-04-2013

Supervisors LMU:

Habilitated Supervisor	Prof. Ulrich Mansmann
Direct Supervisor	PD. Dr. Christian Heumann
3rd LMU Supervisor	Dr. Guenter Froeschl
4th LMU Supervisor	Prof Mathias Siebeck

Supervisor External:

Local Supervisor	Prof Wilfred Mbacham

Reviewing Experts:

1st Reviewer	PD. Dr. Christian Heumann
2nd Reviewer	Prof Ulrich Mansmann

Dean: Prof. Dr. Dr. h. c. M. Reiser, FACR, FRCR

Date of Oral Defence: 16-09-2013

Modeling response and delayance to Parasite clearance time to artemisinin combination therapies

Affidavit

Nji, Akindeh Mbuh

Surname, first name

Street

BP. 8094, Yaounde

Zip code, town

Cameroon

Country

I hereby declare, that the submitted thesis entitled

Modeling response and delayance to Parasite clearance time

Thesis Title

to artemisinin combination therapies

Thesis Title (cont.)

Thesis Title (cont.)

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

The submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

I further declare that the electronic version of the submitted thesis is congruent with the printed version both in content and format.

Place, Date

Signature PhD Student

Dedication

"Dieu OMNES GLORIA"

Acknowledgments

I am very grateful for the support of all the physicians and field personnel who played a great role in the collection of this data. Many thanks to all the team at the Laboratory for Public Health Research Biotechnology (LAPHER) of the university of Yaoundé I with whom i work, who worked tirelessly to bring this project to an end. Special thanks to Aristid Ekollo Mbange and Chedjou Jean Pierre who played a leading role in the molecular analysis of all the samples from the project.

I appreciate the Baptist Health Board and the Biotechnology Center institutional review board who gave the ethical clearance to do this work.

My supervisors have been there to give me the necessary direction to do this work. I thank Prof. Mbacham Wilfred, Prof Ulrich Mansmann, Prof. Christian Heumann and Dr. Guenter Froeschl in this regard.

I thank my wife Perps who have been so patient with my absences from home to write up this work.

Without the capacity development grant by WHO/TDR to Prof Wilfred F. Mbacham, this work would not have been possible.

Yaoundé, 30th April 2013

Abstract

Background: With the emergence of drug resistance in south East Asia to the very efficacious Artemisinin combination Therapies (ACTs) in the treatment of patients with malaria, there is need to understand the relationship between human factors, parasite diversities, environment and delay to parasite clearance in Cameroon. This can inform the control programs against malaria on how to fight ACTs drug resistance.

Objective: This research seeks to assess efficacy and safety of three ACTs and to Model the impact of human factors, drugs, and endemicity to response to therapy and Malaria parasite clearance time.

Methods: The data for this research is from a study –" Artemisinin-Based Antimalarial Combinations and Clinical Response in Cameroon". This study is a non-inferiority study with a 3 arm, open randomised comparative controlled trial. The Target population is children under 120months of age with acute uncomplicated *P. falciparium* malaria. Patients were followed up for 42 days and the principal outcome measure is Adequate Clinical and Parasitological Response(ACPR) at Day 42. Safety profiles of drugs is assessed by Prevalence of adverse events and serious adverse events. We fit the data to two models -A logistic model to understand the response to therapy and a discrete time survival model to explore delayance to parasite clearance. The three drugs we studied are artesunate amodiaquine, dihydroartemisinin piperaquine and artemeter lumefantrine.

Conclusion: Artesunate amodiaquine and dihydroartemisinin peperaquine are safe and are atleast not worse off than artemeter lumefantrine in the treatment of Plasmodium falciparium malaria in Cameroonian children. No patient individual characteristics influence therapy outcome. However the ecological region(site) is important determinant in therapy outcome at 0.1 significance level. The levels of alanine aminotransferase, haemoglobin, creatinine and interaction between ecological region and age group of children are the main driving force in parasite clearance delay.

Keywords: non-inferiority, drug resistance, parasite, clearance, endemicity, randomised, safety

Table of contents

List of figures

List of tables

List of annexes

List of Abbreviations

RFLP	Restriction fragment length polymorphism
pfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
Glurp 1	Glutamate rich protein 1
Pfmdr	*Plasmodium falciparum* multidrug resistance
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
csp	Circumsporozite protein
TBE	Tris borate EDTA
ACTs	Artemisinin based combination therapies
DHAP	Dihydro artemisinin piperaquine
ICAM	Intercellular adhesion molecular I
ELAM-1	Endothelial leucocyte adhesion molecule 1
Msp1/2	Merozoite surface protein 1
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
pfdhps	*Plasmodium falciparum* dihydripteroate synthase
IgG	Immunoglobulins Gamma
SSUr RNA	Small subunit ribosomal ribonucleic acid
AL	Arthemether Lumefantrine (CoArtem)
ASAQ	Artesunate Amodiaquine
NAT	N-acetyl transferase
CYP	Cytochrome P
pRBCs	Parasitized Red blood Cells

TRAP	Thrombospondin adhesive protein
EIR	Entomological Inoculation Rate
WHO	World Health Organization
ALAT	Alanine transaminase
PCR	Polymerase chain reaction
ETF	Early treatment failure
ACPR	Adequate parasitological response
LCF	Late clinical failure
FCT	Fever clearance time
PCT	Parasite Clearance time
Hb	Haemoglobine
AIC	Akaike information criterion
SNP	Single nucleotide polymorphism
PBS	Phosphate Buffer sulfate
EDTA	Ethylene diamine tetraacetic acid

Chapter 1: Introduction

1.1 Background

Malaria is one of the most devastating parasitic diseases of humans that, continues to thrive throughout the world and especially in Sub-Saharan Africa, despite the numerous control efforts realized so far. Malaria is caused by blood infection of protozoan parasites of the genus *Plasmodium,* which is transmitted from one human to another by female *Anopheles* mosquitoes. Five *Plasmodium* species routinely infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* [1]. The high burden of malaria in Africa is due to *P. falciparum*, which adapts and co-spescialises with *Anopheles gambiae* [2][3] the most effective and widespread vector, making it very difficult to control. Presently there are about 450 known anopheles species, of which 60 can potentially transmit malaria, with regard to their vectorial capacity[2].

There was an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years. 109 countries were endemic for malaria in 2008, 45 within the WHO African region[4] . Annually 25 million pregnancies are potentially at risk [5] with related adverse effects like intrauterine growth retardation (IUGR), low-birth weight (LBW) from prematurity, foetal parasite exposure and congenital infection, infant mortality (IM) linked to preterm-LBW and IUGR-LBW [6]. It is estimated that 75000 to 200000 infants' deaths are associated with malaria in pregnancy [6]. It has been estimated that the economic burden of malaria is extremely high, accounting for a reduction of 1.3% in the annual economic growth rate of countries in which malaria is endemic, and that the consequent long-term impact is a reduction of gross national product (GNP) to more than half [7]. To reduce the extreme burden caused by this disease in Africa, the Millennium Development Goal (MDG) were set out with the aim to reduce malaria by 75% by 2015 from its 2005 baseline level with an average comprehensive malaria control cost of US$ 3.0 billion per year, or around US$ 4.02 billion per African at risk [8] .

There are three dominant difficulties in maintaining malaria control[9]: (i) parasite resistance to safe and affordable antimalarials and the spread of unofficial vendors where most of the population go to with elevated risks related to auto-medication

(resistance, side-effects etc) (ii) the almost complete demise of vector control programs in developing tropical and subtropical countries, and (iii) the failure to develop a practical vaccine that prevents malaria. Indeed, genetic diversity due to sequence variations in merozoites (1/2) and circumsporozoites (csp) proteins etc, which is the proper of malaria parasites and especially *P. falciparum* has greatly hampered the production of a vaccine till date. Nevertheless it is essential in clinical trials so as to monitor the spread of resistance especially to artemisinin based combination therapies.

Since its policy change between 2004 and 2006 for malaria treatment (adoption of first and second line treatments for artemisinin-based combination therapies), Cameroon national malaria control programs has undertaken to disseminate this shift in all regions to inform health workers at public mission and private health facilities. However, much still need to be done as there are still gaps in malaria case management in Cameroon [10], as compared to other regions of Africa. Deficiencies in the practices of both public and private providers compromise the effectiveness and cost-effectiveness of malaria case-management. In 2004, malaria was a major public health problem in Cameroon, and it was considered the first cause of morbidity. [11]However, the morbidity and mortality rates now stand at about 35.88% and 24% respectively.[11] This drop may be attributed to the use of Artemisinin-based Combination Therapies (ACTs), Intermittent Preventive Treatment (IPT) in pregnant women, and vector control strategies such as the use of Long Lasting Insecticide Treated Nets (LLITN) and Indoor residual Sprays (IRS) [12].

The discovery in thai-cambodia that ACTs can suffer from resistance is alerting the research community especially in Africa.[13] Molecular Systematic and phylogenetic approaches are now standing among others as predictors of resistance spread. It is generally hypothesized in terms of homology (similarity attributed to descent from a common ancestor) that genes with similar sequences will display similar types of functions or regulations. This will help to contain the spread of resistance, which can be brought about by population movements and vectors transmission [14], to ACTs but also ring alarm for future drug development.

Researchers are now focusing on parasite clearance time after administration of ACTs and some have reported on the fast clearance (short half life) of artemisinins derivatives in Mali [15]. Reports have shown that drug resistant strains can also rapidly be cleared by the system. However delay of parasite clearance time has however been noted with artesunate-mefloquine in southern Cambodia[16] [17].

One of the genes involved in the pharmacogenetics of antimalarials is the NAT2 gene (N-Acetyl transferase 2), which encodes the enzyme N-Acetyl transferase 2, involved in phase 2 of the biotransformation of antimalarial drugs. [18] The cytochrome CYP gene has also been implicated. Many researchers are picking up interest on the inter play of nutrition with disease burden. Indeed, results of dozens of epidemiological studies in recent years strongly suggest that some minerals (zinc), vitamins (vitamin A) and other trace elements in the diet, may reduce the incidence of various degenerative diseases (cardiovascular diseases, malaria, cancer, cataracts, macular degeneration) [19]. In the other hand It has been suggested that the presence of some elements not yet well characterized, in milk favors resistance to malaria in Fulani population compared to non-Fulani [20]. The Fulani therefore clear parasite faster that the non-Fulani. In two ethnic groups, Fulani and Mossi, it was also found that Fulani had lower risks of contracting malaria infections [21].This is ascribed to the absence of a C allele in the rs2706384 gene of interferon regulator factor 1. However, Fulani homozygous individuals to C allele has more chances of contracting malaria. It was demonstrated that some genetic factors in drug metabolism appeared to be substantial contributors to the observed lower efficacy of CoArtem obtained in Cambodia as compared to Tanzania, two different ethnic groups. [22]

There are other contributing factors to the decline of parasite clearance time. In Cameroon where ACTs like AL have been massively deployed and are free of charge, for pregnant women and children modeling or evaluating these responses is crucial as many patients for instance do not always stick to the treatment. This might have implication for alternative therapies; the redesign of drug policy.

1.1.1 Models on drug resistance and efficacy

A few models have been fitted using data from different studies to describe patterns in anti-malarial drug resistance and efficacy. Some of these models have described patterns in drug utilisations, strategies to delay the progress of drug resistance, the role of anti-malarials in elimination malaria, and the impact of artemisinin combination therapy and long acting treatment in reducing malaria transmission [23], [24], [25], [26].

The impact on the choice artemisinin combination therapy and the implementation has been described with data from the Thai borders that have shown high drug resistance and also have areas of different endemicity [26]. This model has shown that anti-malarial drug resistance spreads faster in low transmission than in high transmission settings. This model has also shown that in low transmission settings, it is treatment failure that is the main cause of drug resistance. Artemisinin has been shown to delay the spread when coverage rates are high and that an exponential inverse effect would be seen in terms of spread of drug resistance if coverage is not adequate. The model predicts that the proportion of human population with residual drug levels to be the main determining factor of drug resistance in a setting of high transmission. This model measures rather the spread of drug resistance with the assumption that it already exist.

Hybrid modeling, of three potential benefits of multiple first line treatments (MFTs) have been used to quantify the effectiveness of multiple deployment of artemisinin combination therapies [24]. These models describe the effects reducing the chances of a parasite spreading to other hosts, reducing drug pressure and also reducing parasite fitness to emergence of drug resistance. These models point to the fact that the global emergence of resistance to artemisinin combination therapies is approximately 10 years and that multiple first line therapies in has the potential of ensuring a long term efficacy of artemisinin combination therapies, starting with the partner drug.

Other models using malaria data have been used to predict the impact of malaria transmission with the roll out of artemisinin combination therapies and alternative first line treatments in different levels of malaria transmission [23]. This describes malaria transmission in humans and also mosquito populations with respect to some variables that are likely to have an impact on malaria transmission. This model predicts that

reduction in the prevalence and incidence of infection associated with a complete switch to artemisinin combination therapies would have more impact in areas where there is a low initial transmission that those with a high transmission rate. This model also shows the advantage of long acting treatments over some currently used artemisinin combination therapies in areas of high transmission. This model recommends that in order to make a policy change on an anti-malarial drug, for any community, there is a need to assess the level of malaria transmission in the community and the half life of the drug under consideration. There are other researchers that have looked at the epidemiological models for the spread of drug resistance and others on the evolution of multi drug resistance [27][28]. The epidemiological models which basically use the Macdonald-Ross[29] model of malaria transmission, have shown that malaria drug resistance does not spread except a fraction of people that are infected but not treated does not go below a thresh hold point. The evolution of multi resistance model explores resistance using the parasite population structure. The premise of the multi drug resistance models is that the frequency of mutation change rate in the parasite population depends on the proportion of host treated with drugs and the parasite transmission rate. This model has shown that reducing transmission rate is effective in reducing the spread of drug resistance.

1. 2 Rationale of the study

Anti-malarial drug studies conducted between 1986 and 1992 when monotherapies were still the drugs for treatment of malaria in Cameroon have reported different levels of resistance in different regions. These different levels of resistance, according to the studies depended on what anti-malarial drug was being considered. For example chloroquine resistance was shown to vary between 40-86% in the south and 20-25% in the north. Mefloquine resistance was rather found to be higher in the north (25%) than in the south (2%) . There have been also studies that have shown antifolate failures of 12% and 43% in vivo and in vitro respectively in Yaoundé with resistance to aminoquinolines remaining high in the years between 1994 and 2002. Even though in yaounde, the capital city of Cameroon, amodiaquine and pyronaridine were shown to be efficacious in this same period [30], [31] there were reports in the later years 2005-2010

of declining rates of amodiaquine efficacy or in combination with sulphadoxine pyrimethamine [32], [33]

The replacement of monotherapies by artemisinin combination therapies in the treatment of malaria has greatly improved treatment outcome.[34] Artesunate-amodiaquine and artemether-lumefantrine, are first line and second line treatments of *Plasmodium falciparum* Malaria in Cameroon respectively . These choices for first and second line treatment were made without prior data on the efficacy of these drugs. In Cameroon one of the limitations of new treatment regimens is the frequent stock outs, treatment cost and rational use. In the urban areas there are competing artemisinin combination therapies offered by the private sector and sometime roadside medication vendors[19] Dihydroartemisinin-piperaquine (Aterkin®) has been introduced commercially and is sold in the drug stores. This led to the set objectives to evaluate the efficacy and safety of the ACTs-artesunate-amodiaquine dihydroartemisinin-piperaquine and artemether-lumefantrine as the comparator.

The emergence of drug resistance to the very efficacious artemisinin combination therapies around south east Asia [34] remains a great concern to malaria control. There are yet no methods to tracking this resistance. Even though there are artemisinin resistance containment strategies spearheaded by WHO, combating this resistance will require many unknowns to be addressed first [10]. Therefore, modeling the artemisinin combination therapies response to human, ecological and parasite factors has a potential to unravel what might be playing a role in the artemisinin resistance. The time to parasite clearance with respect to these factors could also be helpful in understanding the progress of artemisinin resistance.

1.3 Objective of study

1.3.1 Primary Objectives

To assess the efficacy of artesunate-amodiaquine, dihydroartemisinin-piperaquine, in comparison with artemether-lumefantrine during 42 days follow up period in children with acute uncomplicated *P. falciparum* malaria, in two different endemic areas.

1.3.2 Secondary Objectives

(i) To assess the efficacy of artesunate-amodiaquine, dihydroartemisinin-piperaquine, in comparison with artemether-lumefantrine during 14 and 28 days follow up period in children with acute uncomplicated *P. falciparum* malaria in two different endemic areas.

(ii) To evaluate the safety of artesunate-amodiaquine, dihydroartemisinin-piperaquine ,in comparison with artemether-lumefantrine during 42 days follow up period in children with acute uncomplicated *P. falciparum* malaria.

(iii) To determine parasite clearance time (PCT) and fever clearance time (FCT) following administration of the three trial regimens. DHAP. ASAQ, AL

(iv) To investigate the treatment response based on WHO criteria (WHO, 2003) in patients in all groups after treatment.

(v) To investigate the Single Nucleotide Polymorphisms (SNPs) in gene markers associated with drug resistance

(vi) To establish the Safety profile and association with metaboliser status

1.3.3 Modelling response to therapy and parasite clearance time

(i) Model the effects of drug use, over prescription, auto medication and presence of multiple drugs, ethnicity and endemic region, baseline vital signs, genetic diversity of parasite strains, safety profiles to response to therapy

(ii)Model the delay in parasite clearance time with respect to drug use, auto medication, ecological region and ethnicity, diversity of parasite strains, safety profiles.

1.4 Significance of Study

Results from this study would help inform policy on the choices of first line treatment against malaria. It would also provide information on the segmentation of Cameroon to identify better ecological and human responses to treatment. This study has the potential of providing information to track drug resistance for better policy to understand when to withdraw or advice for a change of policy.

Chapter 2: Literature Review

2.1 History of Malaria

2.1.1 From mono-therapy to combination therapy

The name malaria comes from the Italian word *Mal aria* that means bad air. It is also called “paludism” from the Latin word “paludis” which means marshes. These two names reflect the early views that the disease is spread by unhealthy mist from marshes. Although people were unaware of the origin of malaria and the mode of transmission, protective measures against the mosquito have been used for many centuries. Inhabitants of swampy regions of Egypt were recorded as sleeping in tower-like structures out of the reach of mosquitoes, whereas others slept under nets as early as 450 B.C. [35]

Ancient treatments were available like infusions of qinghao (*Artemesia annua*) used by Chinese in the last 200 years. Don Francis in 1630 taught native Indians in Peru the excellence of the fever tree to fight malaria. In 1638, the physician of the wife of the Spanish viceroy in Peru (the countess of Chincton) treated her for malaria using the bark of a tree, which prevented shivering. This worked to cure her from the malaria even though he did it for the wrong reason. Linnaeus later named the tree after her. Around the same time the cinchona bark was introduced into Europe and called “Jesuit Powder”. Protestants were suspicious of anything linked to the Jesuits, and refused taking it and dubbed it, “the powder of the devil”. In the 1670s, an Englishman, Robert Talbor used the bark of this same tree to treat Charles II for which he was made a knight. He was later made Chevalier for using the same bark to treat the son of the French King, the Dauphin. [36]

In 1830, Pelletier and Caventou isolated quinine and cinchinine, two alkaloids active against the malaria parasite from this tree. In 1836 Charles Ledger while collecting plants in Peru decided to find the source of the cinchona bark. He came across a species with a high concentration of the active ingredient, quinine, and it was named after him. While the Dutch welcomed it and started plantations in Java, the British turned it down. The bark of this tree was later added to wine in which it metamorphosed into gin and became a delicacy for British colonialists.

In 1889, Laveran, traced the pathogenic agent of malaria for which he was awarded the Nobel Prize for medicine in 1907. This was later elucidated by the classification of three Plasmodium species, *falciparum, vivax* and *malariae* by Golgi and collaborators. Ross only suspected the involvement of the female mosquito in 1897. Grass later confirmed this assertion in 1898. Stephens classified the fourth plasmodium species, *ovale,* in 1922. The discovery of quinine had profound influence on world history because European soldiers survived in Africa as well as the allied forces in the Pacific during World War II. The tropics now became more habitable by colonialists leading to a rapid colonial expansion in Africa. In the United States, it made Westward expansion in the 1830s possible because settlers were given quinine. The capture of the Javanese Cinchona plantations caused the Americans to start a huge project to find new antimalarial drugs. This led to the discovery of an 8-amino - quinoline, called atebrine. Unfortunately, this had only marginal activity against human malaria but with very serious side effects up to temporary insanity. In 1934, the Germans discovered a 4-aminoquinoline compound, which was very active against malaria, and called it Sontochin. In 1943, the French in North Africa informed the Americans of Sontochin. The Americans modified it slightly and renamed it “Chloroquine”. This became the drug of choice for treatment of human malaria. This cheap and available drug however had its hopes dampened when resistance was proven in 1964. This resistance has continued to spread in endemic zones and is also spreading to other antimalarial drugs that have been discovered later on.[36]

Important attributes for the successful implementation of antimalarial drugs are good tolerability and safety (especially in young children), affordability, availability in endemic countries and short course regimens [37]. Drugs that have been used against malaria as monotherapies span from quinine, chloroquine, amodiaquine, mefloquine, piperaquine, lumefantrine, primaquine, atovaquone, to antifolate drugs (sulfadoxine, pyremithamine, proguanil etc). Almost all antimalarials are now to be administered as part of a combination therapy, with each targeting distinct mechanism within the parasite. The main goal is to achieve maximal suppression of parasites and delaying the onset of resistance that has been ascribed to these drugs. Current artemisinin-based combination therapies include artemether–lumefantrine (Coartem, presently the

most commonly used ACT worldwide), artesunate–mefloquine, artesunate–amodiaquine, artesunate–sulfadoxine–pyrimethamine, dihydroartemisinin–piperaquine, and artesunate–pyronaridine [38].

Cameroon, like many other African countries, following the increasing resistance of chloroquine[32]had adopted Amodiaquine and Sulphadoxine-pyremithamine as first- and second- line drugs in 2002 and 2004, respectively. Unfortunately the cure rate of these two drugs was proven to deteriorate as monotherapies in five study sites of Cameroon [32]. In this view there was an increasing need to shift this time to the adoption of combination therapies in 2004 with Artesunate-AmodiAQuine (AS-AQ, Co-Arsucam™) as 1st line drug treatment and in 2006 with Arthemeter-Lumefantrine (AM-LM or AL, Coartem®) as alternative therapy.

Combination therapies are used as single-first line therapies. It has been found that using these combination therapies as multiple-first line therapies (MFTs) will have beneficial effects on the clinical outcome of malaria patients. ACTs can be shared among different population groups, in terms of age for instance (young and adults), at home-based or clinic. Many authors advocate a switch in favor of these MFTs in the malaria treatment policy of countries given the uncertainty behind the emergence of resistance to ACTs. MFTs have the advantage of delaying emergence to resistance/treatment failure and if resistance it reduces the spread [39][24]. This advocacy seems to be governed by research on modelling simulating the use of ACTs as MFTs and their benefits.

2.1.2. First report on Artemisinin combination therapy resistance

Antimalarial combination therapy is defined as the simultaneous use of two or more anti malarial drugs, which may either be co-formulated or co-administered, with different biochemical targets in the parasite or host tissue, whose combined effects are either additive or synergistic .

According to WHO, the objectives of an antimalarial treatment policy are to: ensure rapid cure of the infection; reduce morbidity and mortality, including malaria-related

anaemia; prevent the progression of uncomplicated malaria into severe and potentially fatal disease; reduce the impact of malaria infection on the foetus during pregnancy; reduce the reservoir of infection; prevent the emergence and spread of drug resistance; and prevent malaria in travelers [40]. The combination therapy exploits the difference in time of action of each partner drug due to their different half-lives. In addition, the use of drugs in combination shortens duration of treatment, hence increasing compliance, and decreasing the risk of resistant parasites arising through mutation during therapy[41].

In the last decade artemisinin combination therapy has become a key component of malaria control and elimination efforts. It was of great concern therefore when reports of declining efficacy of artesunate-Mefloquine began to surface from the western and Southern region of Cambodia [42][17], and from the Thai-Myanmar border [13]. It was however difficult to ascertain whether the decline is due to artesunate or mefloquine [43].

Data from Africa indicate that the use of ACTs lead to the selection of parasites resistant to the long-acting partner drugs[44].Mutations in pfatp6 (*P. falciparum* Ca2+ transporting ATPase 6) have been associated with decreased artemether susceptibility in field isolates from French Guyana [45].In this view it is important to monitor the spread of resistance as many molecular phylogenetic analyses have been able to do with drugs like sulfadoxine.

Currently, neither genotypic nor *in vitro* assays can reliably distinguish parasite populations that will respond slowly to the artemisinins. Nevertheless, novel approaches for assessing *in vitro* drug susceptibility are underway in several laboratories focused on drug responses, and changes in transcription and metabolic patterns [46][47][48].

2.2 Epidemiology of Malaria

2.2.1 Human malaria parasites and geographical distribution

Plasmodium species are generally distributed worldwide, but each species varies in distribution in relation to factors such as climatic conditions, blood group types, etc

Plasmodium falciparum: this is the most virulent species with greatest impact on human health in terms of mortality and morbidity. It is associated with the subtertian malignant periodic fevers occurring every 48 hours and the severe and complicated malaria. It parasitizes all red blood cells (RBCs), and this contributes to the higher parasitemia most often observed in infected patients. *P.falciparum* is a pan-tropical distributed parasite mostly prevalent in Africa and some parts of Asia.

Plasmodium vivax: accounts for the relapse cases of malaria, by forming hypnozoites, the dormant form of parasite. Unlike *P. falciparum,* the latter is mostly associated with high morbidity [49] and benign tertian periodic fevers occurring every three days. *P. vivax* prefers young erythrocytes. It is a pan-tropical and temperate distributed parasite, mostly in Asia South and Central America. The absence of *Plasmodium vivax* in black population of West and Central Africa is due to the absence of the Duffy antigen on their red blood cells [50]. These populations are homozygous to the gene responsible for the expression of the Duffy antigen.

Plasmodium malariae: the distribution of *P. malariae* generally coincides with that of *P. falciparum* in areas of endemicity in Africa; they form a mixed infection [51].This parasite causes the quartan malaria with fevers occurring every four days and prefers old erythrocytes, one of the reasons why its parasitaemia is much fewer than that of *P. falciparum* and *P. vivax. P. malariae* is a tropical species mainly distributed in sub-Saharan Africa, most of south East Asia, Indonesia, many islands of the western pacific.

Plasmodium ovale: Like *P. vivax* this parasite also causes the relapse cases of malaria and its tertian benign form. This parasite has the tendency to develop in younger erythrocytes and its Giemsa differentiation from *vivax* is most difficult. However, unlike *P. vivax* it does not modify RBCs as much and produces much fewer merozoites [52]. It is highly distributed in sub-Saharan Africa, New Guinea and Philippines. Lysenko *et al* in 1969 reported for the natural occurrence of this species in sub-Saharan Africa and the islands of the western pacific.

***Plasmodium knowlesi*:** It naturally infects macaques and it is mainly found in Southeast Asia. This species was first identified in a long tailed macaque, *Macaca*

fascicularis. It is now known as a zoonotic species because it can also infect humans [1]. *P. knowlesi* has a 24 hours erythrocytic cycle and the disease progression can be fast [53].

2.2.2 Life cycle and mode of transmission

- **Mode of transmission**

The malaria parasite can be transmitted through several ways which include; transfer of parasitized red cells from an infected mother to the child transplacentally or during labor in which case it is known as congenital malaria [54] during transfusion of blood from infected donors, or through needle-stick injuries [55] often accidentally among health care professionals or due to needle sharing among drug addicts. In addition to these, the malaria parasite is principally transmitted by the bite of an infected female Anopheles mosquito. Sporozoites contained in the saliva of the mosquito (vector) are inoculated into the blood of a human host when the mosquito takes a blood meal. Once in the human host, the parasite continues part of its life cycle which had started in the mosquito host.

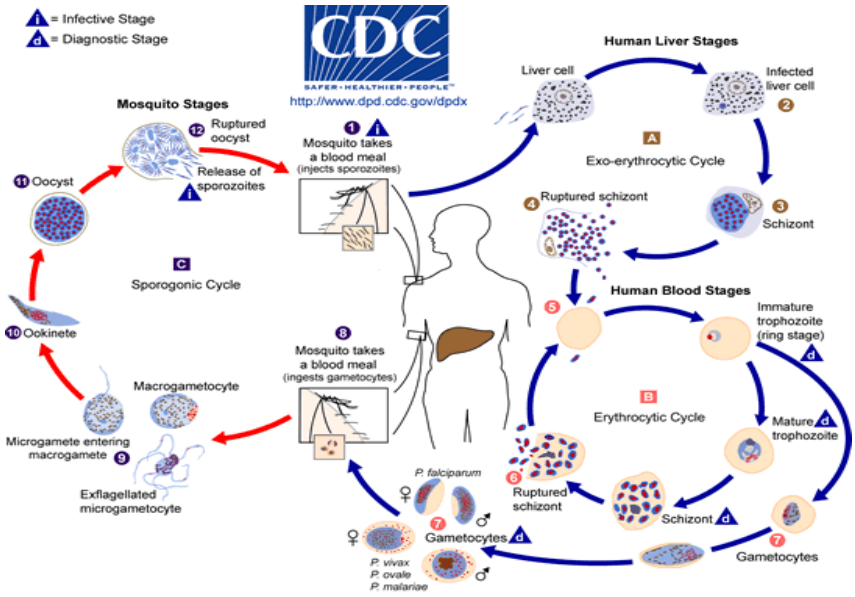


Figure 2.1: Life Cycle of the malaria parasite

Plasmodium life cycle encompasses two main stages that govern the infectious process required for parasite's development within the definitive and intermediate host. The asexual stages constitute two cycles, the exo-erythrocytic schizogony cycle (the liver stage) and the erythrocytic schizogony cycle. The sexual phase splits between humans and mosquitoes. In humans, there is formation of male and female gametocytes that end in mosquito develops into gametes, mating and differentiation of the resulting zygote into multiple forms within the life cycle of these parasites, the key points for microscopic diagnosis are the gametocyte, the ring stage, the mature trophozoites, and the schizont points.

- **Asexual reproduction in the vertebrate host**

This is also known as liver cell schizogony and repeated red cell schizogony cycles and the formation of gametocytes. Infection in the vertebrate host commences when an infected mosquito in the course of feeding injects sporozoites into the peripheral circulation of the host, which finally results in the invasion of hepatocytes. Nevertheless, this is not done directly because new researches on intra-vital imaging have shown that the parasite can settle in the skin for almost six weeks [56] and that nearly one third can leave the injection site and drain to the lymph nodes through Lymphatic systems [57]. Other sporozoites trickle into the blood stream towards the liver where the exo-erythrocytic schizogony can now take place.

- **The exo-erythrocytic stage of malaria parasites in humans**

Schizogony, refers to a replicative process in which parasite undergoes multiple rounds of nuclear division without cytoplasmic division (cytokinesis) followed by a segmentation to form progeny. The sporozoites remain in and may invade the Kuppfer cells in the liver (or the parasite may be phagocytosed) but the former are not able to develop in those cells and so die shortly after invasion. This process of invasion is favored by the motility properties of the sporozoites, driven by molecular molecular mechanisms that involve two molecules; the circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) [45] [46]. After their arrest in the liver sinusoids, sporozoites

detach themselves from the glycosaminoglycans, traverse the Disse space (lying between the sinusoidal cell layer and the hepatocytes) and enter the hepatocytes.

The liver trophozoite develops into a mature schizont (the multinucleated stage of the parasite) and finally a large number of merozoites are released. The mature schizont is 30-70 μm large, has no pigment (there is no hemoglobin in the hepatocyte), and occupies the entire cell cytoplasm. The length of the schizogonic liver cycle is constant for each Plasmodium species to the extent that it can be considered a taxonomic character. The liver cycle ends when the mature schizont ruptures and releases the merozoites into the sinusoids of the liver. Released merozoites can only invade a red blood cell.

Two species of human malaria determine a relapsing infection: P.vivax and P.ovale. In these two species some of the liver trophozoites immediately start the exo-erythrocytic schizogonic cycle which has been described above, while others remain into the liver in a latent (dormant) stage for varying periods of time and are termed hypnozoites. The length of the period of dormancy varies with the sub populations of P.vivax and P.ovale. A single inoculation of sporozoites of a relapsing species contains a mixture of genetically distinct parasites that give rise to discrete subpopulations of exo-erythrocytic trophozoites. The number of relapses, and their periodicity, seems to be a characteristic of the parasite strain.

- **The erythrocytic stage of malaria parasites in humans**

The blood phase of the life-cycle is initiated when the merozoites from liver schizonts are discharged into the circulation [60]. The merozoite is 1 μm in diameter, consisting of a single nucleus and adjacent cytoplasm. As earlier mentioned, apicomplexan possess the capability of infecting RBCs because of their apical complex made of specialized organelles such as rhoptries, densed granules, micronnemes and apicoplast [61]. With these, it invades almost immediately an erythrocyte to enter its trophozoite stage. A vacuole is produced by the parasite which assumes the characteristic ring form (the young trophozoite). Within 12-24 hours, as the parasite grows, the cytoplasm expands,

the vacuole slowly disappears and a characteristic parasitic pigment becomes visible within the cytoplasm. At the end of this phase the trophozoite has a single nucleus, a large cytoplasm, no vacuole, and a variable amount of pigment. The nucleus starts to divide approximately 30 hours after invasion. As nuclear division produces two or more nuclei the parasite enters the stage of a schizont. Nuclear division continues until an appropriate number of merozoites are produced: approximately 36 for *P.falciparum*, 24 for *P.vivax* and *P.ovale*, 12 for *P. malariae*. At the end of this phase the schizogonic cycle is completed, the erythrocyte ruptures releasing the merozoites into the blood stream and determining the typical malaria paroxysm. The merozoites discharged into the circulation invade new erythrocytes to repeat the schizogonic cycle until the process is inhibited by the specific immune response or by chemotherapy.

The erythrocytic stages of malaria parasites has several important implications in clinical practice; first, this is the only stage causing the complex and varying spectrum of symptoms characterizing the disease in humans, secondly, the recognition of parasites in the blood of a patient allows the diagnosis of the infection and the differentiation of the various species of the causing agent. The time required to complete the erythrocytic cycle is a fixed characteristic of the parasite species; *P. falciparum*, and *P. vivax*, have a 48-hour development period, in P.ovale it lasts 50 hours, 72 hours *P. malariae* and 24hours for P. Knowlesi. In practice, however, the typical periodicity of malaria paroxysm cannot be recognized in the initial periods, since most parasite populations are heterogonous and continuous fever therefore results from the completion of asynchronized schizogonic cycles.

- **Gametocytogenesis**

In the course of a schizogonic cycle (within a red blood cell) some of the merozoites become differentiated into sexual forms (the gametocytes). The first stage of the maturation process is the ring form. In *P. falciparum*, the ring form has variable size, the smallest are only 1.2µm in diameter, tend to adhere to the internal surface of the erythrocyte and the nucleus is often divided to show two chromatin dots. The ring form is the only asexual stage usually identifiable in the peripheral blood of patients with *P. falciparum* infection because more mature stages of this species adhere to the

endothelium of post-capillary venules in the tissue. *P. malariae* trophozoites are regular in shape (except those assuming a characteristic band form across the erythrocyte), with a small vacuole, early and abundant pigment, no stippling.

Two types of gametocytes are found in the peripheral blood, the female macro-gametocytes and the male micro-gametocytes. They can be differentiated by the fact that in the male parasite nuclear material is dispersed while in the female parasite it is condensed. These gametocytes are necessary for the perpetuation of the population however, for this to happen (because they cannot leave the blood stream on their own) they need an outside help from the female Anopheles mosquitoes.

- **The sporogonic cycle in the mosquito**

Female mosquitoes seek a blood meal as a protein source for egg production. For the parasites to be transmitted the mosquito needs to bite twice, firstly to become infected, and secondly to infect, after completion of sporogonic development [2]. In the mosquito, the parasite undergoes gametogenesis, fertilization and sporogony. When the mosquito feeds on human, the merozoites drawn from the bloodstream are digested in the stomach of the mosquito while the gametocytes develop in the intestine into mature cells called gametes, the female ovule and the male spermatozoon (gametogenesis). As soon as gametocyte reaches the midgut of the insect the female gametocyte shed the red blood cell and remains free in the extracellular space as a macrogamete. The male gametocyte nucleus divides into eight sperm-like flagellated microgametes each of which also leaves the erythrocyte reaches the midgut and actively moves to fertilize a macrogamete. Exflagellation of the microgametocyte is triggered by factors present in the mosquito midgut and begins about ten minutes after the blood meal. The result of the fertilization process is the zygote, which develops into the elongated, slowly motile diploid ookinete within 18 hours from the blood meal. The ookinete actively penetrates the peritrophic membrane and the epithelium of the midgut and settles beneath the basal lamina of the outer gut wall, where it develops into a non motile oocyst [62]. The product of the mature oocyst is the sporozoites, narrow and curved in shape, actively motile, 10-15 μm in length. The sporozoites actively leave the cyst passing through small perforations without destroying the wall, at least till most of the parasites have

been released, and move into the haemocelomic space of the insect. The sporozoites migrate and reach the salivary glands where they penetrate the basal membrane, pass intracellularly through a secretory cell and settles in the salivary duct with the aid of adhesion molecules like TRAP and CSP [59]. When the mosquito feeds, the salivary fluid (which has anti-clotting properties) and its content of sporozoites are actively injected into the vertebrate host to start another asexual replicative cycle.

2.2.3 Transmission and Endemicity

Endemicity refers to a situation where disease is habitually present within a given geographic area. Malaria endemicity can be classified using the transmission intensity, the entomological inoculation rate (EIR) and clinical parameters.

Transmission intensity: According to MacDonald (1957), malaria intensity can be categorized into two types, which are stable and unstable malaria.

Stable malaria refers to a situation where the population is continuously, exposed to a constant rate of malaria inoculation. The level of infection is sufficiently high to engender a considerable level of clinical immunity (the presence of parasitaemia in the absence of malaria related symptoms) within a population. Unstable malaria appears when a population is exposed to an irregular transmission rate. In unstable malaria conditions, all age groups are at higher risks. The spatial and temporal characteristics of unstable malaria are associated with lower level of clinical immunity within a population and the propensity for epidemics. Travellers, upon return may suffer from drastic malaria disease if not treated. Clinical immunity occurs in the context of premunition often termed partial immunity [9]. It is defined as the presence of an immune response that produces control of but not complete elimination of parasitaemia. Epidemic malaria, which is an extreme form of unstable malaria, can be described as a sharp increase in the frequency of malaria transmission, in excess of normal expectancy.Clinical endemicity: Endemicity in a given setting can be measured by:

(i) Spleen rate (SR): proportion of individuals (usually children) with an enlarged palpable spleen, per 100 individuals of similar ages

(ii) Parasites rate (PR): number of individuals(usually children) with parasitaemia per 100 individuals of similar ages

These last two parameters are important considering their impact or incidence at different level in different areas, and this is often denoted as:

(i) Holoendemic: transmission occurs all year long

(ii) Hyperendemic: transmission is seasonal but intense

(iii) Mesoendemic: regular seasonal transmission

(iv) Hypoendemic: very intermittent transmission

Entomological inoculation rate (EIR), more precise than the previous, is used to measure transmission intensity within a given area and it is now considered the gold standard by WHO [63]. Nevertheless, it lacks some information especially in setting of very low transmission. Therefore, the use of spleen rate and plasmodial rate as indicators is justified. Entomological inoculation rate (EIR) is the number of infected-mosquitoes bites received per person and per unit time(day, week or year). Beier and colleagues [64] reported on the relationships between entomological inoculation rate and malaria *P. falciparum* prevalence from 31 sites in Africa (Table 2.2).

Table 2.1: Relationship between spleen rate, parasitic rate, and EIR and endemicity level in children aged 2 to 9 years

Zones	**Spleen rate**	**Plasmodial rate**	**EIR**
Holoendemic	>75	>75	>100
Hyperendemic	50-75	50-75	11-100
Mesoendemic	10-50	10-50	1-10
Hypoendemic	1-10	1-10	<1

Legend: EIR: entomological inoculation rate

2.4 Genetic Diversity of plamosdium falciparum

Plasmodium falciparum shows high degree of diversity. This parasite is able to change into many different allelic forms of its antigenic proteins while still maintaining the biological function. This increases the ability of the parasite population as a whole to evade immune responses. Diversity of malaria parasite occurs through random mutations when a population undergoes frequent constrictions (i.e. a genetic bottleneck, by drug selection pressure) and subsequent clonal expansion [65]. Furthermore, genetic recombination occurs in mosquitoes during the diploid short phase following fertilisation. When a mosquito ingests gametocytes, from genetically different parasites, meiotic recombination favours the exchange of genetic materials (cross- fertilisation) finally producing new allelic combinations and haplotypes [66]. High malaria transmission areas favour cross-fertilisation where human parasitaemia is frequently polyclonal, whereas low transmission areas favour self-fertilisation with offspring genotypes identical to parent's genotypes [66]. Human population flow also favours genetic diversity of parasite population [67]. However, genetic diversity of *P. falciparum* can be reduced by immune (but not always) or drug pressure, which thus selects parasites that harbour genes conferring resistance to antimalarials drugs. *Plasmodium falciparum* is the most virulent of the five parasites which cause malaria in humans. The inherent variability of *P. falciparum* is particularly prevalent in merozoite surface antigens being targeted for malaria vaccines [68]. This provides multiple effective evasion and drug resistance mechanisms for the parasite. It also represents a major challenge for development of an effective malaria vaccine [69]. Due to the high degree of variation of the surface protein, they have been used to assess the genetic diversity of the parasite population and also to evaluate antimalarial drug efficacy distinguishing recrudescence from reinfection [70] [71].

Genetic diversity is usually expressed in terms of complexity of infection or multiplicity of infection which can be related to endemicity. In effect, the higher the multiplicity of infection the higher the number and differences of circulating *P. falciparum* genotypes and the higher the transmission level.

2.4.1 Immune pressure and evasion of *P.falciparum* from

Different parasite clones may vary significantly in immunogenicity, immune-avoidance mechanisms, susceptibility to drugs, and transmissibility by different Anopheles mosquito vector species.

Extensive sequence variation is a principal characteristic of *P. falciparum* as it evades host's immune system. This evasion is ascribed to the presence of non-synonymous polymorphisms found within the genes concerned by these variations (csp, msp, glurp described later). The malaria parasite may use repetitive, immuno-dominant epitopes as a mechanism to evade the immune response of the human host [72] and many of malaria antigens contain tandem arrays of relatively short sequences. It is important to recall one of the main properties of the genetic code, which is degeneracy. This means that mutations in a codon changes (non-synonymous) or not (synonymous) the encoded amino-acid. Maintenance of degeneracy within a repeat set, and the existence of cross-reacting epitopes in many genes of *P. falciparum* as a consequence of short repetitive sequences and the biased amino acid composition, have been suggested to interfere with the normal maturation of high affinity antibodies. The existence of cross reacting antibodies has often complicated the problem of identifying specific Plasmodium gene products and protective immune responses. In addition, repetitive epitopes may induce T-cell independent B-cell activation [73] suppressing antibody production to more relevant parts of the antigen.

Host immune system leads to diversifying selection, which can be measured in terms of the ratio of non-synonymous to synonymous single nucleotide polymorphisms (SNPs) (pN/pS) [59] [60]. The complexity of the parasite life cycle complicates the development of vaccine as at every stage of the life cycle there is an immune response induced with a number of antigenic proteins [75]. The expression of these multiple specialized proteins during the life cycle is necessary for the parasite to survive in both the mosquito and human hosts' organisms, as well as for the invasion of different types of cells [76]. As noted by Ayala and coluzzi [3] *P. falciparum* has co-adapted and co-specialized with *Anopheles* Gambiae; properties that likely apply in the case of parasite and its human host. It seems as demonstrated by mathematical model integrating

multiple competing parasite strains of varying virulence subjected to selective immune pressure, that the host immune system select for more virulent strain especially in a context where immunity is partial [77].

2.4.2 Immunogenicity

At every stage of the life cycle of malaria parasite there is an induced immune response, meaning the implication of different specialized proteins. It is obvious that these different responses call for different types of epitopes and single nucleotides polymorphisms (SNPs) that can help measuring the immunogenicity (ability of an antigen to induce an immune response) of these proteins. Krzyczmonik and colleagues [74] analysed series of epitopes and SNPs to compare the immunogenicity across developmental stages and different protein classes from Plasmodium. It was found that antigenic proteins produced at the sporozoite/stage-specific level were higher in their number and were under strong immune pressure as indicated by the pN/pS ratio compared to the merzoite for example. This suggests that the immune response induced by sporozoites/stage-specific proteins will as well be higher compared to that of others. Immunogenicity can also be ascribed to compensatory mutations that when selected increase the virulence of the parasite than that of the normal wild type as described below in the presence of continuous drug pressure.

2.4.3 Drug pressure

Resistance is defined as the ability of a parasite to survive or multiply in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication. Three levels of resistance are defined by the WHO; following treatment, parasitaemia clears but a recrudescence occurs; following treatment, there is a reduction but not a clearance of parasitaemia and following treatment, there is no reduction in parasitaemia [78].

Drug pressure is one of those factors that lead to mutation in the malaria parasite. This is mainly related to host parasite interactions and development of protective mechanisms by the parasites. More specifically, because of deletions, insertions or

substitutions in certain genes, single nucleotide polymorphisms (SNPs) have been identified in *P. falciparum* clones which confer resistance to antimalarial drugs. These SNPs in many cases are specifically non-synonymous mutations. Drug pressure can reduce genetic diversity but not always. As a matter of fact in the presence of drug pressure the level of sensitive parasite will decline whereas resistant clones will be selected, which complicates the picture of treatment as clones harboring genes conferring resistance are of clinical interest [37] [79].

Drug pressure can also be detrimental for the parasites in that it inserts mutations that are deleterious for the parasite metabolism as it has been found with resistance to antifolates whereby, mutations at codon 108 and 59 reduce the affinity of the enzymes reductase and synthase enzymes for their natural substrates [80]. Nevertheless, under long term drug pressure some mutations qualified as secondary mutations can surface and complement for the fitness cost due to primary mutations. These secondary mutations like in the case of HIV-1 can increase the virulence or the pathogenicity of the parasite [28]. Apparently not many of these mutations have been categorized as compared to HIV-1. Selection of these compensatory mutations would therefore be beneficial for the parasite to live and this is possible only if the population of *P. falciparum* rises to a sufficient amount 10^{10}-10^{12} that can select for compensatory mutations [81]. A phenomenon termed as competitive Inhibition has been proposed by Bialasiewicz et al [82]. In the context of high malaria transmission with a greater than tenfold differences in the parasitaemia in a mixed infection the species with the greater concentration of DNA will be the only one to be detected. It is likely that this competitive inhibition may take place for the selection of compensatory mutations.

2.5 Molecular diagnosis/genotyping of *Plasmodium falciparum*

From a general point of view, the understanding of the epidemiology of a disease has been hampered by poor knowledge of the pathogen structures [83]. It is worth noting that the pathogenicity or the virulence of many infectious agents depends greatly on the type of genotypes involved as these pathogens can be subject to variations of any sort (gene polymorphism or sequence variation for example). High malaria transmission

areas for example favor cross-fertilization or genetic recombination while low transmission areas favor self-fertilization with offspring genotypes identical to parents' genotypes. Appropriate detection or diagnosis of the incriminated species or strain remains capital as this can bias treatment outcomes in the long/short-term. For the diagnosis of malaria, specifically designed primers with targeted genes have been used to differentiate between human Plasmodium species (speciation PCR). These genes are found in either the nucleus, or the mitochondrion or the plastid (apicoplast) of the genus Plasmodium and generally before a specific primer is designed, a given gene inside one these structures should first have its sequence established [84]. It therefore appears obvious and imperative to study the structural organization (genomics, proteomics) of a pathogen in order to design tools that will help in diagnosis. As a matter of fact Sulaiman and colleagues[83], by studying the phylogenetic relationship between cryptosporidium (Apicomplexan) parasites came to the conclusion that the 70-Kilodalton Heat-Shock Protein (HSP70) gene compared to the 18-Small-Subunit ribosomal RNA gene (SSU rRNA) presented several advantages making a better marker for genotyping.

Molecular phylogenetic stands as a way to predict the function of a gene. It is generally hypothesized in terms of homology (similarity attributed to descent from a common ancestor) that genes with similar sequences will display similar types of functions or regulations. Similarity refers to the extent to which two nucleotides or protein sequences are related. The extent of similarity between two sequences can be based on percent sequence identity and/or conservation.

2.5.1 Structural organization: considerations and implications for genotyping

Care must be taken in distinguishing malaria infection especially as co-infection cases are often misdiagnosed as single, and whereby the related treatment causes rebound of one species. The same apply for multiple genotypes infections where selection of resistant strains occurs favoring its dissemination [85].

One of the principal characteristics of *P. falciparum* is its capability of evading the immune system by eliciting non-synonymous nucleotides polymorphisms (change in the encoded amino acids) [3]. Parasites genes that code for antigenic determinants have been isolated and characterized and notably chosen for vaccine development. These are circumsporozoite protein (csp) encoding surface proteins of the sporozoite, and merozoites for msp-1 and msp-2. The glutamate rich protein, GLURP has also been characterized. The msp-3 has been used for the genotyping of *P. vivax* [86].

- ***The merozoite surface protein 1 (msp-1)***

The merozoite surface protein 1 (msp 1) gene is divided into 17 blocks, based on analysis of sequence diversity; seven highly variable blocks are interspersed with five conserved and five semi-conserved region [87]. A major mechanism for the generation of allelic diversity in the *P. falciparum* msp 1 gene is meiotic recombination in the Anopheles mosquito, which is believed to be dependent on the intensity of transmission. It is suggested that frequent recombination events between msp 1 alleles intermittently generate novel alleles in high transmission areas [68]. This gene is dimorphic in each block, and the alleles have been designated as K1 and MAD20 except in block 2, where there is a third allele known as RO33 [68]. Several studies have reported that msp 1 allelic variants fall under three major types; MAD20, K1 and RO33 but their frequency varies in different geographical areas, even in neighbouring villages [68]. The three alleles establish themselves depending on the presence, type, and number of tripeptide repetitions found in the sequence of this block. Block 2 of the msp 1 gene appears to be subjected to rapid intragenic recombination, and so is highly polymorphic.

- ***The merozoite surface protein 2 (msp-2)***

This gene also known as merozoite surface antigen (msa-2) gene codes for a merozoite surface polymorphic glycoprotein that has been widely studied as one of the major vaccine candidates [87]. The sequencing of DNA has shown that a single copy of msp 2 gene has conserved N- and C terminal domains (blocks 1 and 5), two non-repetitive variable regions (blocks 2 and 4), and a polymorphic central region (block 3) containing variable numbers of tandem repeats, which also vary in sequence and length. Genes in

which polymorphism has arisen through intragenic recombination in repetitive segments are characterized by repeat motifs with length variability differing between strains [87], [88]. Msp 2 has been widely used to characterize *P. falciparum* field isolates, and some authors have reported that it is highly discriminatory and have used it alone to characterize *P. falciparum* populations [89]. However, the use of only one marker, no matter how polymorphic it is, would miss variation at other polymorphic loci, and thus, almost certainly underestimate the magnitude of multiple infections. Nevertheless, the choice of a particular gene marker for typing natural *P. falciparum* clones depends on the question being addressed. Msp 2 alleles, which differ in number and sequence of intragenic repeats, can be grouped into two allelic families, FC27 and 3D7/IC (Figure 2.1) according to the central dimorphic domain as first observed over a decade ago [95].

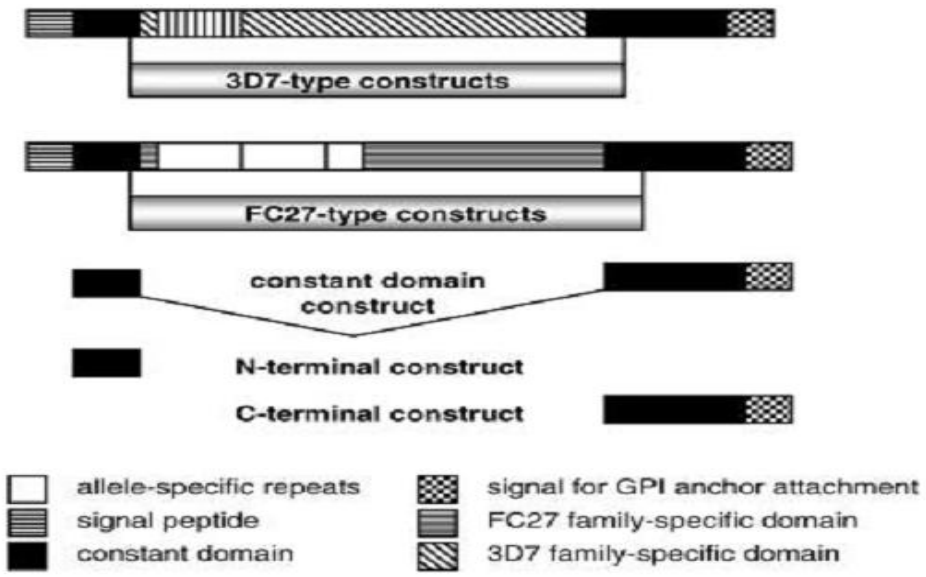


Figure 2.2: Structure of the two families of msp-2 gene, 3D7 and FC27.

- ***The P. falciparum glutamate rich protein (glurp)***

This is another antigen considered to be one of the leading malaria vaccine candidates [90] Glurp is expressed in all stages of the parasite life cycle in humans, including the surface of newly released merozoites. It is highly antigenic with only one allelic family and the gene encoding glurp shows polymorphism in geographically different *P. falciparum* isolates [91]. It is a 220 kD protein expressed in the hepatic, asexual and sexual stages of the parasite life cycle. The protein can be divided into an N-terminal non-repeat region (R25–500 or R0), a central repeat region (R1) and a C-terminal repeat region (R2) [90]. Single nucleotide polymorphisms (SNPs) contribute largely to the variability of *P. falciparum* and provide multiple effective evasion and drug resistance mechanisms for the parasite necessitating the use of molecular techniques to differentiate alleles responsible for recrudescence and re-infection after treatment [92].

These genetic markers are unlinked, i.e. located on different chromosomes, single-copy genes with extensive polymorphism, both with regards to sequence and size, are mostly generated by intragenic repeats that are variable in copy number and length of the repeat unit. As such they have proven to be useful tools both in molecular epidemiology studies in different epidemiological settings as well as to distinguish treatment failures from new infections in antimalarial drug trials [68]. Other genes found in *P. falciparum* genome are microsatellites genes and resistant genes. The latter will be discussed below.

Study the polyclonality (using polymorphic markers) of *P. falciparum* population is of great clinical interest as minority clones may harbor genes conferring resistance to antimalarial therapy that can be selected and therefore contributing to treatment failure [79]. This analysis permits to distinguish between recurrent infections and new infections in clinical trials of antimalarials drugs by using the so called PCR-correction. However this technique (PCR genotyping) has shown drawbacks in that the population of minority clones is underrepresented [93]. These drawbacks lead to misclassification of PCR data in clinical trials and underestimation of the extent of allelic polymorphism in any given infection [94]. A powerful and robust technique, genome sequencing, has

been proposed and used to circumvent these limitations [93]. The authors concluded that the technique provide high quality data useful for drug resistance studies, and robustly represents clonal multiplicity.

2.5.2. Molecular Phylogenetic as a way to predict resistance patterns or spread

As previously mentioned, phylogenetic can help to predict (inference) the function of a given gene after this gene has been aligned with a reference gene. This is how some genes or species have been classified as mutant types with respect to the wild type (reference type). The use of molecular systematic has permitted to classify variants of species on the basis of their sequence homology, which also determines their severity in disease development. This has been the case with the human papillomavirus (HPV) that causes cervical cancer. On the basis of their homology it was found that the HPV16 and 18 are highly oncogenic compared to others, less oncogenic.[95]

The spread of antimalarial resistance since its evolution is worrisome especially knowing that the new medication or ACTs are at stake. Indeed some ACTs (Sulphamethoxypyrazine-pyremithamine/Co-Arinate®) have as partner drugs sulfadoxine or pyremithamine which resistance has been reported in many instances when coupled to amodiaquine or not [32] [96]. Resistance to ACTs has been reported in Asia, Thai-cambodia and in some areas in Africa and the spread of resistance is matter of debate among the scientific communities. Understanding the spatial clustering of *Plasmodium falciparum* populations can assist efforts to contain drug-resistant parasites and maintain the efficacy of future drugs (Taylor et al., 2013). These authors have demonstrated using microsatellite markers, proven to be more sensitive than other markers [97], that populations in the West manifested dhps mutant (resistant) haplotypes with independent lineages and with single-mutant SGKA and AGKA haplotypes. Compared to the East these haplotypes were largely unrelated to the double- and triple-mutant SGEA and SGEG. The authors suggested that there is significant barrier to parasite population flow between the East and the West of Democratic Republic of Congo and *P. falciparum* sulfadoxine resistance is geographically and genetically clustered within the DRC. Another study of the same kind but not confined to one country in 2009 studies the evolutionary origins of dhps

mutations [14]. The latter characterized five major lineages with the geographical distribution of dhps resistant alleles mutations: SGK (Serine-Glycine-Lysine), AGK (Alanine-Glycine-Lysine), and SGE (Serine-Glycine-Glutamate), wild-type alleles AAK (Alanine-Alanine-Lysine) and SAK (Serine-Alanine-Lysine). The authors suggested that there has been dispersal throughout west and central Africa from their original foci, with Cameroon at the confluence of west, central, and southwest African gene pools. These findings show that sulfadoxine-resistant *P. falciparum* has recently emerged independently at multiple sites in Africa and that the molecular basis for sulfadoxine resistance is different in east and West Africa. This latter result may have clinical implications because it suggests that the effectiveness of sulfadoxine as an antimalarial drug may vary across the continent. Furthermore, these findings suggest that economic and transport infrastructures may have played a role in governing recent parasite dispersal across the continent by affecting human migration. Thus, coordinated malaria control campaigns across socioeconomically linked areas in Africa may reduce the African malaria burden more effectively than campaigns that are confined to national territories.

2.6 Anti Malarial Efficacy and in vitro and in vivo tests

Several methods for monitoring antimalarial drug efficacy exist; they include in vivo and in vitro tests and, more recently, molecular markers. In vivo tests are traditionally the “gold standard” method for detecting drug resistance (WHO, 1996). Standardized by WHO these tests reflect the biological nature of antimalarial treatments. Malaria patients are selected, and monitored clinically and biologically for 28 or 42 days after the treatment regimen received. These longitudinal studies measure the incidence of malaria in the real world. The incidence of treatment episodes is an outcome that is highly relevant to public health policy makers, as it reflects not only the burden of disease but also the utilization of health resources.

The advantage of the in vivo tests over the in vitro assays is that they can be conducted in the field with little equipment and personnel and the results are easy to interpret. They reflect the true biological nature of treatment response, which involves a

complex interaction between the parasites, the drugs, and the host response, while in vitro tests measure only the interaction between the parasites and the drugs. Although the information gained from in vivo studies is exactly what is needed to make rational and evidence-based malaria treatment policies, standard in vivo studies remain expensive and time-consuming, and longitudinal clinical efficacy trials are even more so.

In vitro assays are based on the inhibition of the growth and development of malaria parasites by different concentrations of a given drug relative to drug-free controls. As many countries resort to combination therapies to increase treatment efficacy and delay the emergence of drug-resistant parasites, monitoring the efficacy of individual components in drug combinations by in vitro drug sensitivity assays and molecular markers is helpful [98].The in vitro testing plays a role in detecting the early stages of resistance and has become a complementary tool for the surveillance of drug resistance. In vitro assays have the advantage of yielding objective results of parasite responses to drugs without any interference of host factors, including pharmacokinetics, acquired immunity, and patient compliance with therapy. The variations in parasite density and haematocrit (the inoculum effect) as well as the stage-dependent action of antimalarial drugs must be controlled because they cause a significant impact on the outcome of these assays [99].

Parasites are cultured in erythrocytes in the presence of RPMI 1640 media added to already dose 96 well plates. Assays are run in duplicate for each drug, and placed in a candle jar at 37ºC for 72 hours. In vitro drug sensitivity tests are all based on measurement of the effect of drugs on the growth and development of malaria parasites. These are parameters that can be measured in several different ways and each has exceptional characteristics. Parasite growth in drug-exposed cultures is measured relative to drug- free controls. When performed with serial dilution of drugs, these tests will result in sigmoid dose-response curves. These sigmoidals curves sometimes permit to evaluate the synergistic and the antagonistic effects in terms of isobolograms. However, in vitro assays have several problems and drawbacks. (i) They require highly skilled personnel and laboratory equipment. (ii) Parasites isolated from

patients who have taken medication on their own initiative a few days before consultation usually do not grow in vitro (iii) there is no consensus about the determination of the threshold Inhibition Concentration IC50 that distinguishes susceptible from resistant parasites.

Consequently, in vitro tests provide little information on the efficacy of the drug. There is poor correlation between in vivo and in vitro test results, especially in areas of intense transmission, presumably due to the influence of host immunity. The accuracy of the inhibitory concentrations for a given sample is also influenced humoral factors from the donor that can interfere with parasite maturation [100]. Despite these shortcomings, in vitro tests are of value, particularly for testing parasite resistance to new drugs and agents that have not been used previously. They can provide important longitudinal data on changes in parasite response to drugs, which is important collateral information about the emergence and spread of drug resistance. Methods of in vitro drug sensitivity assays include the following: WHO microtest, isotopic (tritiated hypoxanthine uptake) assay, lactate dehydrogenase (pLDH), histidine-rich protein 2(HRP2), SYBER Green I and other fluorescent dyes.

The limitations of in vivo and in vitro methods for measuring drug-resistant malaria and the elucidation of molecular mechanisms of resistance to some antimalarial drugs have led to considerable research on molecular markers for resistance. A number of techniques based on molecular amplification of regions in the parasite genome that are different between species, strains and resistant types have been envisaged.

2.7 Factors affecting Anti malarial Efficacy and Parasite clearance

2.7.1 Human factors (age, immunity)

Genetic diversity is thought to be influenced by age due to variability of acquired immunity in the population whereby acquired immunity is absent in new born children and only acquire partial immunity six months later which is fully developed in adulthood. Till date there is no consensus on the distribution of *P. falciparum* genetic diversity related to age. As immunity increases parasite counts are lower, severe malaria is less common, and parasite clearance is accelerated. In endemic areas this is reflected in the

differing clinical presentations and therapeutic responses with increasing age. Conversely as immunity declines, for example if transmission is reduced, then parasite clearance rates reduce [101]. Naturally acquired immunity has been found to accelerate parasite clearance in response to artesunate and the half-life decreases with age [102]. This parasite clearance is mediated by immunoglobulins IgG that recognize parasitized red blood cells (RBCs) (opsonisation) and have the ability to block cytoadherence or sequestration of parasites. The proportion of IgG was found to inversely correlate with the ½ life of parasite clearance and in an age dependent manner [15] in children in Mali. In fact, artemisinins half-life decreased by 4.1min for every one year increase in age.

A study published in 2011 [103] investigated on the relationship between host candidate gene polymorphisms and clearance of drug-resistant *Plasmodium falciparum* parasites across five large association studies from Burkina Faso, Cameroon, Kenya, Mali and Sudan. Among the 70 SNPs investigated and after adjustments for confounding factors such as age and ethnicity and gender only three polymorphisms were significantly associated with clearance phenotypes of drug-resistant parasites. These polymorphisms were related with the genes coding for interferon regulatory factor 1 (AC or CC genotypes), interleukin 4 receptor gene IL4R (TT or TC), the Der1-like domain family gene Derl3 (AG or GG).

It is generally known that in endemic areas children under five years old are the most affected because of the immune system not yet fully developed. In the other hand it has been suggested that children under six months have a lower incidence of severe malaria compared to older ones because of the presence of antibodies acquired through breast-feeding (probably Immunoglobulins). Maternal milk lacks PABA which is required for the malaria parasite metabolism [20]

In endemic settings individuals are mostly exposed to mixed malaria parasites co-infections. Coinfection with *P. falciparum* and *P. malariae* has been reported in Bangolan, North-West region of Cameroon (Achonduh et al. manuscript submitted) among women and, children as from 2months. Most of these populations had asymptomatic malaria. *Plasmodium falciparum* dynamics or pathogenicity has been reported to be modulated (decrease parasitaemia) by the mild infection *P. malariae*

through non-specific and cross-specific immune response [104]. Clearly, *P. falciparum* population is reduced (competitive inhibition). Conversely [105] reported that *P. malariae* do instead increase *P. falciparum* gametocytaemia, which in this case may raise concern with regard to the spread of the most dangerous of human malaria parasites. It is not however well described whether this decrease or increase in P. falciaprum asexual or gametocytes is age dependent.

2.7.2 The implication of Pharmacogenomics and polymorphism: Principles

The quantitative role of enzymes responsible for drug metabolism, the kinetics of the drug and its ‘’therapeutic window’’, help determine how to adjust drug dose in poor metabolisers or ultra-fast. Not knowing the genotype or phenotype of the patient who is administered a standard drug dose may result in overdose in slow metabolisers and will be more likely to develop toxicity, while ultra-fast metabolisers are under-dosed. Another situation is where the therapeutic effect depends on the formation of an active metabolite, so we do not observe the therapeutic response in poor metabolisers, while we observe an amplified response in ultra-rapid metabolisers [106] Recently several drugs have been withdrawn from the market because of severe side effects associated with taking them..

- **Pharmacokinetics and polymorphisms**

When drugs enter the body, their destiny is determined by the absorption, distribution, metabolism and elimination steps. The majority of pharmacogenetic differences have been characterized at the molecular level until now for genetic variations in enzymes responsible for drug metabolism. The pharmacokinetics was then the first field of clinical research to apply pharmacogenomics and is currently the most active in this regard.

- **Phase I enzymes**

Phase I metabolism is the functionalization phase reactions involving oxidation, reduction and hydrolysis of xenobiotics. Most enzymes involved in the metabolism and elimination of drugs are part of the cytochrome P450 (CYP450). However, many examples of polymorphisms are found in these enzymes [107].

- **Phase II enzymes**

Phase II metabolism in the conjugation phase for glucuronidation reactions, sulfonation and acetylation. N-acetyltransferase type 2 (NAT2), was one of the first phase II enzymes discovered as polymorphic. Indeed, pharmacogenomic differences in NAT2 gene (encoding the N-acetyltransferase type 2), are responsible for a metabolic polymorphism at the N-acetylation of primary metabolites and induce two groups of individuals having two phenotypes: the rapid acetylators and slow acetylators. Other enzymes of phase II metabolism are: glucuronosyltransferases (UPDGTs), glutathione-S-transferases (GSTs), sulfotransferases, the thiopurine methyltransferase (TPMTs) are also polymorphic [107].

- **Metabolism of phase I and phase II enzymes**

The frequencies of genetic variations depend on ethnic populations. It is therefore essential to consider the ethnic characteristics to improve the diagnosis and care of patients by providing information on the structure of genes and regulatory pathways that can lead to impaired response. The potential consequences of polymorphisms in drug metabolism can be: a prolonged pharmacological effect, side effects, lack of activation of the pro-drug toxicity, an increase in the effective dose, drug interactions exacerbated. Indeed, the genetic variability of expression levels or function of these enzymes has a very important impact on the effectiveness of the drug [107].

  - **N-acetyl transferase 2 gene**

The enzyme N-acetyltransferase 2 (NAT-2) is involved in Phase 2 of the biotransformation of xenobiotics and catalyzes the transfer of an acetyl group from acetyl coenzyme A to certain drugs and other xenobiotics with arylamine structure [108]. Therefore, the active enzyme detoxifies drugs such as isoniazid, anti arrhythmic drug proainamide (PA), anti-inflammatory drug 5-aminosalicylic acid (5-ASA), and dapsone, a drug used for the treatment of malaria.

- **Chromosomal Location of NAT2 gene**

The NAT2 gene has 36 alleles and is found in the liver of adults. It is located on the chromosome pair N0 8 [109].

- **Regulation of expression of the NAT2 gene**

Rather et al.[110] have shown by cloning the NAT2 gene that transcription begins at the initiation site (G) in position 233 Pb, and the recognition sites are located in the promoter -10 (TATAAT) and -35 (CTTTTT). The transcript of this gene is induced by xenobiotics [110].

- **NAT2 gene polymorphism**

Longuemaux and colleagues [109] have shown using RFLP-PCR that the NAT2 gene contains 36 alleles and in these alleles come first, haplotype variations of seven mutations (G191A, T341C, A434C, G590A, A803G, A845C and G857A) and five silent mutations (T111C, C282T, C481T, C759T and A803G), 2 substitutions (single-nucleotide), G499A and C190T.

The wild-type allele NAT2 designated NAT2*4 does not have a mutation and is associated with rapid acetylation. NAT alleles NAT2*5 such as, NAT2*6, and NAT2*7*14 NAT2 respectively containing mutations G191A, T341C, A434C and / or are associated with G590A slow acetylation. Alleles associated with rapid acetylation are: NAT2*11, NAT2*12 and NAT2*13. The allelic polymorphism of the NAT2 gene is also based on ethnicity. The different types of NAT2 genotypes are given in Annex 1.

2.7.3 Ecological Factors (ethnicity, endemicity, climate)

Ethnicity has been shown to have an impact on resistance or susceptibility to malaria. The genetic background (lactase persistence) related to their diet has made the Fulani tribe to be resistant to malaria compared to the Dogon tribe who consumes less cow milk than the Fulani do [20]. Milk diet is less rich in P-aminobenzoic acid (PABA), which the parasite needs for its metabolism. In the absence of this, the parasite synthesizes PABA de-novo which still, seems not to be sufficient in the context of Fulani tribes and therefore the parasite is easily suppressed.

It has been found that ethnicity may also have a major impact on drug metabolism and hence drug efficacy and safety. Differences in ethnicity have a deep impact on drug clearance due to pharmacogenetic polymorphism in drug metabolizing enzymes and transporters, or drug targets. Alterations in clearance can have an impact on safety, efficacy and dosing regimen. Certain enzymes polymorphism associated with enhanced or decreased drug metabolism has been characterized with frequencies and types varying among ethnic groups [18]. The latter analysed a series of alleles polymorphisms in genes encoding enzymes involved in the metabolism of antimalarials, namely the cytochrome (CYP) P450 isoenzymes and NAT-2 genes in samples from Cambodia and Tanzania, two different ethnic groups. It was suggested that the CYP3A4*1B allele frequency between the two populations presents a potential explanation for the lower efficacy of arthemeter-lumefantrine (AL) in Cambodia.

Endemicity as mentioned earlier plays a role in the development of acquired immunity and the increase of parasites population dynamics with the consequence on sensitive or minority undectable clones that may harbor genes conferring resistance. More so in high endemic areas people tend to be infected with more than one parasite, which coinfection can modulate the effect of the strongest by lowering is peripheral blood density. Malaria intensity is in the other hand favored by factors such as climate, high atmospheric humidity and social environments or urbanity. These factors in one way or the other facilitate the spread of the disease.

Climate or vegetation favors breeding sites for mosquitoes, which vectorial capacity can be such that more virulent parasites can be transmitted [2]. For the disease to be transmitted the vector survival should be long enough because it is currently affected by the virulence (apoptosis) caused by the parasite [62]. In low transmission areas it was found that *P. falciparum* genotypes that are highly diverse clustered with the rainy season confirming the seasonal distribution of *P. falciparum* genotypes [111]. Most importantly to consider here is the possibility of mosquitoes to transmit parasites that harbor resistance genes and is of concern today to see how much measures are being deployed to study the evolution and the spread of resistance as proposed by some phylogenetic and phylogeography studies.

2.7.4 Parasite factors (resistant genes, life cycle)

These are useful tools that complement phenotypic assays for drug resistance. They also guide the design of strategies to avoid such resistance once it has reached levels of clinical significance. Molecular markers theoretically offer the earliest way to detect emerging drug resistance and intervene accordingly, since they examine fundamental processes in the resistance pathway.

- **Mutations in the dhfr gene and resistance to pyrimethamine**

In vitro resistance to pyremithamine is almost always associated with Ser108Asn domain mutation of DHFR [112][113]. Levels of higher in vitro resistance result from sporadic mutations at codons Asn51Ile, Cys59arg and / or Ile164Leu. The association between the phenotype of in vitro resistance to pyrimethamine and DHFR genotype has been demonstrated by studies of site-directed mutagenesis and transfection experiments. However an alternative mutation causing replacement of a serine by a threonine at codon 108 with a change of an alanine to valine at position 16 may provide a specific resistance to cycloguanil [112][113]. Sequencing of pvdhfr gene in *Plasmodium vivax* showed mutations at position 58 and 117 of the DHFR protein in parasites resistant to pyrimethamine and cycloguanil. These amino acid changes were similar to changes observed in the DHFR protein of *P. falciparum*, respectively with arginine at position 59 and asparagines at position 108.

Recent studies have shown that pyrimethamine and cycloguanil are significantly less active vis-à-vis the mutated forms of DHFR in *P. vivax* [112] These results confirm the results of work on the kinetics of enzyme inhibition of DHFR in *P. vivax*. The affinity is reduced between mutations of Ser58Arg and Ser117Asn of pvdhfr and two antimalarial drugs, pyrimethamine and cycloguanil. This may be the cause of resistance to antifolinic in *P. vivax*.

- **Mutations in dhps gene and resistance to sulfadoxine**

In the same way of *in vitro* resistance to pyrimethamine, resistance to sulfadoxine appears to be associated with Ala437Gly point mutation in field study of DHPS. However, higher levels of resistance to sulfadoxine are associated with additional levels

of codons Ala586Gly, Ser436Phe and Ala613Ser [114], [115].However an additional mutation at codon 540 is correlated with increased levels of resistance to SDX-PYR association [116].

- **Mutations in the Pfdhfr and pfdhps genes and resistance to sulfadoxine-pyrimethamine combination (Fansidar ®)**

Fansidar ® became the first-line drug for the treatment of uncomplicated malaria in Africa after emergence of chloroquine resistance but its effectiveness was short, only 5 years in South East Asia, South America and Africa [37], [114]. Extension of CQ-R to P. falciaprum, led to the increase use of SDX-PYR combination in the treatment of uncomplicated malaria in many countries in Africa [114], [117]. In the case of SDX-PYR combination, these multiple mutations in two genes contribute to resistance in vitro of these two molecules, namely SDX and PYR, taken separately. It appears difficult to establish a series of mutations necessary and essential that cause resistance to the combination of these two molecules.

Thus, Gly-437 mutations followed by Glu-540 are frequently encountered as associated with resistance to SDX-PYR [118]. However, the risk of treatment failure with only one triple mutation 108, 51 and 59 at the PfDHFR gene is low. The targets genes for SDX and PYR are respectively, DHFR and DHPS. Sulphadoxine inhibits dihydropteroate synthase (dhps) gene, whereas Pyremithamine inhibits dihydrofolate reductase (dhfr) gene in the folate pathway of the parasite. This combination acts in synergy. These mutations alter the configuration of the active site and consequently reduce the affinity for active compound.

We might think that mutations in either of these two genes could be the cause of resistance to the association SDX-PYR, but studies have shown that mutations in the Pfdhf gene seems to appear first, followed by mutations on pfdhps gene. Thus, mutations in the pfdhps gene appear in parasites with a double or triple mutation in their gene Pfdhfr selected by the presence of pyrimethamine [119]. Similarly, mutations at codons 437, 540, 581 of pfdhps were mainly observed in areas where prevalence in pfdhfr mutations is high.

- **Genes involved in resistance to chloroquine, quinine, mefloquine and halofantrine**

    - **Genes involved in resistance to chloroquine**

The late appearance of chloroquine resistance in *P. falciparum* suggests a mechanism in which the molecular basis is more complex than the acquisition of a few point mutations as observed for genes involved in resistance to anti-metabolites. Indeed, the results of several studies have shown a multifactorial mechanism that requires simultaneous mutations on several genes located independently of each other on different chromosomes of *P. falciparum*. These are the pfmdr1 genes pfmdr2, pfcg2 and pfcrt.

        - **pfmdr1 and pfmdr2 genes**

Efflux and or lack of accumulation of amino-4-quinolines, by analogy to the mechanism of resistance in cancer cells, were suggested in 1990 ([120]). The phenotype MDR (multidrug resistant) of cancer cells was described for the first time in 1970 by Riehm Biedler who observed the occurrence of simultaneous cross-resistance to anticancer of several chemicals after selecting one resistant line of cancer cells to one class of anticancer. MDR is associated with the decrease in the intracellular accumulation of anticancer drugs as well as resistant cells expressing the P-glycoprotein encoded by the MDR [121] gene. Several observations showed a similar mechanism for chemoresistance in *P. falciparum* as accelerated efflux and / or slowed chloroquine accumulation in chloroquine-resistant parasitized red blood cells (pRBCs) rather than chloroquine- sensitive strains. Initially, it was assumed that changes in pfmdr1 genes and pfmdr2 were related to resistance to chloroquine. This has not been confirmed and instead assumes the involvement of other genetic and molecular factors. No correlation was found between alleles of these two genes and chloroquine resistance in studies of isolates from Sudan. Field studies have shown many exceptions regarding the idea of an association between pfmdr1/2 point mutations or 2 and CQ-R. However, amplification of pfmdr1 has been associated with resistance to mefloquine in *in vitro* studies[121]. These studies also showed that the pfmdr2 gene is neither mutated nor

amplified and its expression in sensitive or resistant parasites is not different, which reduces its role in resistance to antimalarial drugs. Other studies have shown a significant association between amino acid 86 of the pfmdr1 gene and CQ-R [122]and there is a correlation between in vitro response and resistance gene pfmdr1 in only 68% cases [123]. These results show that the codon 86 of the pfmdr1 gene may not be a suitable molecular marker to assess the CQ-R and requires the involvement of other codons of the pfmdr1 gene, but other studies have not shown their implication in the resistance [124]. However, the relation between the pfmdr1 gene and CQ-R suggests the implication of other genetic factors in *P. falciparum*.

- **The gene pfcg2**

Many studies have located the genetic locus governing CQ-R in a region of 400 kb on chromosome 7 of P. falciprum [124]. An extensive study on a restricted area of 36 kb of this locus by using a greater number of RFLP and microsatellite markers identified among the eight potential genes in this locus, polymorphism in the cg2 gene. The Cg2 gene encodes a transmembrane protein of 330 kDa, localized in the membrane of the food vacuole and in the plasma membrane of *P. falciparum* [125] However, other studies have shown that the relationship between cg2 polymorphism and CQ-R is strong but not absolute, suggesting the possible contribution of other genes in the resistance.

- **The pfcrt gene**

A more detailed analysis of the locus of 36 kb including cg2 gene revealed the pfcrt gene (chloroquine resistant transmembrane protein) consisting of 13 exons. This gene is located on chromosome 7 as cg2. Complex and polymorphic, pfcrt is absolutely linked to the CQ-R [126]. The pfcrt gene product is a transmembrane protein (integrin), it could play a role as a carrier of chloroquine. Many in vivo and in vitro studies have shown since 2000 that there is a correlation between the K76T mutation and CQ-R [127] [126] [124]. These studies have shown that whenever there is a treatment failure, there is an absolute selection of the mutant allele of the gene pfcrt K76T among most children with the mutant allele with an adequate clinical response to chloroquine [124].

This discrepancy observed in other studies is probably due to the polyclonality of most infections [93]. However, in areas where there is a high rate of CQ-R, the mutant allele of the gene pfcrt is ubiquitous and this does not seem to affect the clinical response of the immune individuals for which chloroquine is an effective treatment and where there is complete parasite clearance. All these studies show that the gene pfcrt may be a key determinant in the phenotype of resistance or sensitivity to chloroquine. The combination genotype pfcrt-resistance phenotype is more evident in vitro than in vivo studies, however, the correlation between pfcrt and CQ-R in vitro is not perfect because either the polyclonality [30]or because of the influence of multiple mutations in the pfcrt gene on the phenotype of the CQ-R. Eleven codons of crt protein can mutate in a CQ-R parasite [126]However, mutations in the gene pfcrt generally form a single haplotype in Asia and Africa. A different genotype from those determined so far has been observed in a native South American strain, New Guinea [123].

- **Role of P-glycoprotein homologue Pgh1 the Resistance**

Protein P-glycoprotein belongs to the family of ABC transported. It is overexpressed in MDR cells and act as efflux pumps cytotoxic drugs. Several observations showed that a similar mechanism could be at the origin of drug resistance in *P. falciparum* as efflux accelerated and / or decelerated chloroquine in erythrocytes parasitized by a CQ-R strain, but not by a CQ-S strain accumulation. Modulation of the CQ-R by verapamil calcium channel blocker partially reverse drug resistance in neoplastic cells (Martin et al., 1987). The presence in *P. falciparum* of a homologous gene to MDR cancer cells, called pfmdr1 gene [126]or the detection of P-glycoprotein parasite called pgh-1 [112] was associated with CQ-R.

Aside from genes conferring resistance that harbour some parasites clones some factors by ‘’virtue’’ of the nature and intrinsic to the parasite may account for the decrease of parasite clearance. Decrease in *P. falciparum* population has been reported in the context of mixed infection with *P. malariae*. The latter modulates the dynamics of *P. falciparum* population through non-specific and cross-specific immune response

[104]. The intrinsic characteristic related to parasites here can be explained by the capacity or the ability defined in its immunogenicity property of the parasite to trigger specific immune response. *P. malariae* and *P. falciparum* are different in that, *P. malariae* can be eliminated by *P. falciparum*-specific effectors, but is unlikely to activate or stimulate proliferation of *P. falciparum*-specific effectors. The possibility of cross-immunity here is offered by the degree to which both share epitopes but in the limit of effectors stimulation or activation [104]. The model (blood-stage dynamics of a mixed-species malaria infection) that explained these mechanisms is based on the consideration of the prepatent period of *P. falciparum* and *P. malariae*. In facts the model showed that at least 25 days are required to greatly suppress *P. falciparum*. In another instance using a dynamic model integrating the dynamic of *P. falciparum* and *P. vivax* coinfection and the effect of antimalarial [85] it was demonstrated that when the infection is misdiagnosed as *P. vivax* single infection treatment for *P. vivax* can lead to a rapid surge of *P. falciparum*. This finding as suggested by the authors can be applicable in the case of mixed-genotypes (drug susceptible and drug reistance) whereby following treatment resistant genes can be selected and easily spread.

Understanding the complexity of human malaria parasite life-cycle is of key interest as drugs are specifically designed to target specific parasite stages. Parasite clearance time can be influenced by factors related to the parasite itself. For example it is well known that artemisinis derivatives target the ring-stage but it is not well understood how this is possible for mature ring-stage parasites that have the ability to sequester to organs [101] [15].

When parasitized red blood cells (pRBCs) rupture at schizonts/mature trophozoites stage, the plasmodials antigens released stimulate the lymphocyte Th1, which secretes interferon IFN-γ. INF-γ and malaria toxins Glycosylphosphatidylinositol (GPI), released on merozoites rupture, activate the macrophages that secrete cytokines (TNF-α, IL-1, IL-6). These are responsible for many of the signs and symptoms including paroxysms [128] TNF alpha concentation > 100 pg/ml of serum is highly associated with cerebral malaria and death [129]. Another toxic product is the malarial pigment haemozoin produced by the polymerization of heme, a by-product of host haemoglobin proteolysis

by the malaria parasite. Haemozoin is known to severely inhibit the function of monocytes and macrophages after its ingestion [130]. The above-cited cytokines have double effects. Firstly, their parasiticide action facilitates parasites clearance at low concentration [128] either immediately or by the intermediate of free oxygenated radicals or nitric oxide (NO). Secondly, the cytokines stimulate endothelial cells of capillaries and cerebrals post-capillaries veinules, which express adhesion molecules like ICAM-1, VCAM-1, and E-selectine. These molecules later on interact with the parasite ligand, *Plasmodium falciparum* ertythrocyte membrane protein1 (PfEMP-1), expressed at the level of protuberances (Knobs) of parasitized red blood cells (pRBCs), facilitating adhesion of pRBCs with an attempt to escape host immunity system (destruction by the spleen), therefore disappearing from the peripheral blood (Figure 2.2C). However, there is a possibility for these pRBCs of trafficking in other blood vessels where sequestration is not possible probably due to the presence of some antibodies that target the pfEMP1. These antibodies then prevent sequestration by a mechanism known as opsonisation, meaning that pRBCs are labelled by these antibodies for phagocytosis in the spleen (Figure 2.2B) (Beaudry et al. 2011). These antibodies may contribute to the clearance of ring-stage parasites, a process which does not depend on artemisinin clearance in the context of artemisinin treatment. Dihydroartemisinin (DHA), the active metabolite of all artemisinins, causes ring-stage parasites to undergo pyknosis (Figure 2.2A). This mechanism is possible by the production of free radicals from enoperoxide that alkylate and oxidize the proteins and lipids of intraerythrocytic parasites [132]. These circulating pyknotic forms are eventually “pitted” from red blood cells (RBCs) as they pass through endothelial slits in the spleen, which returns the previously infected, intact RBCs to the peripheral blood [133]. This process occurs in all patients treated with artesunate and is likely the predominant mechanism of parasite clearance in most cases.

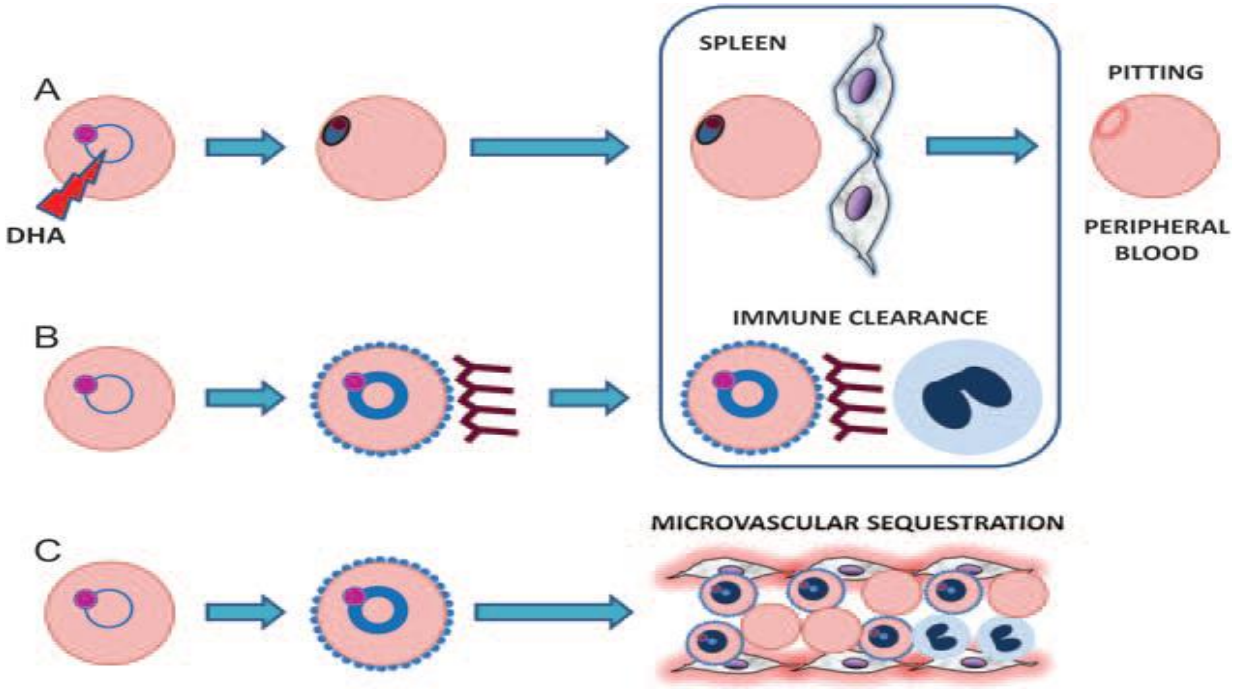


Figure 2.3: Clearance of ring-stage Plasmodium falciparum parasites from peripheral blood during a parasite clearance rate study.

Studies have also been carried out for resistant parasites clearance time [134]. The authors found that chloroquine resistant parasites carrying the K76T mutant allele are cleared in an age dependent manner suggesting the implication of key genetic or immunity elements that still need to be identified. As stated earlier IgG and IgM to be part of the immune elements contributing to the short parasite clearance time reported after artemisinin treatment [15].

Chapter 3: Materials and Methods

3.1 Research Design

The study design was a 3-arm, open, randomised controlled trial. A total of 720 children of either gender, with acute uncomplicated *P. falciparum* infection, who fulfill all of the inclusion and have none of exclusion criteria, and provide signed informed assent (by at least one parent or legal guardian) were enrolled in the study (appendix I).

Enrolled patients were randomised to receive the three treatment, i.e, Study Arm-A: artesunate-amodiaquine, Study Arm-B: dihydroartemisinin-piperaquine and Study Arm-C: artemether-lumefantrine at the ratio of 2:2:1. Patients administered treatment by directly observed treatment (DOT) by the study nurse during a 3-day period and full clinical and laboratory assessments and observation of early adverse effects were assessed. On treatment completion after the third day, participants were required to report to the study clinic on days 7, 14, 21, 28, 35 and 42 or at any other time when clinical sign(s)/symptom(s) of malaria is suspected. Those who failed to keep these follow up appointments were visited by a community health worker. Appendix II summarises the activities during recruitment and follow-up. During the first three days, the study clinician made a clinical assessment of the patient once a day and recorded all observations in the Trials Register and Hospital Patient File before transfer into the case report form (CRF). Blood smear for examination of parasitaemia, haematology and biochemistry were investigated during follow up and/or on the day of reappearance of parasitaemia (recrudescence/reinfection).

3.1.1 Study population

- **Description of Study Population**

Children of either gender, aged between 6 months (> 5kg) and 10 years, with acute uncomplicated *falciparum* malaria were recruited from the outpatient clinics of participating health facilities. The study population was selected from malaria positive patients during routine practice at the study sites.

- **Study Sites**

The study was conducted at two geographically distinct sites, i.e., Mutengene (Littoral-Forest) and Garoua (Sahel-savanna). These sites fall within two of the three major geo-ecological zones of Cameroon as described by MARA maps [22]

Mutengene is situated at coordinates $04^{o}01'N$, $09^{o}11'E$. The climate is equatorial with a rainfall of 10,000 mm per annum and a temperature average of $25^{o}C$. The vegetation is semi-mangrove and tropical wet forest. The study site is limited to the south and south-east by the sea and to the north and north east by mount Cameroon, an active volcano that is 4,100m above sea level. The population works predominantly on palm and rubber estates that are owned by the Cameroon Development Corporation (CDC). Garoua is in the North of Cameroon and lies at the coordinates, $06^{o}24'N$, $10^{o}46'E$. Garoua serves as a river port in years when the rainfall is abundant. Situated in the river Benue basin, it receives an average annual rainfall of 380 mm. It has about 4 months of rainy season. Temperature here averages about 31oC for most of the year and the vegetation is guinea-savannah. The population is predominantly Muslim and is comprised mainly of cattle raisers. A few have taken to trading with neighbouring Nigeria.

- **Sample Size Estimation**

Assuming that artesunate-amodiaquine was successful (cure rates) in above 94% of the patients, then p was set at 94%. To demonstrate with 95% confidence ($\alpha = 0.05$) that artesunate-amodiaquine or dihydroartemisinin-piperaquine was acceptable if they are at worst 10% (d) inferior in the occurrence of failures, we would accept a 10% risk (β) or (90% power) to rule out the null hypothesis of the lack of inferiority.

Using the formula with f(α, β) statistics[135]:

$$N = [2p \times (100-p) \times f(\alpha, \beta)]/10^2$$

$$= (2 \times 94 \times 6x\ 10.5)/\ 10^2$$

$$= 118 \text{ individuals in the smallest arm}$$

Considering that other trials have reported loss to follow-up and withdrawal rates of 10% in 28days follow-up periods, we Assumed a 20% loss to follow up and withdrawal for a 42 days follow-up which allows a size of 142. Randomization in the ratio of 2:2:1 for artesunate-amodiaquine: dihydroartemisinin-piperaquine and artemether-lumefantrine will be done. A minimum of 142 cases for the artemether-lumefantrine arm, and 284 cases for each of the two tested arm (Arsucam® and Duo-cotecxin®) will be required, to make a total of 710 cases for both study sites. For purposes of block randomisation, a sample size of 720 was preferred.

3.1.2 Study procedure and sampling

- **Screening and Recruitment**

In conformity with routine malaria diagnosis at the study sites, the same flow of patients was maintained. On the morning of the study day (day 0), the investigator's medical team approached potential candidate children with acute uncomplicated falciparum malaria. Randomisation of patients to trial allocation and pre-treatment investigations (clinical and laboratory assessments) was be started when subject inclusion/exclusion criteria have been met and written informed assents are obtained. Informed assents for study participation was obtained from their parents/guardians (signatures or thumb impressions). The parents/guardians was informed verbally as well as in writing about the nature of the study, the anticipated risks and benefits, the discomfort to which the subjects was exposed, as well as the right to interrupt the participation at any time on their own free-will. The contents of the subject information/informed assent sheet will be explained (Appendix II). The information procedure was also attended by an independent witness to assure that the contents of the written subject information/informed assent sheet have been explained to the subjects. A literate witness will also sign the informed assent form in addition to a non-literate participant. All subjects screened for the study were registered in Identification of Screened and Enrolled Participants Log to indicate their demographics.

- **Inclusion and Exclusion criteria**

The criteria for inclusion of participants for the study were as follows:

- Children of either gender, aged between 6 months (> 5kg) and 10 years.
- Children Suffering from acute uncomplicated *P. falciparum* malaria confirmed by microscopy using Giemsa-stained thick film with an asexual parasite density of 1,000 to 100,000 parasites/µl.
- Children presenting with fever (axillary temperature ≥ 37.5oC) or having a history of fever in the preceding 24 hours.
- Ability to ingest tablets orally (either suspended in water or uncrushed with food).
- Willingness to participate in the study with written assent from parent/guardian. Parental authorization was obtained for children less than 8 years old and documented assent of parents/guardians for children 8-10 years.
- Willingness and ability to attend the clinic on stipulated regular follow-up visits.

A child was excluded from being enrolled to the study if any of the following “danger signs of severe malaria”: were observed:

- Not able to drink or breast feed
- Persistent vomiting (>2 episodes within previous 24 hours)
- Convulsions (>1 episode within previous 24 hours)
- Lethargic/unconscious
- Signs/symptoms indicating severe/complicated malaria according to WHO criteria (WHO definition).
- Concomitant illnesses, underlying chronic hepatic or renal disease, abnormal cardiac rhythm, hypoglycaemia, jaundice, respiratory distress,
- Serious gastrointestinal disease, severe malnutrition (W/H < 70%) or severe anaemia (haemoglobin < 5 g/dl).
- Known hypersensitivity to the study drugs.

- **Randomisation**

Block-randomisation stratified per centre was used to allocate the patients to the three trial arms. The block size was 30 and blinded only to the treating physicians but not the pharmacy attendant who administers the drug. The actual randomization was computer generated by the statistician attached to the project. The numbers so generated were placed each in an opaque envelope. Each envelope contained a paper with the assigned random trial arm (Study Arm-A, B, or C) but numbered serially.

Randomisation was done only after informed assent for study participation and patient inclusion and exclusion criteria have been met. Each study participant was assigned the next lowest randomisation number (patient's study ID number). This ID number was the subject's unique identifier and was used to identify the subject on the CRF and in labelling all study-related laboratory samples.

- **Parasitological Assessments**

During follow up, finger-prick blood samples was collected from patients for malaria examination every 8 hours in the first three days and thereafter followed up weekly on days 7, 14, 21, 28, 35, 42, and at any time (unscheduled visit) when parasitaemia/clinical signs and symptoms of malaria reappears. Parasitaemia was considered cleared if 2 consecutive negative smears occured during the first 3 days. Re-infection was distinguished from recrudescence through the analysis of the msp1, msp2 and glurp genes.

- **Clinical Assessments**

Clinical assessments was performed in all visits on patients in all trial arms and at any time when parasitaemia/clinical signs and symptoms of malaria reappeared. During hospital visits, vital signs including body temperature was recorded.

- **Molecular Markers of Recrudescence/Re-infection**

Finger-prick blood was collected in filter papers during follow up on days 7, 14, 21, 28, 35, 42 and at any time when parasitaemia/clinical signs and symptoms of malaria

reappeared. The analysis of molecular markers was done only when blood smears for malaria were found positive.

- **Evaluation of Trial Response**

Patient's trial outcome was classified according to the WHO guidelines (WHO 2003) with application as follows:

(1) **Early Treatment Failure (ETF)**

Development of danger signs or severe malaria on day 1, 2 or 3 in the presence of parasitaemia; or Parasitaemia on day 3 with axillary temperature > 37.5 o C; or Parasitaemia on day 3, with 25% of count on day 0; or adverse events > grade 3 requiring change in treatment on days 0-2.

(2) **Late Clinical Failure (LCF)**

Development of dangers signs or severe malaria after day 3 in the presence of parasitaemia without previously meeting any of the criteria of ETF; or Presence of parasitaemia and axillary temperature >37.5 o C (or history of fever in past 24 hours), on any day from days 4 onwards, without previously meeting any of the criteria of ETF.

(3) **Late Parasitological Failure (LPF)**

Presence of parasitaemia on any day from days 7 onwards and axillary temperature <37.5 o C, without previously meeting any of the criteria of early treatment or late clinical failure.

(4) **Adequate Clinical and Parasitological Response (ACPR)**

Absence of parasitaemia on day 42 irrespective of temperature without previously meeting any of the criteria of early treatment failure or late clinical failure or late parasitological failure

3.1.3 Ethical Considerations

Ethical approval was obtained for the study protocol, any amendments, the informed assent or any other aspect of study required by Ethics Committee both in Cameroon

and the Ethics Review Committee of the World Health Organisation (WHO-ERC) before initiation of the trial.

All artemisinin derivatives including artemether, artesunate and artemisinin have been proven safe with minimal adverse reactions. As a consequence we did not for see any problems as result of the children taking the drugs under study. We however were aware from prior trials with these drugs that artesunate amodiaquine may cause itches or rashes while artemether lumefantrine and dihydroartemisinin may cause some headaches. To minimise these risks, the partipants were monitored and treated immediately when any of the adverse events were observed.

Patients included in the study received potential benefit directly from treatment with either one of the three trial regimens (artemether-lumefantrine, artesunate-amodiaquine and dihydroartemisinin-piperaquine). These ACTs have proven effective for treatment of acute uncomplicated falciparum malaria in different areas of the world with cure rate of more than 90% and fever and parasite cleared within 48 hours after treatment. Indirect benefit to the participants was that they had an opportunity to have a detailed medical and physical examination, laboratory tests, follows up visits at home and treatment of common illness of infants and children in the home of the participants. Finally, it was our hope that knowledge gained from the study will provide useful information on policy-decision on effective treatment regimens in Cameroon.

A data safety and monitoring board (DSMB) was put in place to monitor the data from the study for any safety concerns and to take appropriate action

3.2 Molecular analysis

3.2.1. Extraction of malaria parasite and human DNA

The following genes were assessed:

- *Plasmodium falciparum Pfmsp2* gene to distinguish between recrudescence and reinfection
- *Plasmodium falciparum pfmdrI N86Y* gene conferring drug resistance,

- *NAT 2* human gene for therapeutic response

DNA (both for human genomic DNA and parasite) was extracted from blood spots on filter papers (Whatmann 3MM) by the chelex boiling method described by Plowe et al., (1995). The necessary materials were sterilized by autoclaving, and the working areas including other equipment were disinfected with 10% bleach and 70% alcohol.

The blood spots were carefully excised from the filter papers, transferred into eppendorf tubes containing 1ml of 0.5% saponin in 1X sterile PBS for cell lysis. The tubes were inverted several times and kept at 4^0C overnight. The next day, the brown solution was discarded and the filter papers washed with 1ml of 1X PBS and kept at 4^0C for 15-30minutes. The supernatant was then discarded and the filter paper in each tube was transferred into their corresponding 1.5ml eppendorf tubes, each containing 50µl of 20% chelex-100 plus 150µl of DNAase free water, previously placed in the heat-block set at 100^0C a few minutes before, and not forgetting to clean the pair of forceps between samples.

After allowing samples in the heating block for 10 minutes the samples are agitated for 30 seconds and placed once again in the heating block for a further 10minutes. This is repeated twice for each tube. The tubes were centrifuged at 13200rpm for 3minutes and the supernatant collected in fresh microfuge tubes. Centrifugation was repeated for 3 minutes and the supernatant collected into fresh tubes, taking care not to pick up any chelex crystal. The solution (DNA) was then stored at -20^0C until use.

3.2.2 PCR genotyping of pfmsp2 gene (Adjusted cure rate to differentiate between recrudescence and reinfection)

The genetic fingerprinting technique nested Polymerase Chain Reaction, based on the polymorphic antigen loci pfmsp2, pfmsp1 and Glurp gene (*Plasmodium falciparum* merozoite surface protein 2) was used in this study to determine wheter *P. falciparum* parasites recurring in a patient's peripheral blood after antimalarial treatment (Failure, Day X) are genetically identical to, or different from the parasites present prior to treatment (Baseline, Day zero). Numbers of studies have found that among the above

cited markers pfmsp2 is so far the highly polymorphic with allelic variants (bands) ranging from 4, 5 to 6 in endemic settings. Here the pfmsp2 gene marker was used. If identical to pre-treatment parasites, the recurrent infection is considered to be a recrudescence; if different it is considered to be newly emerging from the liver (hypnozoites, dormant form).

In this assay the product of the first amplification was used as the template for the second amplification in which a different set of primers was used (nested PCR).

The final volume of each PCR mixture in each tube was 25µl. The reaction mixture of each tube consisted of 18.25µl of molecular biology water, 2.5µl of 10X thermopol buffer, 0.5µl of 10nM dNTPs (New England Biolabs), primers (S3 and S4) (Table 3.1), obtained from MR4, USA and 0.25µl of Taq polymerase, (New England Biolabs USA). Each tube for the outer PCR contained 22µl of PCR reagents which was made up to 25µl by adding 3µl of the corresponding DNA. The tubes were then placed in the T3 thermal cycler (Biometra, U.K) with the following conditions; pre-denaturation 94^0C for 3minutes followed by 40 cycles of denaturation at 94^0C for 30seconds, annealing at 42^0C for 60seconds, elongation 65^0C for 2minutes and final extension at 72^0C for 3minutes.

For the nested PCR, each tube contained 24µl of PCR which was made up to 25µl by adding 1µl of amplicon from the outer PCR. Each experiment included a negative control and a positive control containing *P. falciparum* genomic DNA. The tubes were then introduced in the thermal cycler machine (T3 Biometra) and subjected to the following conditions; pre-denaturation at 94^0C for 3 minutes, followed by 30 cycles of denaturation at 94^0C for 30seconds, annealing at 50^0C for 60seconds, elongation at 72^0C for 2 minutes and final extension at 72^0C for 3minutes.

Table 3.1: Primer sequences for msp 2 gene

Gene	PCR	Primers	Sequence (5'→3')
Msp-2	Outer	S_2	GAG GGA TGT TGC TGC TCC ACA G
		S_3	GAA GGT AAT TAA AAC ATT GTC
	Nested	S_1	GAG TAT AAG GAG AAG TAT G
		S_4	CTA GAA CCA TGC ATA TGT CC

Legend: PCR = Polymerase chain reaction, msp-2 = merozoite surface protein

3.2.3 Amplification of *pfmdr* gene

The final volume of each PCR mixture in each tube was 25µl. The reaction mixture of each tube consisted of 18.25µl of molecular biology water, 2.5µl of 10X thermopol buffer, 0.5µl of 10nM dNTPs (New England Biolabs), primers (MDR1 and MDR2) (Table 3.2), obtained from MR4, USA, and 0.25µl of Taq polymerase (New England Biolabs USA). Each tube for the outer PCR contained 22µl of PCR reagents which was made up to 25µl by adding 3µl of the corresponding DNA. The tubes were then placed in the T3 thermal cycler (Biometra, U.K) with the following conditions; pre-denaturation 94^0C for 3minutes followed by 40 cycles of denaturation at 94^0C for 30seconds, annealing at 56^0C for 30s, elongation at 60^0C for 1 minute and final extension at 60^0C for 3minutes.

For the second amplification (inner PCR), the reaction was performed in a 25µl reaction mixture each containing 20.25 µl of nuclease free water, 2.5 µl of 10X Thermopol buffer, 0.5µl of 10mM dNTPs, 0.25µl of each primer (MDR3 and MDR4) 2.5µM, 0.25 µl of Taq DNA polymerase (New England Biolabs), and 1µl of amplicon from the outer PCR amplification. All were put in the different microfuge tubes which had been previously labelled, as well as the positive control tube. The mixtures were subjected to the following amplification conditions; pre-denaturation at 94^0C/3min, followed by 30 cycles of denaturation at 94^0C/30s, annealing at 48^0C/30s, elongation at 64^0C/60s and final extension at 64^0C/3min.

Table 3.2: Primer sequences for the amplification of pfmdr 1 gene

Gene	PCR	Primers	Sequence (5'→3')	Amplicon Size
Pfmdr I	Outer	MDR1	GCGCGCGTTGAACAAAAAGAGTACCGCTG	Ca. 300 bp
		MDR2	GGGCCCTCGTACCAATTCCTGAACTCAC	
	Nested	MDR3	TTTACCGTTTAAATGTTTACCTGC	
		MDR4	CCATCTTGATAAAAAACACTTCTT	

Legend: PCR = Polymerase chain reaction, *Pfmdr* = *Plasmodium falciparum* multidrug resistance

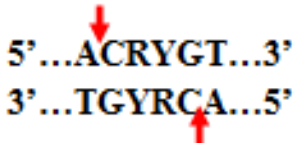
The amplicons obtained here were later subjected to restriction digest as described below.

3.2.4 Restriction Fragment Length Polymorphism (RFLP) analysis for Pfmdr1 mutation (N86Y) conferring resistance

Some clones (genetic diversity) are of clinical interest as they may harbor genes conferring drug resistance and thus be selected by treatment, causing therapeutic failure. In this study mutation analysis (SNP) in the resistance gene pfmdr1 was done to differentiate between pure mutant types (86Y), pure wild types (N86) and mixed infections (N86Y). The presence of polymorphism in a DNA fragment can result in the loss or the gain of a restriction site in that fragment (Syaffruddin et al., 2005). Thus, a gain of restriction site for the enzyme *Afl III* indicate the presence of polymorphism.

- ***Afl III* digestion**

Afl III digests specifically DNA sequences carrying the mutant allele 86Y(Tyr). This enzyme is extracted from the E. coli bacterial strain that carries the *Afl III* d'*Anabaena flos-aquae* and recognizes the following DNA sequence:



Digestion was done into a total reaction mixture of 20 µl in a sterile 1.5ml eppendorf tube containing: 1.5µl of *Afl III* restriction enzyme (5Units/ µl) (New England Biolabs), 3µl of 10X buffer 3 (NEB 3) and 0.2µl of 100X bovine serum albumin (BSA). The volume was made up to 12µl by adding 7.3µl of nuclease free water. A total of 8µl of amplicon from nested PCR was added into each tube and the mixtures incubated in a thermocycler at 37^{o}C for 20 hours.

3.2.5 PCR genotyping of the NAT-2 gene

Genetic differences in the capability of individuals to metabolize drugs can explain the inter-individuals variability observed within a population; resulting in different and diverse therapeutic response. Genetic polymorphism (SNP) has been found in the NAT 2 gene where it influences the biotransformation rate of individuals, which in this case can be categorized as slow acetylators, fast acetylators and intermediate acetylators. NAT 2 gene is an enzyme involved in the metabolic pathway of phase II biotransformation/elimination of xenobiotic. The identification of SNPs herein may be relevant in establishing the genetic profile for toxicity with a usual dose of a given drug, establishing the genetic profile for favorable response to a given drugs, just to cite a few.

A single run conventional PCR was used for the amplification of NAT-2 gene based on the methodology proposed by Chen et al., 2007 with slight modifications. The reaction mixture of each tube consisted of 36.5µl of molecular biology water, 5µl of 10X thermopol buffer, 1.0 µl of 10mM dNTPs (New England Biolabs), 0.5 µl of each primers (NAT-1F and NAT-1R) (Table 3.3), obtained from MR4, USA, and 0.5µl of Hot start Taq polymerase (5Units/ µl) (New England Biolabs USA). Each tube of PCR contained 44µl of PCR reagents which was made up to 50µl by adding 6µl of the corresponding DNA. The tubes were then placed in the T3 thermal cycler (Biometra, U.K) with the following conditions; pre-denaturation 95^{0}C for 15 minutes followed by 40 cycles of denaturation at 95^{0}C for 50 seconds, annealing at 55^{0}C for 50s, elongation at 72^{0}C for 50 seconds and final extension at 72^{0}C for 3minutes.

Table 3.3: Primer sequences for the amplification of NAT-2 gene

Gene	Primers	Sequence (5'→3')	Amplicon Size
NAT-2	NAT-1F	CCAATAAAAGTAGAAGCGA	535 basepair
	NAT-1R	CTCTTCCAGGACCTCCA	

3.2.6 Restriction Fragment Length Polymorphism (RFLP) analysis for the NAT-2 gene polymorphism

As earlier stated the presence of polymorphism in a DNA sequence can result in a gain or loss of restriction site. In this study we analyzed 4 SNPs namely:

- NAT 2∗4 wild type (fast acetylator)
- NAT 2∗5 mutant type (slow acetylator)
- NAT 2∗6 mutant type (slow acetylator)
- NAT 2∗7 mutant type (slow acetylator)

The polymorphisms were analyzed by using the following restriction endonucleases:

- Kpn I: the absence of a restriction site is characteristic of the presence of the NAT 2∗5 mutation as compare to the wild type, which possesses a restriction site for the same restriction enzyme
- Taq I: characterizes the absence of one restriction site on the mutant type NAT 2∗6 as compare to the wild type NAT 2∗4, which possesses two restriction sites for the same restriction enzyme
- Bam HI: the absence of a restriction site is characteristic of the presence of the NAT 2∗7 mutation as compare to the wild type, which possesses a restriction site for the same restriction enzyme

- Intermediate acetylator: Is characterized by the presence of a wild type and the presence of a mutant type; NAT 2∗4/ NAT 2∗5, NAT 2∗4/ NAT 2∗6, NAT 2∗4/ NAT 2∗7

If there is complete digestion of an allele by the three restrictions enzymes Kpn I, Taq I, and Bam HI then it permits to rule out the absence of all the mutant types and consequently this is considered to be NAT 2∗4 allele. The expected possible combinations are described latter in results (Table 4.4).

- **RFLP analysis by Bam HI enzyme**

Bam HI enzyme is extracted from the E. coli bacterial strain that carries the Bam HI gene from *Bacillus amyloliquefaciens* H and recognizes the following DNA sequence:

Digestion was done into a total reaction mixture of 20 µl in a sterile 1.5ml eppendorf tube containing: 7.3µl of nuclease free water, 3µl of 10X buffer 3 (NEB 3), 0.2µl of 100X bovine serum albumin (BSA) and 1.5µl of *BamHI* restriction enzyme (20 Units/ µl) (New England Biolabs), and The volume was made up to 20µl by adding 8µl of amplicon from PCR. The mixture was incubated in a thermocycler at 37^{o}C for 16 hours.

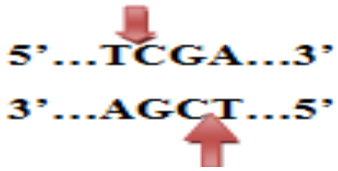
- **RFLP analysis by Kpn I enzyme**

Kpn I enzyme is extracted from the E. coli bacterial strain that carries the Kpn I gene from *Klebsiella pneumoniae* and recognizes the following DNA sequence:

Digestion was done into a total reaction mixture of 20 µl in a sterile 1.5ml eppendorf tube containing: 7.3µl of nuclease free water, 3µl of 10X buffer 1 (NEB 1), 0.2µl of 100X bovine serum albumin (BSA) and 1.5µl of *Kpn I* restriction enzyme (10 Units/ µl) (New England Biolabs), and The volume was made up to 20µl by adding 8µl of amplicon from PCR. The mixture was incubated in a thermocycler at 37^{o}C for 16 hours.

- **RFLP analysis by Taqαl enzyme**

Taq alpha I enzyme is extracted from an E. coli bacterial strain that carries a *Taq alpha I* overproducing plasmid and recognizes the following DNA sequence:



Digestion was done into a total reaction mixture of 20 µl in a sterile 1.5ml eppendorf tube containing: 7.3µl of nuclease free water, 3µl of 10X buffer 4 (NEB 4), 0.2µl of 100X bovine serum albumin (BSA) and 1.5µl of *Taq alpha I* restriction enzyme (20 Units/ µl) (New England Biolabs), and The volume was made up to 20µl by adding 8µl of amplicon from PCR. The mixture was incubated in a thermocycler at 65^{o}C for 16 hours.

3.2.7 Agarose gel electrophoresis of PCR products and digests

The final PCR products and digests were revealed using agarose gel electrophoresis. A 1.5 to 2% gel was prepared by weighing 0.75-1.0g of agarose (Seakem Nusieve) into 50ml of 1X TBE buffer in a 250ml duran bottle. The mixture was placed on the bunsen burner for about 15minutes, after which it is allowed to cool down to about 60^{0}C. 2.5µl of EtBr was then added to the gel, swirled gently and carefully poured into the gel casting tray, and then allowed for about 45mins to solidify. A total 10µl of the final product of PCR was carefully mixed with 2.5µl DNA loading buffer and loaded into the wells that had been immersed into the electrophoresis tank containing TBE buffer. The molecular weight marker (10µl) was also loaded into one of the wells. The set up was connected to current after closing the lid. The DNA migrated with respect to charge to give different

band sizes. The distance migrated by the DNA was visually monitored by tracking dyes (bromophenol blue). The band sizes were visualized with the aid of a U.V trans-illuminator and photographed using a digital camera. The distance migrated by each gene was measured against that of molecular weight marker. The molecular weight of each gene was determined graphically from the curve of $\log_{10}$ of molecular weight marker against the distance migrated by interpolation.

3.3 Data analysis and Modeling

3.3.1 Data management

As a quality assurance measure, data was double entered using Microsoft Office Access 2007. Statistical software SPSS version 17 (Somers, NY) was used for data management and processing. Data was analysed using the software R version 2.11.1. The main analysis for efficacy parameters was done using both the intention-to-treat (ITT) and per-protocol(PP). For the modeling stage the per protocol dataset was used.

3.3.2 Descriptive analysis of Primary objectives

PCR-adjusted parasitological cure rates on Day 42 were compared using $\chi 2$ test as well as the odds ratios for likelihood of cure with 95% CIs. Both the intention to treat and per protocol dataset were used.

3.3.3 Descriptive analysis of secondary objectives

First the PCR Adjusted results was shown indicating samples that we considered recrudescence or re-infection. Also the molecular results indicating the distribution of the study population according to whether they are slow or fast metabolisers were assessed.

PCR-adjusted therapy outcome per treatment arm were assessed for day 14 and 28 and compared across the different treatment arms by $\chi 2$ test. Kaplan Meier curves for FCTs and PCTs were plotted to determine any difference by site and treatment.

Similarly, safety variables were analysed using ITT. Comparison of categorical variables between the two trial groups vs control arm was analysed using $\chi 2$ test. Comparison of continuous variables such as changes in laboratory parameters following treatment)

between groups was assessed by the Wilcoxon signed-rank test for data conforming to non-normal distribution or by the paired t-test for data conforming to normal distribution.

3.3.4 Non-inferiority of ASAQ and DHP to AL

We showed non-inferiority of ASAQ and DHAP compared to AL by constructing a 2 sided 100(1-2α)% confidence interval (CI) for the difference of cure rates of ASAQ and DHAP when compared to AL. We construct the the 95% CI of the difference in cure rates of patients in the AL arm compared to DHP and ASAQ by using the formula:

$$95\%CI = (p_1 - p_2 \pm 1.96\sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}})$$

Where p1,p2 are respectively the cure rates ofdrug1 and drug 2 and q1 and q2 are 1-cure rates of drug1 and 2 respectively. n1 and n2 are the number of patients under drug 1 and 2 respectively. We then compare the upper bound of these 95% CI with the non-inferiority margin (10%). If the upper bound is less that 10%, we conclude non-inferiority.

3.3.5. Explorative multivariate modelling

-Variable selection

The covariates used for the two models are the site(Garoua or Mutengene), the drug(AL, DHP or ASAQ), the residence type(urban or rural), the ecological type(sahel or forest or savannah), the sex(male or female),age group(<=60years,>60years), weight and temperature. Given that some suspected liver function test and haematological parameters were assessed only on the day 0,7and 42, we consider only the day 0 values with the assumption that their influence on response is constant all through the visit days. To this end, we include, alanine amino transferase level(abnormal rage, normal range), creatinine levels(abnomal, normal) and neutrophil levels(abnormal, normal), haemoglobin level(low, normal), acetelator status(slow metaboliser , fast

metaboliser). All normal or abnormal ranges are according to the Cameroonian population as stipulated in the protocol of the study.(see annex5)

-Model selection strategy

Selection of Model would be by the Akaike Information Criterion(AIC) using the step function in R 2.11.1 in preference to stepwise model selection which usually have an inherent biases in parameter estimation, inconsistencies among model selection algorithms, and an inappropriate focus or reliance on a single best model[136]. The model with the least AIC value would be selected.

Modeling Treatment outcome(Adequate Clinical and parasitological response)

Given that treatment outcome is measured only at one time point (day 42) according to the per protocol data set, we fit a logistic regression to find out the influence of the covariates on outcome of treatment.

A simple logistic regression with one predictor can be represented by:

$$\text{logit}(Y) = natural\log(odds) = \ln\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta X. \quad \textbf{...(1)}$$

where π is the probability that the outcome of interest(in this case cure) occurs and β is the coefficient of the predictor(covariate) X and α is the y intercept . In this representation x can be either continuous or categorical. but the response which is whether the patient is cured (yes/no) can only be categorical. The value of β, determines the direction of the relationship between X and the natural log odds of Y. Odds ratios can easily be calculated from this model by taking the exponential of the coefficient (β)of a binary variable.

We start up with a full model comprising of all the covariates under investigation and by use of a step function in R 2.11.1, we access several models while comparing their Akaike Information criterion (AIC) value. We select the model with the least AIC value as our best model.. The only assumption for a logistic regression is the binomial

distribution assumption for the conditional mean for the outcome(cure or not cured). This assumption is however tenable as the binomial distribution assumption is robust as long as the sample is independent. This is the case in our study and so we do not check this assumption. Parameter estimates are considered significant at 0.05 level.

- **Modeling delayance to parasite clearance**

In this model, Response variable is the time to parasite clearance. We start by first plotting Kaplan Meier survival curves to understand the evolution of the different factors that are suspected of playing in role in the time to complete clearance of parasite. This would help feed the modeling stage.

Time in this study is the visit days(0, 1, 2, 3, 7, 14, 21, 28, 35 and 42). Parasites can get cleared at a particular time and only get recorded on a visit day. Therefore only an interval of time when parasites are cleared is recorded. The methods of survival analysis using time as a continuous variables are not very appropriate in this setting. . We rather fit a discrete time survival model with day of visit as the discrete time.

One important question in a time to event setting is the distribution of the hazard with time(hazard function). These are simply the probabilities of having the event at time t given no event before t. Given that the hazard function are probabilities, cox(1972) reparameterised these probabilities to have a logistic dependence on predictors and timeThe discrete hazard model is given by:

$$hij = \frac{1}{1+e^{-[(\alpha_1 D_{1ij} + \alpha_2 D_{2ij} + ... + \alpha_J D_{Jij}) + (\beta_1 Z_{1ij} + \beta_2 z_{2ij} + ... + \beta_P Z_{Pij})]}} ....(2)$$

where [D_{1ij} , D_{2ij} , . . . ,D_{Jij}] are sequence of dummy variables with values [d_{1ij} , d_{2ij} , . . . ,d_{Jij}] indexing time periods. J refers to the last period observed for anyone in the sample. [α_1 , α_2 ,..., $\alpha_{J]}$ represent the baseline level of hazard in each time period and the slopes[β_1 , β_2 ,... , $\beta_{p]}$ describe the effects of predictors on the baseline hazard function particularly on a logistic scale.Taking log transformation for the equation(2) above , we obtain(Singerwillet et al,1993)

$$\log_e\left(\frac{h_{ij}}{1-h_{ij}}\right) = (\alpha_1 D_{1ij} + \alpha_2 D_{2ij} + ... + \alpha_J D_{Jij}) + (\beta_1 Z_{1ij} + \beta_2 z_{2ij} + ... + \beta_P Z_{Pij}) ...(3)$$

Hazard funtion transformed this way gives us the conditional log odds that an event will occour in each time point(visit day) given that the individual did not get the even in the previous time point(visit) as a linear function of α_j specific to time j, and the values of the predictors at time j multiplied by the appropriate slopes(β_p).

This model is fitted using glm function in R.2.11.1 software with logit function as link function.

To fit this model using logistic regression, we first convert the data to a person- time data set such that each subject(record) has one record corresponding to the discrete observed time and therefore the number of records for each subject corresponds to the number of discrete observed times till the event is observed as shown in figure 3.1

As in the case of modeling response to therapy, we start off with a full model with all the covariates selected and then by using the step function , we access several models and select the model with the least AIC values.

Figure 3.1: Example Data transformed to person-data time for fitting discrete time survival model using logistic regression

Subjnumber	Sitecode	Drug	Age_group	Temp	Par	cleared	Visit day
3	1	ASAQ	0	38	52,400	0	0
3	1	ASAQ	0	36	240	0	1
3	1	ASAQ	0	36	0	1	2
4	1	AL	0	38	14,320	0	0
4	1	AL	0	37	160	0	1
4	1	AL	0	37	0	1	2

"cleared" is the event status variable and "visit day" is the observed discrete time

we assess hazard conditional probabilities by plotting predicted probabilities per time point and per predictor.

Based on the fitted conditional hazard probabilities the survival probability at time j can be obtained by equation(4).

$$\hat{s}_j = \prod_{k=1}^{k} (1 - \hat{h}_k) \ldots (4)$$

Where h_k is the conditional probabilities at time j. The estimated values of S_j where j=1,2,..K is used to plot the survival function.

To fit the Discrete time model, we assume that the linear-logistic model is a valid representation of reality(linearity),that all heterogeneity is across individuals is accounted for by the variation of the values of the covariates and that the logit -hazard profiles correspond to all possible values of every predictor are distinguished only by their relative elevation(proportionality). We proof linearity assumption by fitting a generalised additive model and smoothing out the continuous variables degrees of freedom of these continuous variable close to 1 indicate linearity. Also show assumption of no unobserved heterogeneity we fit a mixed model with each subject as a random effect and a very small variation accounted by the random effect will show that all the covariates in our model cover almost all the variation in the data. The proportional odds sequence of discrete time models indicates that the the proportional assumption is valid

Chapter 4: Results

4.1 Patient Flow and baseline characteristics

4.1.1 Patient flow

In total, 2500 children were screened from which 720 were randomised and enrolled into the three treatment arms (Figure 4.1). Of these, 697(23 never completed treatment) treatment outcomes were available in the intention-to-treat (ITT) while 623 outcomes were available in the per protocol analysis.

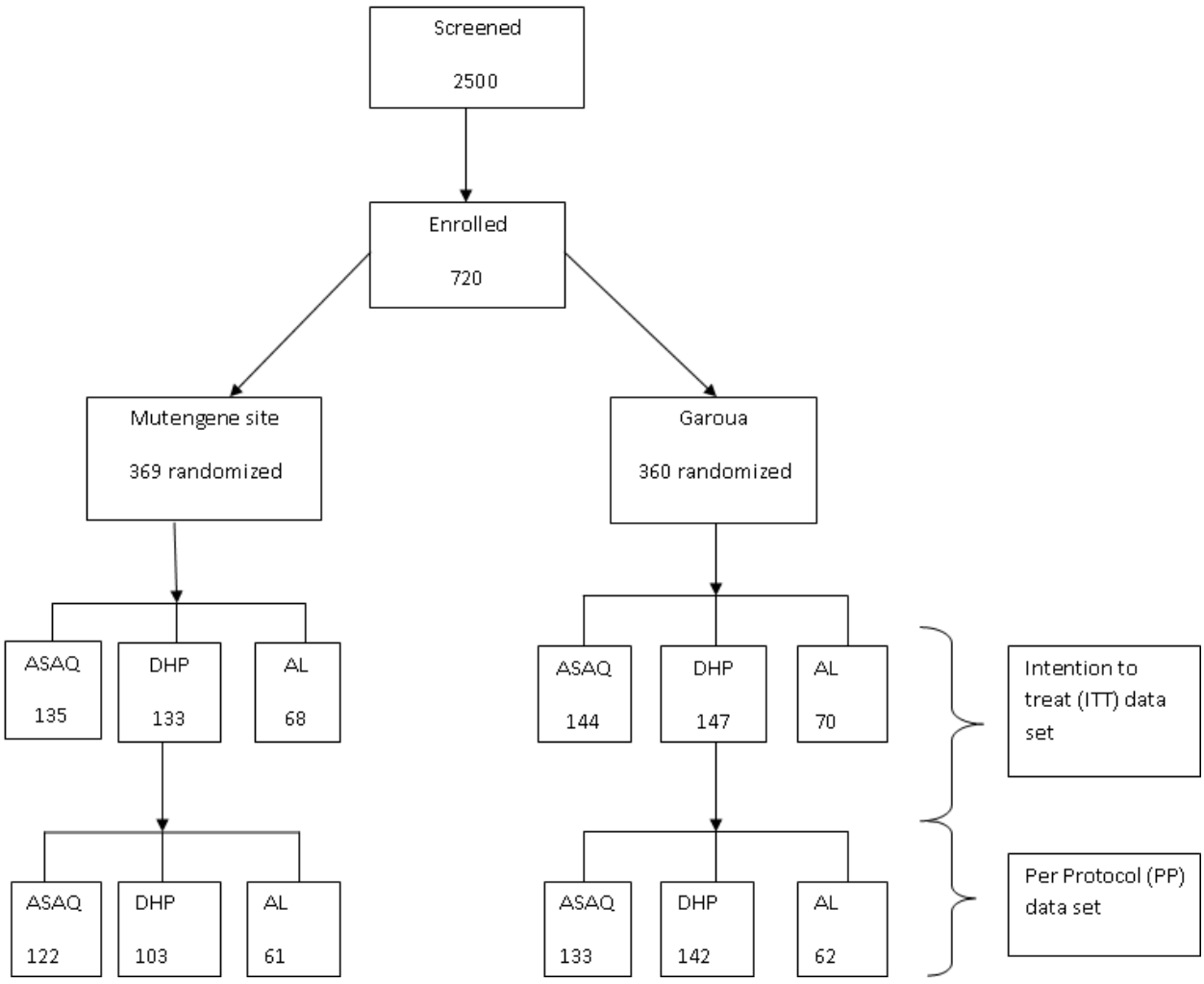


Figure 4.1:Study profile showing the number of patients recruited into each arm

4.1.2 Patient baseline characteristics

There was no significant difference in baseline characteristics (Table 4.1) across the 3 treatment arms.

Table 4.1: Baseline characteristics of randomised Study Participants

Characteristics	ASAQ	AL	DHP	P-value
Age*(months)±SD	55.35±34.5	57.97±33.8	54.88±32.9	0.67
Weight*(kg)±SD	16.9 ±7.3	17.8±7.7	16.9±6.8	0.41
Auxillary temperature*(°C)±SD	38±1.1	38±1.1	37.8±1.1	0.5
Haemoglobin*(g/dl)±SD	10.4±2.14	9.9±2.1	9.9±2.1	0.44
Parasite density**(/µl)	13555(1000-100000)	14808(1000-100000)	13690(1000-100000)	0.71
Creatine mg/l	0.65±0.46	0.73±1	0.68±0.72	0.48
ALAT IU/l	30±52	25±23	26±28	0.81
Absolute neutrophil count /µl	47±18	48±19	47±18	0.76
sex(male:female)	(145:131)	(63:75)	(142:137)	0.5

* mean ±SD(standard Deviation)
** Geometric mean and range
ASAQ = artesunate amodiaquine; DHP = dihydroartemisinin piperaquine; AL = artemether lumefantrine

4.2 Descriptive analysis of Primary objectives

4.2.1 Efficacy

The PCR- unadjusted cure rates for the intention to treat analysis were 80.4% (111/138) for AL, 81.4% (227/279) for ASAQ and 80.4% (225/280) for DHP (p=0.9). Per protocol analysis of unadjusted PCR cure rates on day 42 were 91% (112/123) for AL, 89.4% (228/255) for ASAQ and 89.4% (219/245) for DHP (p=0.86) . The intention to treat analysis of PCR adjusted cure rates on day 42 were 92% (127/138) for AL, 91% (260/279) for ASAQ and 89% (251/280) for DHP (p=0.31). PCR adjusted per protocol analysis cure rates on day 42 were 96.7% (119/123) for AL, 98.1% (250/255) for ASAQ and 96.3% (236/245) for DHP (p=0.75).

The PCR adjusted per protocol odds of being cured being in the AL compared with ASAQ arm is 0.987[95%CI(0.951,1.024)] and the odds of being cured being in the AL compared with DHP arm is 1.004[95%CI(0.964,1.046)]

4.3 Descriptive analysis of secondary objectives

4.3.1 Molecular analysis results

-PCR-Adjusted cure rate: distinction between recrudescence and re-infection

Out of the 79 (22 from Mutengene, and 57 from Garoua) patients that failed therapy as confirmed by Microscopy on the field, an analysis of pfmsp2 gene was done to distinguish between recrudescence and re-infection. Sixteen recrudescence cases were identified with 10 from Mutengene and 6 from Garoua. A typical agarose gel electrophoresis permitted to differentiate between identical genotypes with the same base pair size at baseline D0 and failure day DX (recrudescence) and different allelic variants with different base pair sizes on D0 and DX (new infection) (Figure 4.2).

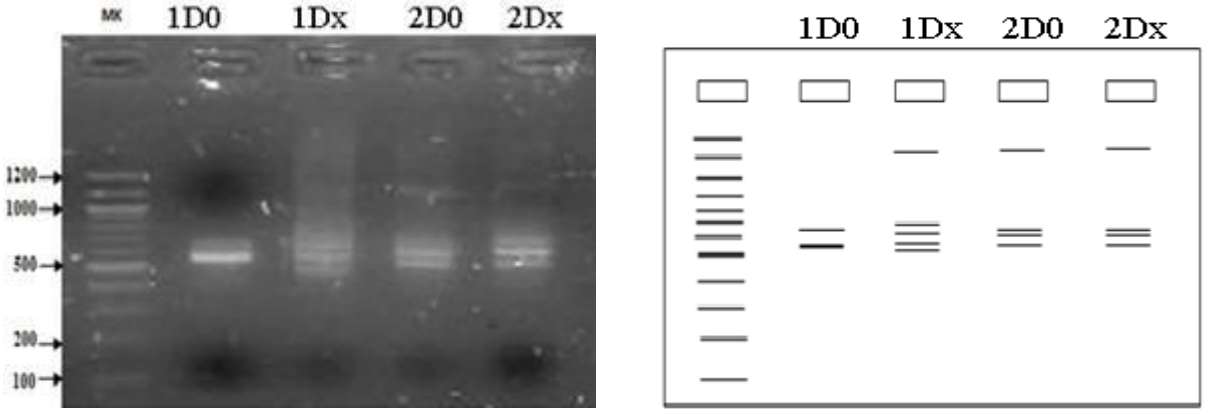


Figure 4.2:Agarose (2%) gel electrophoresis of nested PCR products of the pfmsp2 gene

Legend: samples 1D0, 1DX are new infections and samples 2D0, 2DX are recrudescence.

-RFLP analysis of pfmdr 1 N86Y polymorphism

One of the parameters suspected of causing therapeutic failure is the presence of polymorphism in the pfmdr1 N86Y gene of *Plasmodium falciparum*. Only the 16 patients that presented recrudescence (true failures to treatment) were analyzed for this polymorphism. The PCR-Restriction Fragment Length Polymorphism (RFLP) analysis of the 16 recrudescence samples permitted to differentiating between pure mutant types (86Y), pure wild types (N86) and mixed infections (N86Y) (Figure 4.3). There were 4 samples with pure mutant types (86Y), 1 for mixed (N86Y), 11 wild type (N86). The effect of Gene Polymorphism of the parasite seems not to be the main driving force to drug resistance in the study population. This variable is left out in further modelling

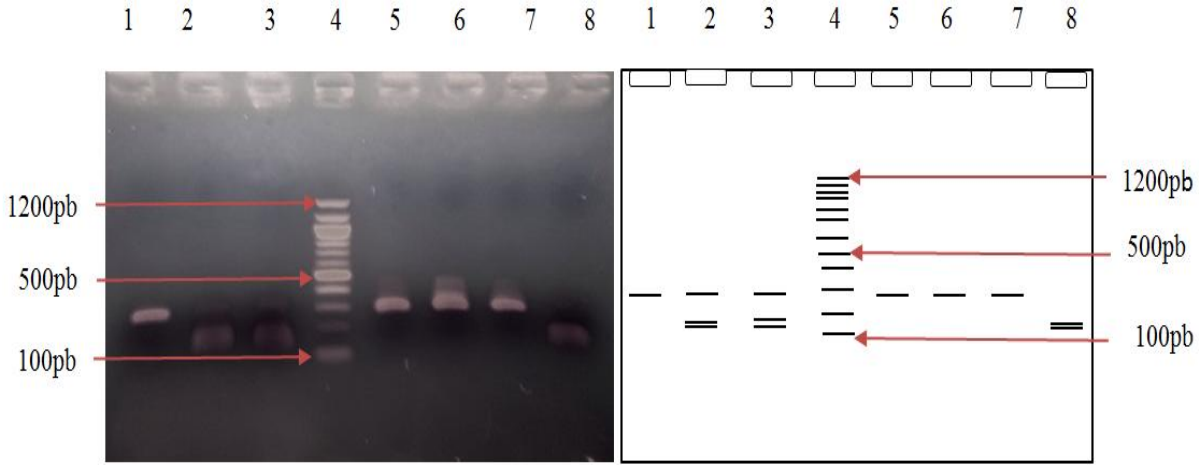


Figure 4.3:Agarose (2%) gel electrophoresis of PCR digests of Pfmdr 1 gene.

Legend: samples 2, 3 are mixed infections, (Asn)N86/86Y(Tyr); samples 5,6,7 are wild types strains infections(N86); sample 8 is a pure mutant type (86Y) and sample 1 is the positive control for wild type (Undigested). Asn= Asparagin; Tyr = Tyrosine; pfmdr 1 = *Plasmodium falciparum* multidrug resistance; MK= 100 base pair marker.

-Slow versus fast metabolisers (PCR-RFLP analysis of NAT-2 gene)

After a successful PCR amplification yielding a 535 basepair, samples were subjected to the action of the following enzymes: BamHI for NAT 2∗7 mutant type, Kpn I for NAT 2∗5 mutant type and Taq alpha I for NAT 2∗6 mutant type. The following profile was obtained (Figure 4.4).

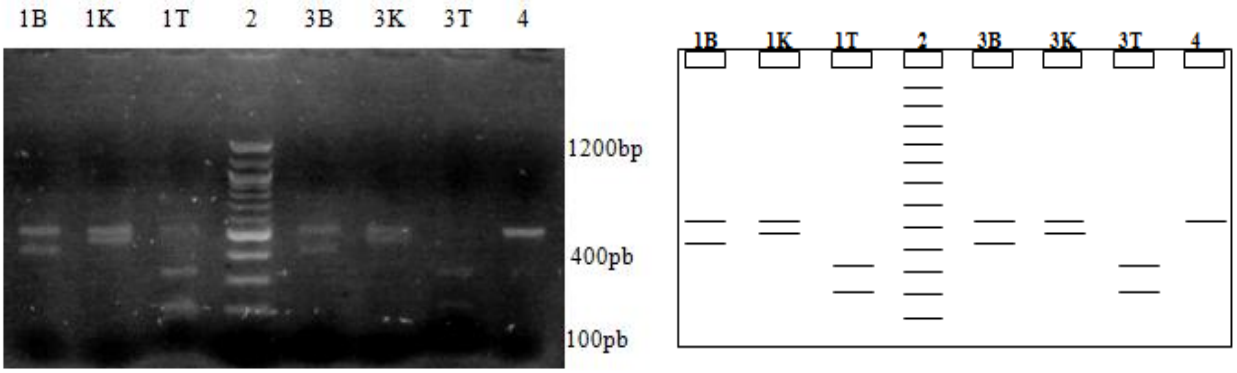


Figure 4.4:Agarose (2%) gel electrophoresis of NAT-2 gene digests.

Legend: After the action of BamHI, KpnI and TaqI : 535bp and 428bp for 1B and 3B (NAT2*7) ; 535bp and 483bp for 1K and 3K (NAT2*5) ; 330bp, 205bp and 170bp for 1T and 3T (NAT2*6) ; (4) Undigested control for wild type; (2) 100 basepair (bp) molecular weight marker. The numbers represent the samples and the letters BKT stand respectively for BamHI, KpnI and Taq alpha 1.

The band pattern obtained after digestion with these three enzymes was combined for interpretation of phenotypes and genotypes (part of annex 1).

- Distribution of fast and slow metabolisers per site and treatment arm

In the two population groups, Mutengene presented slow and fast metaboliser phenotypes higher than in Garoua whereas the intermediate phenotype was more represented in Garoua(figure 4.5a). Merging the intermediate phenotype to slow, the distribution of metaboliser status were similar across the treatment regimens(figure 4.5b).

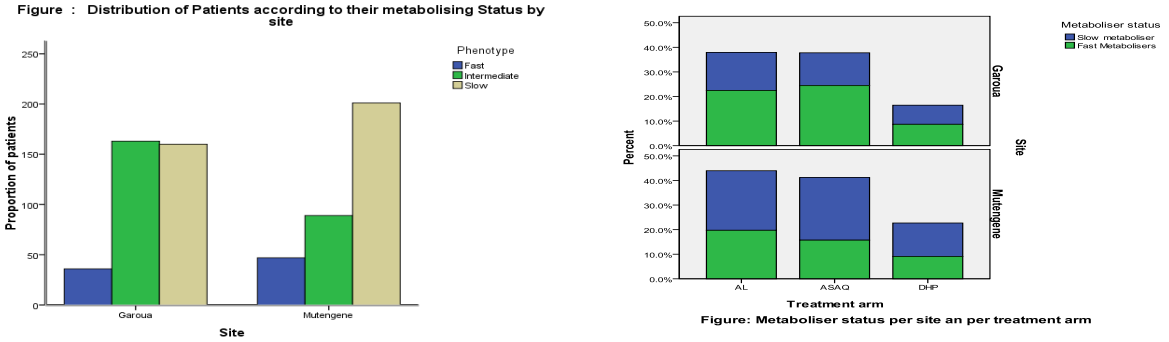


Figure 4.5:Distribution of fast and slow metabolisers by site and treatment arm

legend; DHP = Dihydroartemisinin piperaquine, AL = Arthemeter lumefantrine, AS-AQ = artesunate amodiaquine. Fast metaboliser=both fast and intermediate phenotype ; slow metaboliser=slow phenotype

4.3.2 Efficacy and therapy outcome on day 14 and 28

Comparing therapy outcomes for the different treatment arms show no significant difference for both day 14 (p=0.51) and 28 (p=0.78) respectively. No early treatment failure was observed amongst patients in the artemether lumefantrine and artesunate amodiaqine arm (Table 4.3) on day 14 and 28. All failures in DHP and AL arms had occurred by D14 and no more before D28. On the contrary, 7 more failures occurred between D14 and 18 for ASAQ.

Table 4.2:: PCR adjusted Efficacy outcomes(per protocol analysis) for Day 14 and Day 28

	Day 14				Day 28			
outcome	ASAQ	DHP	AL	P-value	ASAQ	DHP	AL	P-value
ACPR	250	236	119		243	236	119	
ETF	0	2	0	0.51	0	2	0	0.78
LCF	2	4	1		7	4	1	
LPF	2	3	3		4	3	3	

ASAQ = artesunate amodiaquine; DHP = dihydroartemisinin piperaquine; AL = artemether lumefantrine
ACPR = adequate clinical and parasitological response, ETF = early treatment failure; LCF = late clinical failure, LPF = Late parasitological failure.

4.3.3 Safety of the three treatment arms

There was a significant change in the Haemoglobin(g/dl) level in patients in the DHP (p=0.001)and AL(P=0.001)(Table 4.3) between Day 0 (D0) and Day 7 (D7), while no significant difference was observed in children who were administered ASAQ.

Table 4.3:Difference in patients mean haemoglobin levels((g/dl) on day 0 and day 7 by treatment arm

Treatment	D0	D7	D7-D0	*P-value
ASAQ	10.4	9.9	0.49	0.35
DHP	9.9	9.5	0.50	0.001
AL	9.9	9.3	0.55	0.001

Out of a total of 249 adverse events recorded across the different follow-up days, one of them was a serious adverse event in which the child was suffering from severe fatigue. This child was in the artemether lumefantrine arm. Most of the adverse events were cough, vomiting and lack of Appetite. The distribution of adverse events according to their intensity irrespective of the possible cause show that most of them were mild and less severe (Table 4.4)

Table 4.4: Number of adverse events irrespective of causality with respect to treatment and intensity

	Intensity				
Treatment	1	2	3	4	Total
ASAQ	56	36	6	1	99
DHP	71	34	4	1	110
AL	24	15	0	1	40
Total	151	85	10	3	249

Intensity(1=mild,2=moderate,3=severe,4=very severe)

Amongst those adverse events whose cause was suspected to be the drug, there was no statistical significant difference (p value=0.83) when comparing the three treatments with respect to the intensity of adverse event (Table 4.5).

Table 4.5:Number of adverse events with drug as the probable cause with respect to treatment and intensity

	Intensity				
Treatment	1	2	3	4	Total
ASAQ	37	25	3	1	66
DHP	46	23	2	1	72
AL	15	8	0	1	24
Total	98	56	5	3	162

Intensity(1=mild, 2=moderate, 3=severe, 4=very severe)

There was no difference between liver function test-creatinine, aminotransferase (ALAT), aspartate aminotransferase (ASAT) across the different treatment arms

4.4 Non Inferiority testing

The 95% confidence interval for the difference in cure rates of ASAQ with AL(PAL-PASAQ) is 0.014 ±1.96(0.000435)=[-0.015, -0.013] and the 95% confidence interval for the difference in cure rates of DHAP with AL(PAL-PDHP) is 0.004±1.96(0.000405)= [0.003, 0.005]. All the upper bounds of these intervals are less than the inferiority margin of 0.1 and therefore we have proven the non-inferiority of DHAP and ASAQ when compared to AL.

4.5: Parasite clearance time and Fever Clearance time

4.5.1: Proportion of patients with parasitaemia with respect to time

Proportion of patients with parasitaemia with respect to time were similar across the three study arms (Figure 4.6).

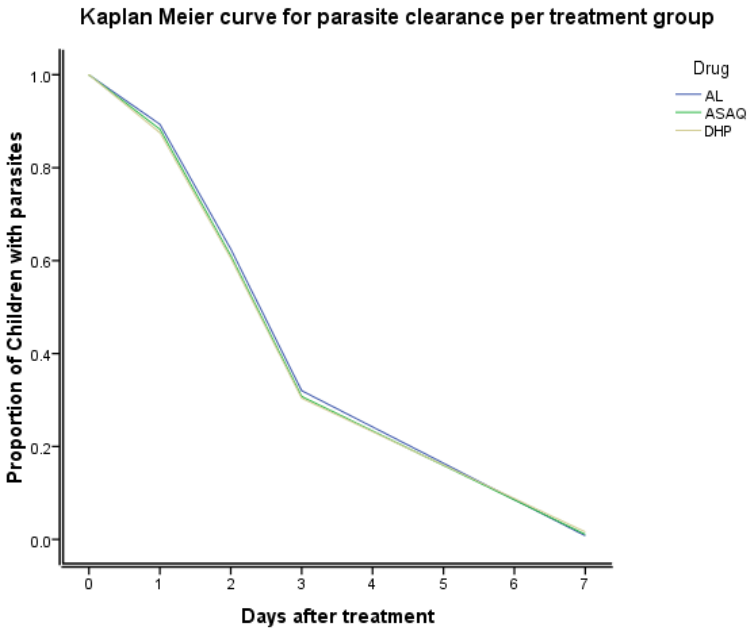


Figure 4.6:Parasite clearance time per treatment arm

There was however a little site effect on parasite clearance. About 18% of children in Garoua clear their parasites by day one compared to 8% in Mutengene.(figure 4.7) Comparing the proportion of patients with parasite clearance with respect to time across the different treatment arms and site did not show any appreciable difference.

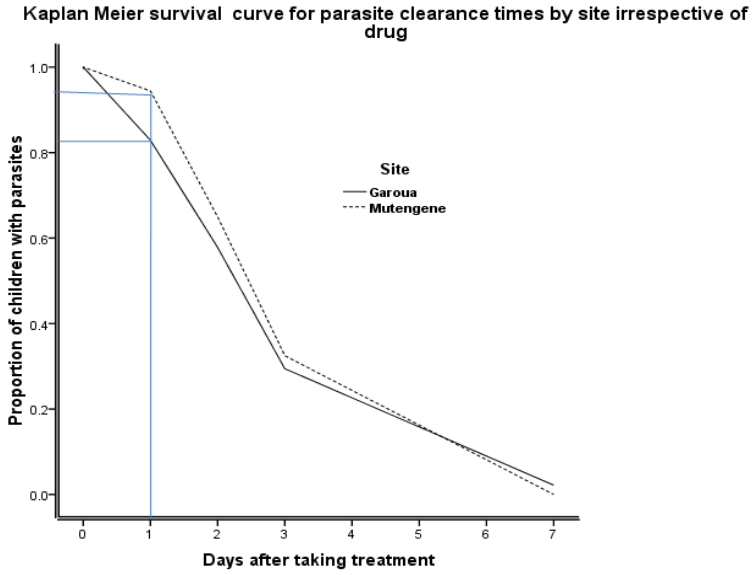


Figure 4.7:Parasite clearance time per site and treatment arm

4.5.2 Proportion of patients with fever with respect to time

The Kaplan Meier curve for fever clearance shows a pretty similar pattern across the three treatment arms. However a pair wise comparison using the log rank test showed a significant difference between the ASAQ and DHP(p value=0.04).

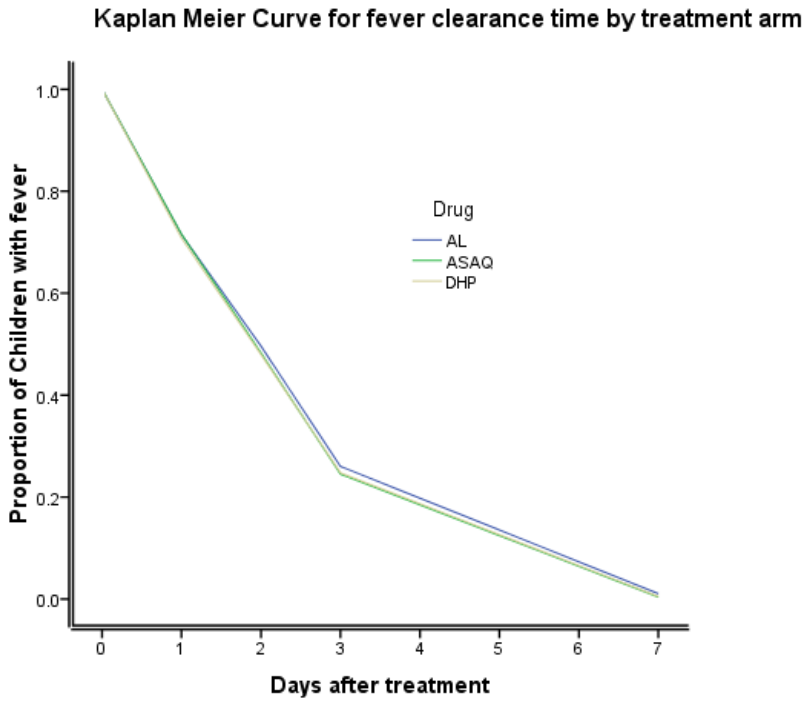


Figure 4.8:proportion of patients with fever with respect days after treatment.

Patients in ASAQ-arm cleared fever better than those in DHP arm However, no significant difference (p=0.21) was found between the treatment groups with respect to fever clearance time (FCT) at day 3

4.6 Modeling treatment outcome and delayance t o parasite clearance

4.6.1 Exploratory Data analysis

Exploring the relationship between the continuous variables that would be used to fit the multivariate model on therapy outcome, indicate that only weight and age of patients have linear relationship (Figure 4.9).

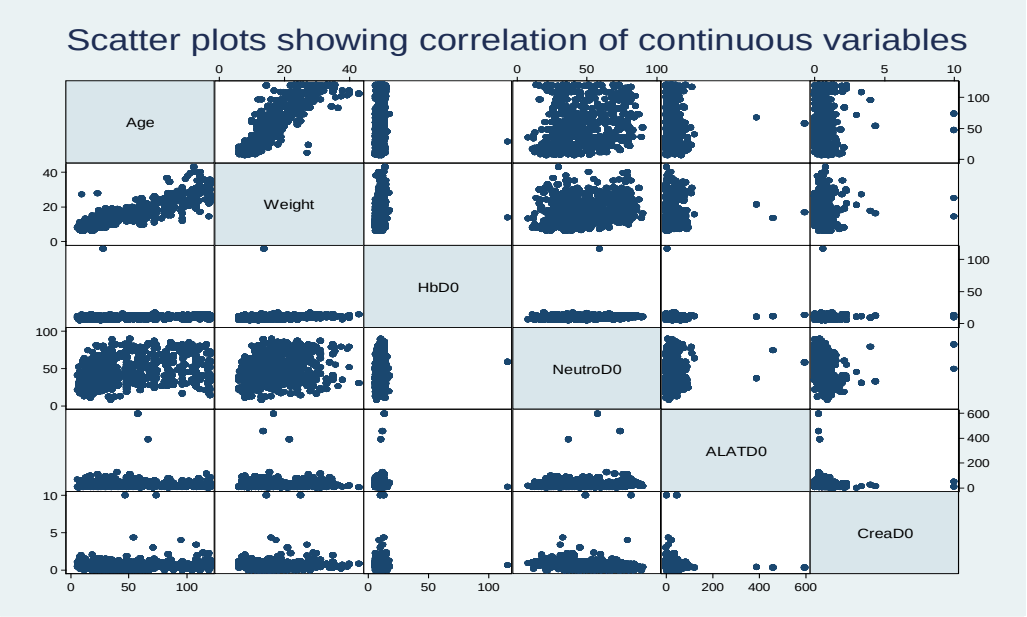


Figure 4.9:Correlation between continuous variables

A look at the distribution of the outcome (adequate clinical response) with the different continuous variables. The box plots below all show that only the neutrophil levels on day zero and age of patients show a difference in their distribution when comparing with the outcome status. Patients who have a positive outcome (cured) coded as "1" are older and have more of their neutrophil levels above the median neutrophil level than that their counterparts who are not cured coded as "0"(Figure 4.10).

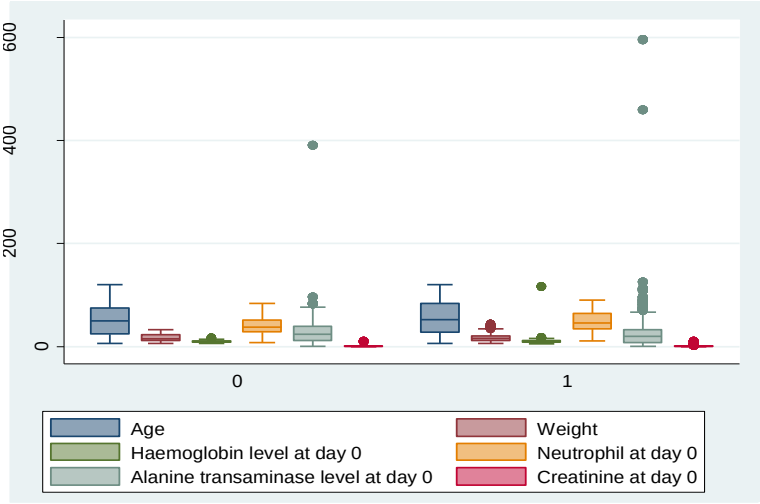


Figure 4.10:Relationship between the outcome (ACPR) and continuous variables

4.6.2 Multiple logistic regression

The full model with all covariates as described in methods section(not shown here) was fitted and non of the suspected predictors were significant at 0.05 level of significance. However the full model showed age group >5years to have an effect at 0.1 significant level.

Using the step function in R 2.11.1 as way of looking for the best fitting model, we compared the AIC values and taking the model with least values. The final model was fitted and is shown in Table 4.7

Table 4.6:Final model of Treatment outcome using logistic regression:

	Final model
Predictors	**β(S.E)**
Age_group>5yrs	0.39(0.50)
Site(Mutengene)	**0.99(0.60).**
ASAQ	0.24(0.74)
DHP	0.37(0.68)

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The final model showed no significant covariate at 0.05 level of significance However, at 0.1 level of significance, the site effect is significant. The odds of getting cured given that you are in Mutengene site is 2.69($e^{(.99)}$)

4.5.1 Exploratory Data analysis using Kaplan Meier survival estimates

The Kaplan-Meier Survival estimates in the following results, indicates the probability of patients still persisting with parasitaemia at the different times (days after treatment).

Figure 4.12 shows a distinct difference in the probability of survival on day across the three treatment arms with dihydroartemisinin piperaquine having the least probability of survival. However on day two, all of the treatments have a probability of lower than 0.1

with patients in dihydroartemisin arm still having the least probability of survival (having parasites still persisting) when compared to the other drugs in the study.

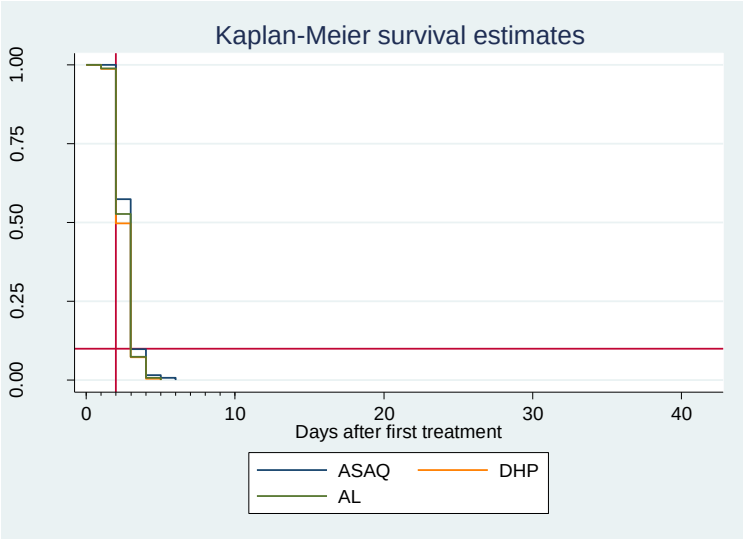


Figure 4.11:Kaplan-Meier survival estimates for persistence parasitaemia

On day one, patients who have genes NAT2 genes that make them fast metabolisers have less probability of surviving (parasites still persisting) than their slow metabolisers counter parts. This trend does not continue to day two where patients have the same probability of survival irrespective of their metaboliser status (Figure 4.13).

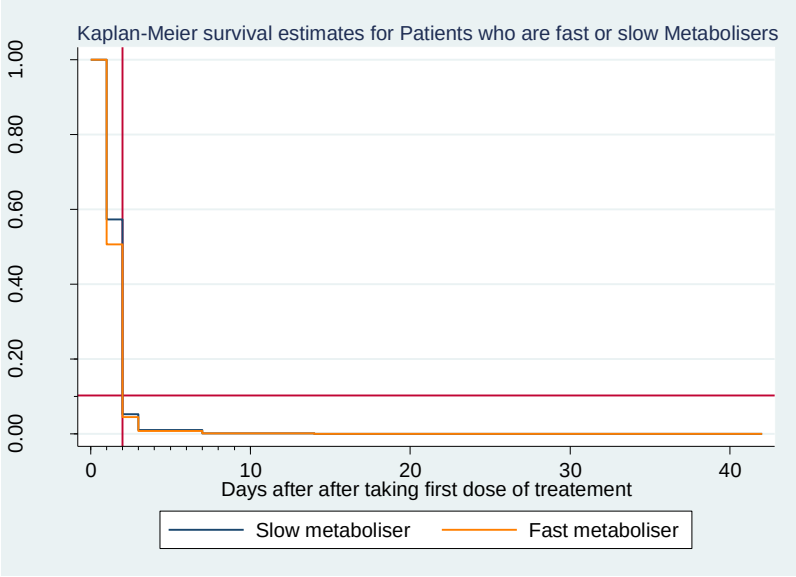


Figure 4.12:Kaplan-Meier survival estimates for fast and slow metaboliser

When age of patients is categorised into patients below 5 years of age and those above 5 years of age, there was no comparative difference between these groups with respect to their survival rates on any of the follow-up (Figure 4.14).

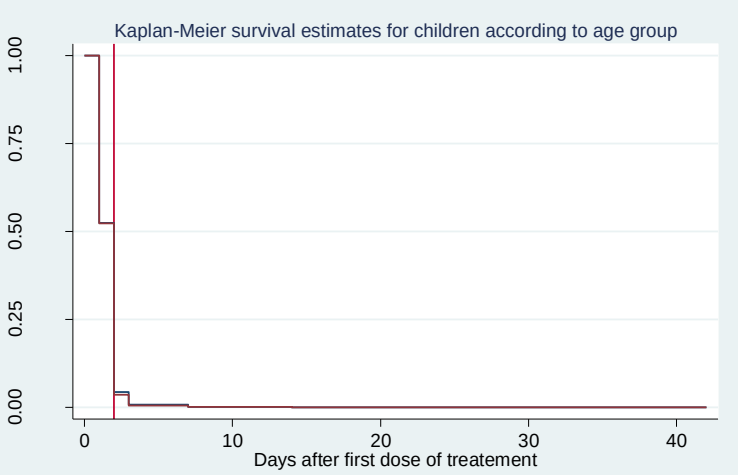


Figure 4.13:Kaplan-Meier survival analysis for children according to age groups.

Grouping the patients under study into whether they had the normal range of haemoglobin (haemoglobin levels<10g/l) levels or not seem to be time dependent. One day after treatment, the kaplan meier survival shows that those with low haemoglobin levels have a lower probability of persisting with parasite than those on those with normal haemoglobin ranges (Figure 4.15)

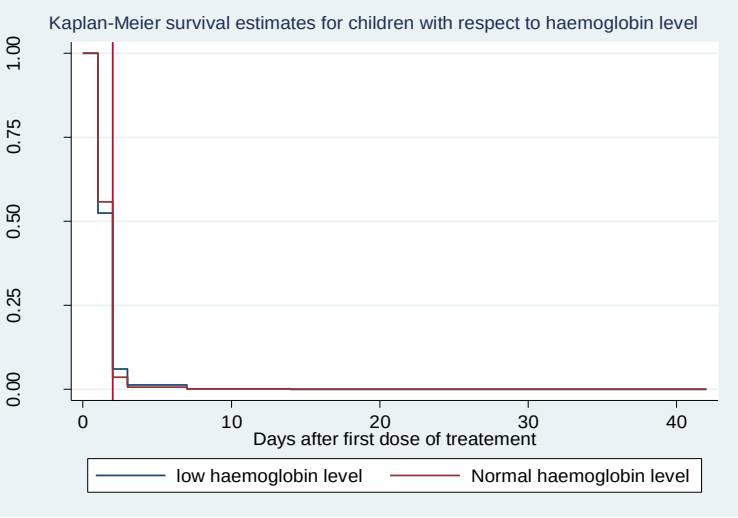


Figure 4.14:Kaplan-Meier survival analysis estimate with respect to haemoglobin level

When the patients are categorized into normal and abnormal with respect to the normal range in Cameroon (42-72)% neutrophil levels, the Kaplan-Meier survival curves show that those with abnormal neutrophil levels have a higher probability of persistent parasites in the first two days after treatment (Figure 4.16).

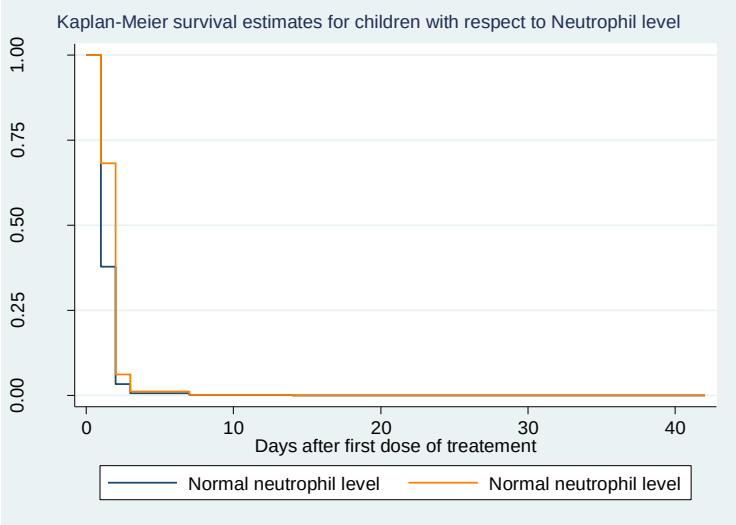


Figure 4.15:Kaplan-Meier survival estimates for children with respect to neutrophil level

Kaplan Meier survival estimates of the trend in parasite clearance with respect to site show that patients in Garoua have a lower probability of having their parasites persist on day one than their Mutengene site counterpart. The trend continues to day two but is not as appreciable as in day one (Figure 4.17).

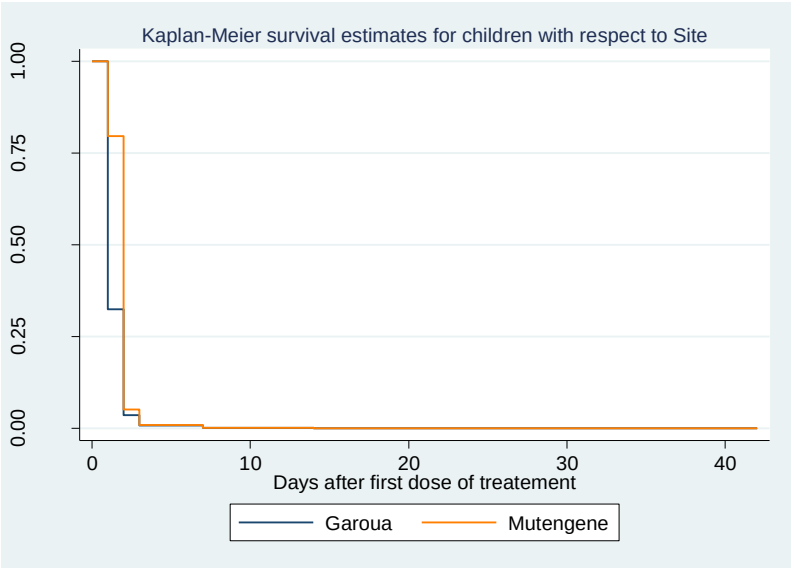


Figure 4.16:Kaplan-Meier survival estimates for children with respect to site

4.5.2 Fitting the discrete time survival model

The results of the discrete time fitted model in (Table 4.8), show the main effects of time(model 1) and the full model incorporating time and all the variables chosen for modeling(model2) and the final model which is gotten through assessing different models and judging best fit by the Akaike Information criteria.

Model 1 shows that day 1, 2, 3, 7 and 14 are the important days which influence parasite clearance. All analysis that follow would look at trends in parasite clearance using these days as time point.

In the final model, the age group, site, ALAT level on first hospital visit, creatinine level on first hospital visit, and the interaction between age group and site are important in determining time to parasite clearance(significance level of 0.05). However, at significance level of 0.1, the haemoglobin level is significant.

Table 4.7:Discrete time survival model parameter estimates with standard errors for Model 1,2 and 3

	Model1	Model 2	Final Model
Predictors	**β(S.E)**	**β(S.E)**	**β(S.E)**
day1	4.48(0.42)***	4.63(0.43)***	4.72(0.42)***
day2	6.41(0.44)***	7.14(0.48)***	7.20(0.46)***
day3	6.01(0.61)***	6.75(0.65)***	6.74(0.64)***
day7	5.06(0.61)***	5.04(.63)***	5.09(0.62)***
day14	6.66(1.14)***	6.60(1.16)***	6.64(1.14)***
day21	-8.99(535.41)	-9.77(533.41)	-9.52(535.41)
Sex(male)		-0.10(0.15)	
Age_group>5yrs		-0.76(0.40).	-0.68(0.22)**
Site(Mutengene)		4.05(1.29)**	-2.15(0.25)***
ASAQ		-0.14(0.36)	
DHP		-0.17(0.36)	
fast metaboliser		0.03(0.16)	
normal neutrophils level day0		-0.13(0.16)	
Normal ALAT level day 0		0.46(0.22)*	0.45(0.22)*
Ecotype(Forest)		1.49(1.23)	
Ecotype(Sahel)		-1.26(1.70)	
Urbanicity(urban)		-0.03(0.26)	
Weight		0.01(0.02)	
Temp		-0.09(0.11)	
Hb level>10g/dl		0.26(0.16)	0.29(0.16).
Normal Creatinine level at day0		-0.64(0.21)**	-0.64(0.20)**
Age_group>5yrs*ASAQ		0.12(0.41)	
Age_group>5yrs*DHP		-0.01(0.41)	
Site(Mutengene)*ASAQ		0.36(0.41)	
Site(Mutengene)*DHP		0.69(0.42).	
Age_group>5yrs*Site(Mutengene)		1.00(0.31)**	1.08(0.31)***
AIC	**1206.7**	**1145.6**	**1125.2**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

A table of odds ratios of the final model show that those with normal ALAT ,Hb levels on first day of visit have a higher chance of clearing their parasites earlier than their counter parts with abnormal levels

Table 4.8:Hazard Odd ratios and 95% CI for variables in the final model

Odd ratios and 95% CI for significant variables of final model

Predictors	Odd ratio estimate	95% CI
Age_group>5yrs	0.51	(0.33,0.78)
Site(Mutengene)	0.12	(0.07,0.18)
Normal ALAT level day 0	1.56	(1.02,2.40)
Hb level>10g/dl	1.34	(0.98,1.82)*
Normal Creatinine level at day0	0.52	(0.35,0.78)
Age_group>5yrs*Site(Mutengene)	2.94	(1.62,5.38)

*not significant

However, having normal Creatinine levels is associated with delay in parasite clearance than those with abnormal creatinine levels(usually lower levels than minimum normal level).Parasite clearance is delayed in mutengene (OR=0.12) in general influence is modified whether the child is older than 5years or not.

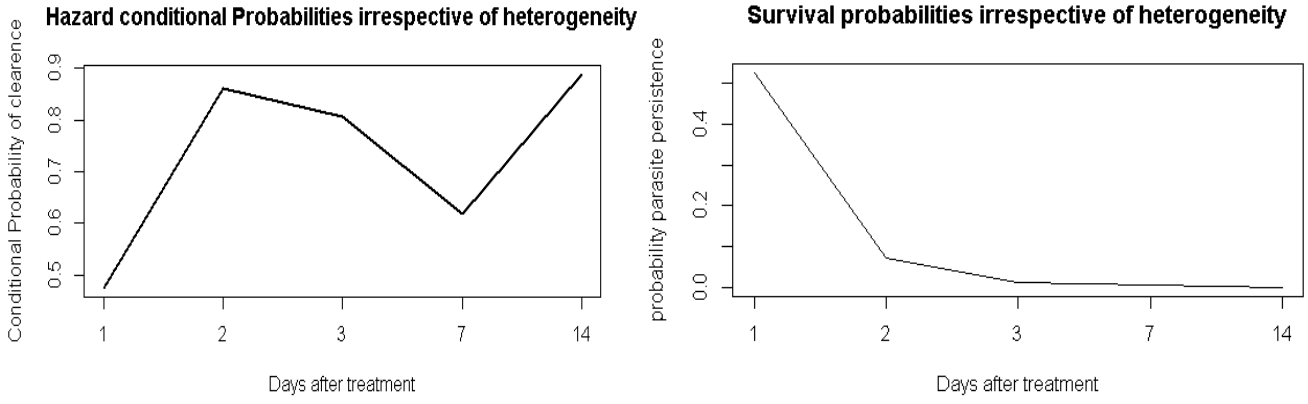


Figure 4.17:Hazard and survival probability profiles of the time effect on parasite clearance

high chance of children clearing their parasites if they had not already done so on day 1. By day 3, most children have cleared their parasite(see survival function-figure 4.18b)

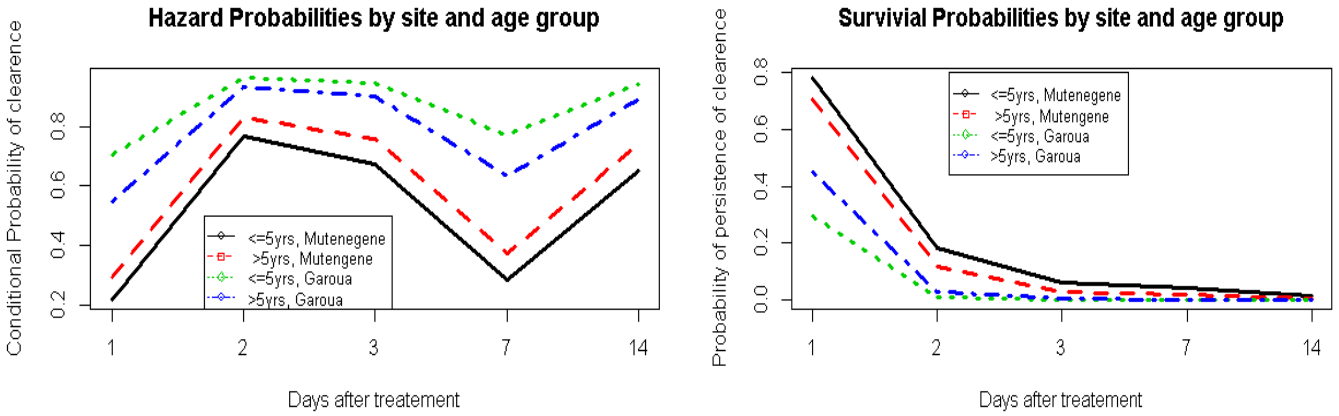


Figure 4.18:Hazard and survival probabilities by site and age group

Mutengene lags behind Garoua in parasite clearance especially from day 1 and 2. Older children in Mutengene has a better parasite clearance prognosis than the younger ones(figure 4.19a). In Garoua(see figure 4.19b) it is rather younger children who clear their parasites earlier(day 1 especially) than the older children.

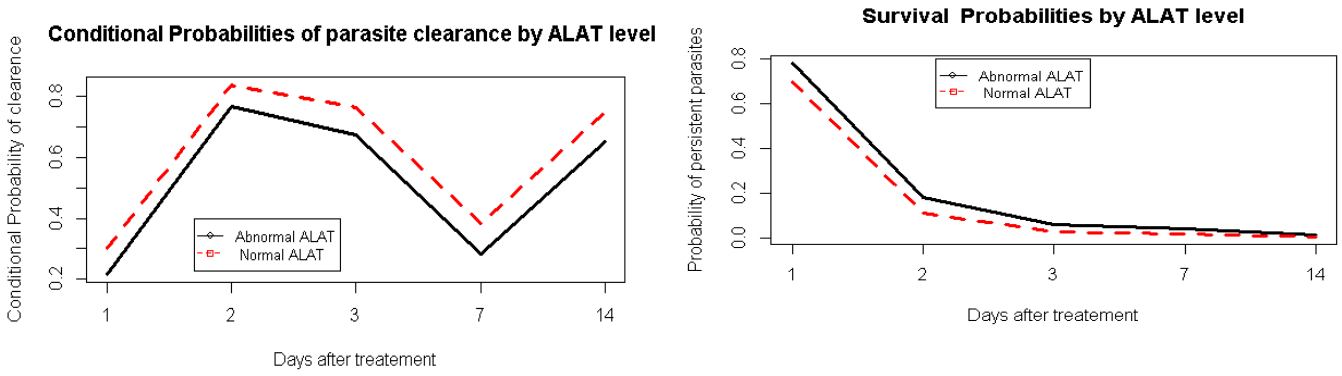


Figure 4.19:Hazard and survival probabilities by ALAT level

The alanine aminotransferase(ALAT) level influences time to parasites clearance. Those with normal normal ALAT levels on first day of visit remain consistent with a higher chance of clearing their parasites by each visit day on condition that they did not clear on a previous visit day. For day 2 , for example, the probability of clearing parasites normal ALAT level is 83% while for abnormal ALAT level is 76% given that the parasites were not cleared on day 0.

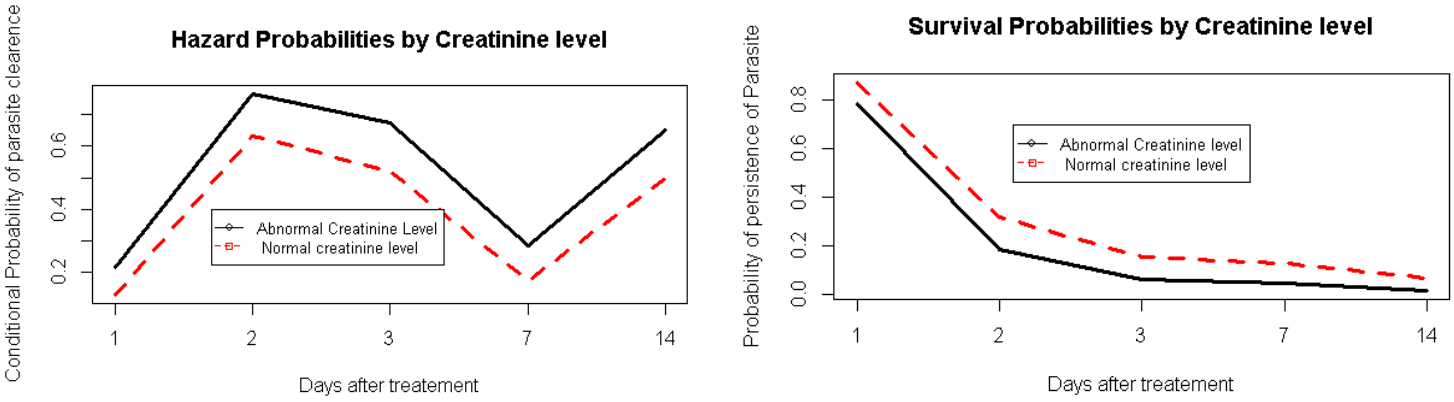


Figure 4.20:Hazard and survival probabilities by creatinine levels

Patient creatinine levels also influence how fast malaria parasites get cleared. Those with a Cameroonian specific normal creatinine level (see annex on normal ranges) have lower chance of clearing their parasites by each visit day given they had not cleared in the previous day than those that have abnormal creatinine levels.

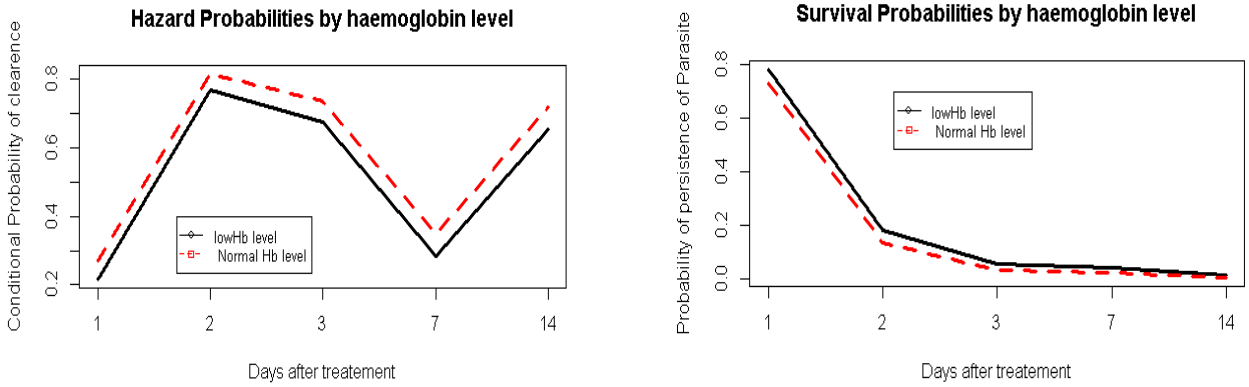


Figure 4.21:Hazard and survival probabilities by haemoglobin levels

Even though haemoglobin levels is not a very important(significant only at 0.1 level of significance) covariate that influences time to parasite clearance, we show that there is a trend in which those with low haemoglobin levels lag behind those with a normal haemoglobin level in clearing parasites(figure 4.22)

4.5.3 Discrete time survival Model Diagnostics

We fitted a generalised additive model using the variables of the full model while smoothing weight and temperature which are the only continuous covariates. Weight had degree of freedom of 1.001 while temperature had a degree of 3.077. Even though the smoother for temperature has more than 1 degree of freedom, it is not significant(p value=0.16). Therefore the linearity assumption of the predictors is tenable.

Fitting a mixed model to the final selected predictors and with subjects or individual patients as random effects, we get the variance captured by random effects to be 0.00000078 with standard deviation of 0.00028 which is very negligible. Therefore the assumption of no unobserved heterogeneity in the model is valid. Therefore there is evidence that our assumptions for this discrete time survival model hold.

Chapter 5: Discussion

Efficacy, safety and Non inferiority of Drugs

Artemisinin-based combination therapies (ACTs) continue to gain ground as the most efficacious treatment for uncomplicated *Plasmodium falciparum* malaria. Many studies are looking at the comparative efficacy of the different ACTs in settings where the treatment is most likely to be used[137]. These studies seek to better inform malaria experts and health policy makers on the preferred ACTs or alternatives [137] for the different malaria endemic countries[100-102] . This study has compared the efficacy of ASAQ, DHP and AL in the same population during the same period.

Results presented herein show high cure rates for the different treatment arms for 14, 28 and 42 follow-up days of follow up. These results are consistent with results from studies in other malaria endemic countries in Sub-saharan Africa [101] [102]. The high cure rate is consistent in the two sites with different ecologies and climatic conditions. The high cure rates of these anti-malaria drugs and the effective use of insecticide treated bed nets could significantly reduce morbidity and mortality in Cameroonian population (NMCP Cameroon Report 2012)

Although the difference is not significant, studies have shown that the dietary cow milk which is one of the main nutrition source for the Fulani community is speculated to have an immuno protective activity with the potential to act also against drug resistant forms of the malaria parasite [20], [140]. This study shows similar ACTs cure rates in Mutengene and Garoua and therefore supports the nationwide implementation of ACTs irrespective of geography and ethnicity and brings an added advantage towards malaria elimination.

The authorities in the Ministry of Health in Cameroon are fighting the illicit sale of medication by road vendors and unauthorized agents. There is still a wide circulation of competing drugs to those enforced by the government for treating malaria. The situation is worsened with stock outs of antimalarials at the different recognized distribution centers [141]. Patients are obliged to search for alternatives without proper information

on the source of anti-malarial, its efficacy and tolerability. The non-inferiority of the study drugs compared to widely used artemether lumefantrine will enable care givers to make informed prescription decisions. The use of alternative available efficacious and safe drugs is helpful in delaying the antimalarial drug resistance that is beginning to emerge [24]

With the reports of emergence of drug resistance to the very efficacious artemisinin combination therapies, there is need to monitor the efficacy and safety of these drugs closely. One way to monitor anti-malaria drug resistance in the absence of validated molecular markers and appropriate *in vitro* models is by analyzing parasite clearance times [142]. Parasite clearance time curves represented by the proportion of patients that clear parasites with respect to the time from onset of treatment did not show any appreciable delay across the three study drugs. There is still the need for planned monitoring of the *in vivo* parasite clearance times for the different ACTs currently used in Cameroon.

The proportion of patients with a temperature below 37^0C after the 3rd day of first treatment and who remain so for the next 48 hours show similar patterns across the different study drugs. However pair wise comparison showed a difference between Patients in the ASAQ treatment compared to the DHP treatment group.. This difference however only suggests that patients under ASAQ treatment as compared to DHPand AL get relieved of the symptom much faster. This does not have any implications on the efficacy of the drugs.

The change in liver function tests: creatinine, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) did not show any significant difference between day 0 and day 7 values across the three drug regimens under study. However, there is a significant difference in the day 0 and day7 haemoglobin levels of patients in the AL and DHP. This therefore suggests that patients in the ASAQ treatment group are more likely to achieve convalescence before their counterparts in the AL and DHP treatment group. Though there is need for further research, this result also suggests that Iron tablets alongside AL, DHP might be considered in management of malaria patients.

Treatment Outcome model

Patients' ability to fight diseases plays a major role in any therapy outcome. In some studies, characteristics like age, sex play great role. Given that the outcome is measured only on day 42 for per protocol analysis, we go for a logistic regression.

Malaria has be shown to associated with anemia (low haemoglobin levels) especially during pregnancy [143]. However some artemisinin combination therapies like ASAQ and AL increase haemoglobin levels from day 0 to day 28 at different rates [144]. Even though haemoglobin levels are low in patients with malaria, the sickle cell haemoglobin plays a protective role against malaria [145]. High haemoglobin level is protective against high parasitaemia which also reduces the risk of severe malaria [145].

In our Logistic model, low haemoglobin levels have not been associated with an unfavorable treatment outcome to the drugs.

singlet oxygen have been shown to be the neutrophil product responsible in supressing the growth of parasistes. [146]. Neutrophils , which play a great role in adverting infections in humans according to our model do not(not significant) influence treatment outcome. All the drugs under study have not shown any association with neutropenia(low neutrophil levels). Therefore, there is a synergy between high Neutrophils level and treatment in the treatment outcome. Children under the age of 10 who are the cohort under this study are exposed to many infections due to the fact that their immunity is still for the most part innate and therefore, a combined effort by health practitioners (in Cameroon especially) needs to be directed to fight other infections common to this age group to save these efficacious drugs .

Alanine aminotransferase(ALAT) which is one of the regular liver function test in any drug tolerability study was not selected in our final model as a predictor for therapy outcome. Aline aminotransferase is indicative of liver injury. Dihydroartemisinin which is one of the drugs in this study has been shown to have been associated with elevated levels of alanine amintransferase [41] . The liver which is a vital organ of the human

system is responsible for metabolism and is very crucial in drug intake to the target cells. A healthy liver will therefore favour a positive outcome of any treatment including all anti malarials. Alanine amino transferase not influencing therapy outcome according to our model might mean that our study population tolerate the drugs so well .

The drugs which patients are prescribed also play a great role in therapy outcome. In our study, the treatment arm to which the patient is, has little or no predictibility to whether the patients gets cured or not. This result is inconsistent with studies that show that first line treatments to malaria must not be global but be on a region basis [147]. In Cameroon, there is a high drug pressure with many drug vendors that are not licensed [141] there is is need for health care givers to be aware that AL,DHP and ASAQ could be used as first line drugs and does not jeopardise cure. Drug regulation authorities would save the very efficacious drugs by stringently regulating the type of anti-malarials sold with preference with these three(AL,ASAQ,DHP)

Parasite clearance time

In this study we sought to model parasite clearance time in two ecologically regions (Mutengene and Ngaoundéré) in children age six months to 10 years following treatment with Arthemeter-Lumefantrine (AL), artesunate-amodiaquine (ASAQ) and Dihydroartemisinin piperaquine (DHP). With the delay in the parasite clearance time that might have contributed to artesunate-mefloquine resistance at the Thai-Cambodia border ways , circumventing or delaying the onset and spread of resistance have been proposed among which is the implementation of multiple first line therapies [39] [24].

There are many contributing and interrelated factors that are associated to delay of parasite clearance . Integrating these factors in models gives better estimation of each contributing factor to the delayance. Others have proposed and used models which express best the parasite clearance rate and using clearance time as a continuous variable(white et al). In our study, we fitted a discrete time survival model and the interaction of age group and site was significant. However, the relationship between age group and site is not consistent across the two sites. In mutengene,older children(>5yrs)

have a higher probability of clearing the parasite than their younger(<=5yrs). In Garoua, the relation is reversed to that in mutengene with younger children clearing parasites faster. This is inconsistent with studies that have suggested age to be an inadequate surrogate marker [148] for immunity especially in low malaria transmission areas. This model has shown that patients in Mutengene lag behind those in Garoua especially at one and two days after treatment in clearing their parasites. This might be due to that fact that patients in Mutengene which is a high malaria transmission rain forest area have persistent parasitaemia.

Patients who are fast metabolisers are more prone to have parasite persisting on day one probably owing to the availability of plasma drug [148]. This was not evaluated in our study. However when comparing time to parasite clearance with respect to patients metaboliser status, it was not significant.

Patients with normal ALAT on their first visit to the hospital would clear their parasites faster than those with abnormal levels. This trend is also true for normal haemoglobin levels even though it is only significant at 0.1 significant level. Abnormal creatinine levels(most have lower creatinine levels than normal) have shown to favour faster parasite clearance. Abnormal ALAT levels and elevated creatinine levels are indicative of an infection in the case of ALAT and start of failure of kidneys in case of creatinine. This therefore means a better prognosis in terms of parasite clearance would be achieved if health givers can treat all co infections as well. Abnormal haemoglobin levels would also compromise the time to parasite clearance.

Chapter 6: Conclusion and Recommendation

6.1 Conclusion

Even though at the start of this study, the government policy for first line treatment of plasmodium falciparium malaria was artemether lumefantrine, this research has shown that artesunate amodiaquine and dihydroartemisinin piperaquine are also highly efficacious with cure rates of 98.1% and 96.3% respectively. Better still, this study has shown that these two drugs(artesunate amodiaquine and dihydroartemisin piperaquine) are at least not worse off(with a 10% margin of inferiority) than artemeter lumefantrine in the treatment of *Plasmodium falciparum* malaria in the cameroonian children.

The three drugs in this study are comparatively safe. However those who were treated with dihydroartemisin piperaquine and artemeter lumefantrine show a reduction in haemoglobin levels compared to the levels of the first day of treatment. There was no significant difference when comparing the frequency of the adverse events(mostly vomiting, cough and lack of appetite) that were suspected to be caused by drug, across the different treatments.

The characteristics or variables considered in our outcome model do not influence cure rates. However, those in Mutengene at 0.1 significant level are at least two times(OR=2.7) as likely to be cured than those in Garoua.

There is a delayance in Parasite clearance in Mutengene compared to Garoua especially on day 1 and 2. This delayance is further modified in each site by age group.

Abnormal creatinine, alanine aminotransferase , and haemoglobin levels before taking AL,ASAQ and DHP for the treatment of malaria compromise the time to parasite clearance in the blood stream.

6.2 Recommendation

The national malaria control program of Cameroon which is the Main malaria control policy maker should expand their first line treatment of plasmodium malaria to include artemether lumefantrine, dihydroartemisinin piperaquine and artesunate amodiaquine which have been shown to be very efficacious against malaria.

Due to the drop in haemoglobin levels after treatment with these drugs, policy should include association of iron replenishing drugs and vitamin B12 with these anti-malarials.

Given that creatinine and alanine aminotransferase levels play arole according to this study in the time to parasite clearance,control efforts to curb other infections that are common with children under the age of 10 years is crucial. This will boost efficacy and therefore prolong the time to appearance of drug resistance to these very efficacious anti malarials

A more in-depth study on why patients in forest region(mutengene) lag behind those in the sahel savannah region(Garoua) of Cameroon is crucial. This would help understand how other factors that were not considered in this study could usher in drug resistance.

REFERENCES

[1] B. Singh, L. Kim Sung, A. Matusop, A. Radhakrishnan, S. S. G. Shamsul, J. Cox-Singh, A. Thomas, and D. J. Conway, “A large focus of naturally acquired Plasmodium knowlesi infections in human beings.,” *Lancet*, vol. 363, no. 9414, pp. 1017–1024, 2004.

[2] A. Cohuet, C. Harris, V. Robert, and D. Fontenille, “Evolutionary forces on Anopheles: what makes a malaria vector?,” *Trends in Parasitology*, vol. 26, no. 3, pp. 130–136, 2010.

[3] S. M. Rich and F. J. Ayala, “Population structure and recent evolution of Plasmodium falciparum.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 13, pp. 6994–7001, 2000.

[4] World Health Organization, “The Africa Malaria report,” 2008.

[5] J. G. Beeson, “Of mothers and malaria,” *Trends in Parasitology*, vol. 18, no. 9, p. 420, 2002.

[6] R. W. Steketee, B. L. Nahlen, M. E. Parise, and C. Menendez, “The burden of malaria in pregnancy in malaria-endemic areas.,” *The American journal of tropical medicine and hygiene*, vol. 64, no. 1–2 Suppl, pp. 28–35, 2001.

[7] J. Sachs and P. Malaney, “The economic and social burden of malaria.,” *Nature*, vol. 415, no. 6872. pp. 680–685, 2002.

[8] World. health Organisation, “World health oranisation Malaria Report ,” 2011.

[9] D. L. Doolan, C. Dobaño, and J. K. Baird, “Acquired immunity to malaria.,” *Clinical microbiology reviews*, vol. 22, no. 1, pp. 13–36, Table of Contents, 2009.

[10] C. Sayang, M. Gausseres, N. Vernazza-Licht, D. Malvy, D. Bley, and P. Millet, “Treatment of malaria from monotherapy to artemisinin-based combination therapy by health professionals in urban health facilities in Yaoundé, central province, Cameroon,” *Malaria Journal*, vol. 8, no. 1, p. 176, 2009.

[11] C. C. A. Malaria(CCAM), “POLICY BRIEF ON SCALING UP MALARIA CONTROL INTERVENTIONS IN CAMEROON,” 2008.

[12] R. W. Steketee and C. C. Campbell, “Impact of national malaria control scale-up programmes in Africa: magnitude and attribution of effects.,” *Malaria journal*, vol. 9, no. 1, p. 299, 2010.

[13] A. P. Phyo, S. Nkhoma, K. Stepniewska, E. a Ashley, S. Nair, R. McGready, C. Ier Moo, S. Al-Saai, A. M. Dondorp, K. M. Lwin, P. Singhasivanon, N. P. J. Day, N. J. White, T. J. C. Anderson, and F. Nosten, "Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study.," *Lancet*, vol. 379, no. 9830, pp. 1960–1966, 2012.

[14] R. J. Pearce, H. Pota, M.-S. B. Evehe, E.-H. Bâ, G. Mombo-Ngoma, A. L. Malisa, R. Ord, W. Inojosa, A. Matondo, D. a Diallo, W. Mbacham, I. V van den Broek, T. D. Swarthout, A. Getachew, S. Dejene, M. P. Grobusch, F. Njie, S. Dunyo, M. Kweku, S. Owusu-Agyei, D. Chandramohan, M. Bonnet, J.-P. Guthmann, S. Clarke, K. I. Barnes, E. Streat, S. T. Katokele, P. Uusiku, C. O. Agboghoroma, O. Y. Elegba, B. Cissé, I. E. A-Elbasit, H. a Giha, S. P. Kachur, C. Lynch, J. B. Rwakimari, P. Chanda, M. Hawela, B. Sharp, I. Naidoo, and C. Roper, "Multiple origins and regional dispersal of resistant dhps in African Plasmodium falciparum malaria.," *PLoS medicine*, vol. 6, no. 4, p. e1000055, 2009.

[15] T. M. Lopera-mesa, S. Doumbia, S. Chiang, A. E. Zeituni, D. S. Konate, M. Doumbouya, A. S. Keita, K. Stepniewska, K. Traore, S. A. S. Diakite, D. Ndiaye, J. M. Sa, J. M. Anderson, M. P. Fay, C. A. Long, M. Diakite, and R. M. Fairhurst, "Plasmodium falciparum Clearance Rates in Response to Artesunate in Malian Children With Malaria : Effect of Acquired Immunity," *The Journal of infectious diseases*, vol 207, no. 11 pp. 1–9, 2013.

[16] K. ARTAVANIS-TSAKONAS, J. E. TONGREN, E. M. RILEY "The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology.," Clinical & Experimental Immunology, vol 133, no. 2 pp. 145–152, 2003.

[17] W. O. Rogers, R. Sem, T. Tero, P. Chim, P. Lim, S. Muth, D. Socheat, F. Ariey, and C. Wongsrichanalai, "Failure of artesunate-mefloquine combination therapy for uncomplicated Plasmodium falciparum malaria in southern Cambodia.," *Malaria journal*, vol. 8, p. 10, 2009.

[18] E. M. Hodel, S. D. Ley, W. Qi, F. Ariey, B. Genton, and H.-P. Beck, "A microarray-based system for the simultaneous analysis of single nucleotide polymorphisms in human genes involved in the metabolism of anti-malarial drugs," *Malaria Journal*, vol. 8, p. 285, 2009.

[19] A. N. Zeba, H. Sorgho, N. Rouamba, I. Zongo, J. Rouamba, R. T. Guiguemdé, D. H. Hamer, N. Mokhtar, and J.-B. Ouedraogo, "Major reduction of malaria morbidity with combined vitamin A and zinc supplementation in young children in Burkina Faso: a randomized double blind trial.," *Nutrition journal*, vol. 7, p. 7, 2008.

[20] a I. Lokki, I. Järvelä, E. Israelsson, B. Maiga, M. Troye-Blomberg, A. Dolo, O. K. Doumbo, S. Meri, and V. Holmberg, "Lactase persistence genotypes and malaria

susceptibility in Fulani of Mali," *Malaria Journal*, vol. 10, no. 1475–2875 (Electronic), p. 9, 2011.

[21] V. D. Mangano, G. Luoni, K. A. Rockett, B. S. Sirima, J. Forton, T. Clark, G. Bancone, E. S. Akha, S. Pubblica, S. Parassitologia, and R. La, "Interferon regulatory factor-1 polymorphisms are associated with the control of Plasmodium falciparum infection," Genes and Immunity, vol. 9, no. 2, pp. 122–129, 2010.

[22] E. M. Hodel, *The effects of pharmacogenetics on pharmacokinetics of artemisinin-based combinations in malaria patients.",* PhD Thesis, University of Basel, Faculty of Science 2009.

[23] L. C. Okell, C. J. Drakeley, T. Bousema, C. J. M. Whitty, and A. C. Ghani, "Modelling the impact of artemisinin combination therapy and long-acting treatments on malaria transmission intensity.," *PLoS medicine*, vol. 5, no. 11, p. e226; discussion e226, 2008.

[24] D. L. Smith, E. Y. Klein, F. E. McKenzie, and R. Laxminarayan, "Prospective strategies to delay the evolution of anti-malarial drug resistance: weighing the uncertainty.," *Malaria journal*, vol. 9, p. 217, 2010.

[25] L. J. White, R. J. Maude, W. Pongtavornpinyo, S. Saralamba, R. Aguas, T. Van Effelterre, N. P. J. Day, and N. J. White, "The role of simple mathematical models in malaria elimination strategy design," *Malaria Journal*, vol. 8, no. 1, p. 212, 2009.

[26] W. Pongtavornpinyo, S. Yeung, I. M. Hastings, A. M. Dondorp, N. P. Day, and N. J. White, "Spread of anti-malarial drug resistance: Mathematical model with implications for ACT drug policies," *Malaria Journal*, vol. 7, no. 229, p. 229, 2008.

[27] S. Mok, M. Imwong, M. J. Mackinnon, J. Sim, R. Ramadoss, P. Yi, M. Mayxay, K. Chotivanich, K.-Y. Liong, B. Russell, D. Socheat, P. N. Newton, N. P. J. Day, N. J. White, P. R. Preiser, F. Nosten, A. M. Dondorp, and Z. Bozdech, "Artemisinin resistance in Plasmodium falciparum is associated with an altered temporal pattern of transcription.," *BMC genomics*, vol. 12, no. 1, p. 391, 2011.

[28] K. Theys, K. Deforche, J. Vercauteren, P. Libin, D. A. van de Vijver, J. Albert, B. Asjö, C. Balotta, M. Bruckova, R. J. Camacho, B. Clotet, S. Coughlan, Z. Grossman, O. Hamouda, A. Horban, K. Korn, L. G. Kostrikis, C. Kücherer, C. Nielsen, D. Paraskevis, M. Poljak, E. Puchhammer-Stockl, C. Riva, L. Ruiz, K. Liitsola, J.-C. Schmit, R. Schuurman, A. Sönnerborg, D. Stanekova, M. Stanojevic, D. Struck, K. Van Laethem, A. M. Wensing, C. A. Boucher, and A.-M. Vandamme, "Treatment-associated polymorphisms in protease are significantly associated with higher viral load and lower CD4 count in newly diagnosed drug-naive HIV-1 infected patients.," *Retrovirology*, vol. 9, p. 81, 2012.

[29] J. C. Koella and R. Antia, “Epidemiological models for the spread of anti-malarial resistance.,” *Malaria journal*, vol. 2, p. 3, Feb. 2003.

[30] J. Bickii, L. K. Basco, and P. Ringwald, “Assessment of three in vitro tests and an in vivo test for chloroquine resistance in Plasmodium falciparum clinical isolates.”, *Journal of clinical microbiology*, vol. 36, no. 1, pp. 243–247, 1998.

[31] L. K. Basco, “Molecular epidemiology of malaria in Cameroon. XIX. Quality of antimalarial drugs used for self-medication.” ,*The American journal of tropical medicine and hygiene*, vol. 70, no. 3, pp. 245–250, 2004.

[32] E. A. Mbacham WF, Njuabe MT, Evehe MS, Moyou R, “Antimalaria Drug Studies in Cameroon Reveal Deteriorating Fansidar and Amodiaquine Cure Rates." Journal of the Cameroon Academy of Sciences, 5. 58-64, vol. 5, pp. 58–64, 2005.

[33] W. F. Mbacham, M. B. Evehe, P. M. Netongo, I. M. Ali, N. E. Nfor, A. I. Akaragwe, P. N. Mimche, A. Nji, C. F. Djoko, B. Tawe, B. Gawa, T. Asongna, B. Toh, B. Atogho-tieudeu, N. Nge, R. Ebeng, J. Ahmadou, C. Kuaban, J. Bickii, V. Penlap, V. P. Titanji, and N. Njikam, “Mutations within folate metabolising genes of Plasmodium falciparum in Cameroon,” Malaria Journal vol. 8, no. 19, pp. 4749–4754, 2009.

[34] P. J. Guerin, S. J. Bates, and C. H. Sibley, “Global resistance surveillance: ensuring antimalarial efficacy in the future.,” *Current Opinion in Infectious Diseases*, vol. 22, no. 6, pp. 593–600, 2009.

[35] Centre for disease control, “Malaria history.” [Online]. Available: http://www.cdc.gov/malaria/history/index.htm. [Accessed: 15-Jan-2013].

[36] J. JARAMILLO-ARANGO, *The Conquest of Malaria*. 1950.

[37] I. Petersen, R. Eastman, and M. Lanzer, “Drug-resistant malaria: molecular mechanisms and implications for public health.,” *FEBS letters*, vol. 585, no. 11, pp. 1551–1562, 2011.

[38] A. Attaran, K. I. Barnes, C. Curtis, U. Alessandro, C. I. Fanello, M. R. Galinski, G. Kokwaro, S. Looareesuwan, M. Makanga, T. K. Mutabingwa, A. Talisuna, J. F. Trape, and W. M. Watkins, “Viewpoint WHO , the Global Fund , and medical malpractice in malaria treatment,” vol. 363, pp. 237–240, 2010.

[39] M. F. Boni, D. L. Smith, and R. Laxminarayan, “Benefits of using multiple first-line therapies against malaria.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 37, pp. 14216–14221, 2008.

[40] WHO, “WHO Malaria report,” 2008.

[41] E. A. Ashley and N. J. White, “Artemisinin-based combinations,” *Current Opinion in Infectious Diseases*, vol. 18, no. 6, 2005.

[42] P. Lim, A. P. Alker, N. Khim, N. K. Shah, S. Incardona, S. Doung, P. Yi, D. M. Bouth, C. Bouchier, O. M. Puijalon, S. R. Meshnick, C. Wongsrichanalai, T. Fandeur, J. Le Bras, P. Ringwald, and F. Ariey, “Pfmdr1 copy number and arteminisin derivatives combination therapy failure in falciparum malaria in Cambodia.,” *Malaria journal*, vol. 8, p. 11, 2009.

[43] M. A. Travassos and M. K. Laufer, “Resistance to Antimalarial Drugs : Molecular , Pharmacologic , and Clinical Considerations,” *Malaria Journal*, vol. 65, no. 5, pp. 64–70, 2009.

[44] A. Martensson, B. Ngasala, J. Ursing, M. Isabel Veiga, L. Wiklund, C. Membi, S. M. Montgomery, Z. Premji, A. Farnert, and A. Bjorkman, “Influence of consecutive-day blood sampling on polymerase chain reaction-adjusted parasitological cure rates in an antimalarial-drug trial conducted in Tanzania.,” *The Journal of infectious diseases*, vol. 195, no. 4, pp. 597–601, 2007.

[45] R. Jambou, E. Legrand, M. Niang, N. Khim, P. Lim, B. Volney, M. T. Ekala, C. Bouchier, P. Esterre, T. Fandeur, and O. Mercereau-Puijalon, “Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6.,” *Lancet*, vol. 366, no. 9501, pp. 1960–1963, 2005.

[46] R. G. Davis, A. Kamanga, C. Castillo-Salgado, N. Chime, S. Mharakurwa, and C. Shiff, “Early detection of malaria foci for targeted interventions in endemic southern Zambia.,” *Malaria journal*, vol. 10, p. 260, 2011.

[47] S. Mok, M. Imwong, M. J. Mackinnon, J. Sim, R. Ramadoss, P. Yi, M. Mayxay, K. Chotivanich, K.-Y. Liong, B. Russell, D. Socheat, P. N. Newton, N. P. Day, N. J. White, P. R. Preiser, F. Nosten, A. M. Dondorp, and Z. Bozdech, “Artemisinin resistance in Plasmodium falciparum is associated with an altered temporal pattern of transcription,” *BMC Genomics*, vol. 12, no. 1, p. 391, 2011.

[48] K. Mint Lekweiry, A. Ould Mohamed Salem Boukhary, T. Gaillard, N. Wurtz, H. Bogreau, J. E. Hafid, J.-F. Trape, H. Bouchiba, M. S. Ould Ahmedou Salem, B. Pradines, C. Rogier, L. K. Basco, and S. Briolant, “Molecular surveillance of drug-resistant Plasmodium vivax using pvdhfr, pvdhps and pvmdr1 markers in Nouakchott, Mauritania.,” *The Journal of antimicrobial chemotherapy*, vol. 67, no. 2, pp. 367–374, 2012.

[49] A. Das, M. Sharma, B. Gupta, and A. P. Dash, “Plasmodium falciparum and Plasmodium vivax: so similar, yet very different.,” *Parasitology research*, vol. 105, no. 4, pp. 1169–1171, 2009.

[50] R. Carter and K. N. Mendis, “Evolutionary and Historical Aspects of the Burden of Malaria Evolutionary and Historical Aspects of the Burden of Malaria,” Clinical Microbiology reviews , vol. 15, no. 4, PP. *564-594*

[51] W. E. Collins and G. M. Jeffery, “Plasmodium malariae: parasite and disease.,” *Clinical microbiology reviews*, vol. 20, no. 4, pp. 579–592, 2007.

[52] W. E. Collins and G. M. Jeffery, “Plasmodium ovale : Parasite and Disease Plasmodium ovale : Parasite and Disease,” vol. 18, no. 3, 2005.

[53] C. Putaporntip, T. Hongsrimuang, S. Seethamchai, T. Kobasa, K. Limkittikul, L. Cui, and S. Jongwutiwes, “Differential prevalence of Plasmodium infections and cryptic Plasmodium knowlesi malaria in humans in Thailand.,” *The Journal of infectious diseases*, vol. 199, no. 8, pp. 1143–1150, 2009.

[54] E. Serra-Casas, C. Menendez, C. Dobano, a. Bardaji, L. Quinto, J. Ordi, B. Sigauque, P. Cistero, I. Mandomando, P. L. Alonso, and a. Mayor, “Persistence of Plasmodium falciparum Parasites in Infected Pregnant Mozambican Women after Delivery,” *Infection and Immunity*, vol. 79, no. 3, p. 1399, 2011.

[55] A. D. Kitchen and P. L. Chiodini, “Malaria and blood transfusion,” *Vox Sanguinis*, vol. 90, no. 2, pp. 77–84, 2006.

[56] L. M. Yamauchi, A. Coppi, G. Snounou, and P. Sinnis, “Plasmodium sporozoites trickle out of the injection site.,” *Cellular microbiology*, vol. 9, no. 5, pp. 1215–1222, 2007.

[57] R. Amino, S. Thiberge, S. Shorte, F. Frischknecht, and R. Ménard, “Quantitative imaging of Plasmodium sporozoites in the mammalian host.,” *Comptes rendus biologies*, vol. 329, no. 11, pp. 858–862, 2006.

[58] L. D. Sibley, S. Hâkansson, and V. B. Carruthers, “Gliding motility: an efficient mechanism for cell penetration.,” *Current biology : CB*, vol. 8, no. 1, pp. R12–4, 1998.

[59] R. L. Coppep and V. Brown, “Adhesive proteins of the malaria parasite and Victor of resulting.”

[60] P. Perlmann and M. Troye-Blomberg, “Malaria and the immune system in humans.,” *Chemical immunology*, vol. 80, pp. 229–242, 2002.

[61] L. H. Bannister, J. M. Hopkins, R. E. Fowler, S. Krishna, and G. H. Mitchell, “A Brief Illustrated Guide to the Ultrastructure of Plasmodium falciparum Asexual Blood Stages,” *Parasitology Today*, vol. 16, no. 10, pp. 427–433, 2000.

[62] A. Ghosh, P. Srinivasan, E. G. Abraham, H. Fujioka, and M. Jacobs-Lorena, “Molecular strategies to study Plasmodium–mosquito interactions,” *Trends in Parasitology*, vol. 19, no. 2, pp. 94–101, 2003.

[63] W. health O. technical Report, “No Title Parasitological confirmation of malaria diagnosis,” 2009.

[64] J. C. Beier, G. F. Killeen, and J. I. Githure, “Short report: entomologic inoculation rates and Plasmodium falciparum malaria prevalence in Africa.,” *The American journal of tropical medicine and hygiene*, vol. 61, no. 1, pp. 109–113, 1999.

[65] P. L. Sutton, E. H. Clark, C. Silva, and O. H. Branch, “The Plasmodium falciparum merozoite surface protein-1 19 KD antibody response in the Peruvian Amazon predominantly targets the non-allele specific, shared sites of this antigen.,” *Malaria journal*, vol. 9, p. 3, 2010.

[66] R. E. L. Paul and K. P. Day, “Mating Patterns of Plasmodium falciparum,” *Parasitology Today*, vol. 14, no. 5, pp. 197–202, 1998.

[67] C. Rogier, B. Pradines, H. Bogreau, J.-L. Koeck, M.-A. Kamil, and O. Mercereau-Puijalon, “Malaria epidemic and drug resistance, Djibouti.,” *Emerging infectious diseases*, vol. 11, no. 2, pp. 317–321, 2005.

[68] N. Sakihama, H. Ohmae, B. Bakote’e, M. Kawabata, K. Hirayama, and K. Tanabe, “Limited allelic diversity of Plasmodium falciparum merozoite surface protein 1 gene from populations in the Solomon Islands.,” *The American journal of tropical medicine and hygiene*, vol. 74, no. 1, pp. 31–40, 2006.

[69] S. K. Pierce and L. H. Miller, “NIH Public Access,” vol. 182, no. 9, pp. 5171–5177, 2010.

[70] Steve M. Taylor, Malcolm E. Molyneux, FRCP; David L. Simel, MHS; Steven R. Meshnick, Jonathan J. Juliano, " Does this patient have malaria?", JAMA vol. 304, no. 18, PP. 2048-2056, 2010.

[71] Timothy J. C. Anderson et al, “Microsatellite Markers Reveal a Spectrum of Population Structures in the Malaria Parasite Plasmodium falciparum" Molecular biology and evolution , vol 17, no. 10, Pp. 1467-1482, 2000.

[72] R. F. ANDERS, “Multiple cross-reactivities amongst antigens of Plasmodium falciparum impair the development of protective immunity against malaria,” *Parasite immunology*, vol. 8, no. 6, pp. 529–539.

[73] L. Schofield, “On the function of repetitive domains in protein antigens of Plasmodium and other eukaryotic parasites,” *Parasitology Today*, vol. 7, no. 5, pp. 99–105, 1991.

[74] K. Krzyczmonik, Michał Świtnicki, Szymon Kaczanowski “Analysis of immunogenicity of different protein groups from malaria parasite Plasmodium falciparum.” Infection Genetics and Evolution,vol 12, no. 8, PP. 1911–1916

[75] Stephen M. Todryk & Adrian V. S. Hill“Malaria vaccines: the stage we are at” Nature Reviews Microbiology 5, 487-489 (July 2007).

[76] L. Florens, M. P. Washburn, J. D. Raine, R. M. Anthony, M. Grainger, J. D. Haynes, J. K. Moch, N. Muster, J. B. Sacci, D. L. Tabb, A. a Witney, D. Wolters, Y. Wu, M. J. Gardner, A. a Holder, R. E. Sinden, J. R. Yates, and D. J. Carucci, “A proteomic view of the Plasmodium falciparum life cycle.,” *Nature*, vol. 419, no. 6906, pp. 520–526, 2002.

[77] D. Gurarie, “Evolution of malaria virulence in cross-generation transmission through selective immune pressure.” Nature proceedings, 23rd June 2007

[78] World Health Organization, “WHO briefing on Malaria Treatment Guidelines and artemisinin monotherapies,” April, 2006.

[79] N. B. Gadalla, S. E. Elzaki, E. Mukhtar, D. C. Warhurst, B. El-Sayed, and C. J. Sutherland, “Dynamics of pfcrt alleles CVMNK and CVIET in chloroquine-treated Sudanese patients infected with Plasmodium falciparum.,” *Malaria journal*, vol. 9, p. 74, 2010.

[80] and D. O. Plowe CV, Djimde A, Wellems TE, Diop S, Kouriba B, “No Title,” *American Journal of tropical Medicine and Hygiene*, vol. 55, no. 5, pp. 467 – 471, 1996.

[81] I. M. Hastings, S. Paget-McNicol, and a Saul, “Can mutation and selection explain virulence in human P. falciparum infections?,” *Malaria journal*, vol. 3, p. 2, 2004.

[82] S. Bialasiewicz, D. M. Whiley, M. D. Nissen, and T. P. Sloots, “Impact of competitive inhibition and sequence variation upon the sensitivity of malaria PCR.,” *Journal of clinical microbiology*, vol. 45, no. 5, pp. 1621–1623, 2007.

[83] I. M. Sulaiman, U. M. Morgan, R. C. Thompson, a a Lal, and L. Xiao, “Phylogenetic relationships of Cryptosporidium parasites based on the 70-kilodalton heat shock protein (HSP70) gene.,” *Applied and environmental microbiology*, vol. 66, no. 6, pp. 2385–2391, 2000.

[84] A. B. Erry, R. F. Abre, F. B. E. Ical, S. C. Assaing, and J. M. Agnaval, “Revue Générale CONTRIBUTION OF PCR-BASED METHODS TO DIAGNOSIS AND MANAGEMENT OF IMPORTED MALARIA,” Medicine tropicale, vol 65, pp. 176–183, 2005.

[85] D. P. Mason and F. E. Mckenzie, “BLOOD-STAGE DYNAMICS AND CLINICAL IMPLICATIONS OF MIXED *PLASMODIUM VIVAX–PLASMODIUM FALCIPARUM* INFECTIONS,” American Journal of tropical medicine and hygiene, vol. 61, no. 3, pp. 367–374, 2008.

[86] H. Joshi, “Markers for population genetic analysis of human Plasmodia,” Journal of vector borne diseases, Vol 40, september & December, pp. 78–83, 2003.

[87] G. N. Kiwanuka, “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.,” *Journal of vector borne diseases*, vol. 46, no. 1, pp. 1–12, 2009.

[88] N. K. Ghanchi, A. Mårtensson, J. Ursing, S. Jafri, S. Bereczky, R. Hussain, and M. a Beg, “Genetic diversity among Plasmodium falciparum field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2.,” *Malaria journal*, vol. 9, p. 1, 2010.[89] A. B. A Farnert, “LIMITED ADVANTAGE OF MULTIPLE CONSECUTIVE SAMPLES FOR GENOTYPING *PLASMODIUM FALCIPARUM* POPULATIONS DURING THE FIRST DAYS OF TREATMENT,” American Journal of tropical Medicine and Hygiene2, vol. 73, no. 1, pp. 204–206, 2005.

[90] V. L. S. Lusingu J.P Alifrangis M, Mmbando B, Theisen M, Kitua A.Y and L. M. M. and T. T.G, “Cytophilic antibodies to Plasmodium falciparum Glutamate Rich Protein are associated with malaria protection in an area of holoendemic transmission.,” *Malaria journal2*, vol. 4:48, 2005.

[91] J. C. L. Pratt-Riccio L Calvalho L, and Theisen M., “Antibody Response Profiles Induced by Plasmodium falciparium Glutamate-Rich Protein in Naturally Exposed Individuals from a Brazillian Endemic for Malaria,” *American Journal of Tropical Medicine and Hygiene,*, vol. 73, no. 6, pp. 1096–1103, 2005.

[92] F. I. Irion A Abdulla S, Smith T, Mull R, Tanner M, Hatz C, and Beck H.P, “Distinction of recrudescence from new infections by pcr-rflp analysis in a comparative trial of cgp 56 697 and chloroquine in Tanzanian children,” *Tropical Medicine and International Health*, vol. 3, no. 6, pp. 490–494, 1998.

[93] T. Robinson, S. G. Campino, S. Auburn, S. a Assefa, S. D. Polley, M. Manske, B. MacInnis, K. a Rockett, G. L. Maslen, M. Sanders, M. a Quail, P. L. Chiodini, D. P. Kwiatkowski, T. G. Clark, and C. J. Sutherland, “Drug-resistant genotypes and multi-clonality in Plasmodium falciparum analysed by direct genome sequencing from peripheral blood of malaria patients.,” *PloS one*, vol. 6, no. 8, p. e23204, 2011.

[94] C. J. Sutherland, A. Alloueche, L. McRobert, R. Ord, J. Leggat, G. Snounou, M. Pinder, and G. a T. Targett, “Genetic complexity of Plasmodium falciparum

gametocytes isolated from the peripheral blood of treated Gambian children.," *The American journal of tropical medicine and hygiene*, vol. 66, no. 6, pp. 700–705, 2002.

[95] E.-M. de Villiers, C. Fauquet, T. R. Broker, H.-U. Bernard, and H. zur Hausen, "Classification of papillomaviruses.," *Virology*, vol. 324, no. 1, pp. 17–27, 2004.

[96] W. F. Mbacham, M. B. Evehe, P. M. Netongo, I. A. Ateh, P. N. Mimche, A. Ajua, A. M. Nji, D. Irenee, J. B. Echouffo-tcheugui, B. Tawe, R. Hallett, C. Roper, G. Targett, and B. Greenwood, "Efficacy of amodiaquine , sulphadoxine-pyrimethamine and their combination for the treatment of uncomplicated Plasmodium falciparum malaria in children in Cameroon at the time of policy change to artemisinin-based combination therapy," Malaria Journal, vol 9, issue 34 pp. 1–8, 2010.

[97] D. Ajzenberg, A. L. Bañuls, M. Tibayrenc, and M. L. Dardé, "Microsatellite analysis of Toxoplasma gondii shows considerable polymorphism structured into two main clonal groups.," *International journal for parasitology*, vol. 32, no. 1, pp. 27–38, 2002.

[98] World Health Organisation," MalariaReport, "2010

[99] G. Q. Zhang, Y. Y. Guan, B. Zheng, S. Wu, and L. H. Tang, "*Molecular assessment of Plasmodium falciparum resistance to antimalarial drugs in China",Tropical medicine and International Health,* vol. 14, no. 10. 2009, pp. 1266–1271.

[100] W. T. and P. CV, " Chloroquine resistant malaria" Journal of Infeciont, Disease,vol. 184, pp. 770–776, 2001.

[101] N. J. White, "The parasite clearance curve," *Malaria Journal*, vol. 10, no. 1, p. 278, 2011.

[102] A. W. Maiga, B. Fofana, I. Sagara, D. Dembele, A. Dara, O. B. Traore, S. Toure, K. Sanogo, S. Dama, B. Sidibe, A. Kone, M. A. Thera, C. V Plowe, O. K. Doumbo, and A. A. Djimde, "No Evidence of Delayed Parasite Clearance after Oral Artesunate Treatment of Uncomplicated Falciparum Malaria in Mali," *The American Journal of Tropical Medicine and Hygiene* , vol. 87 , no. 1 , pp. 23–28, 2012.

[103] M. Diakite, E. a Achidi, O. Achonduh, R. Craik, A. a Djimde, M.-S. B. Evehe, A. Green, C. Hubbart, M. Ibrahim, A. Jeffreys, B. K. Khan, F. Kimani, D. P. Kwiatkowski, W. F. Mbacham, S. O. Jezan, J. B. Ouedraogo, K. Rockett, K. Rowlands, N. Tagelsir, M. M. Tekete, I. Zongo, and L. C. Ranford-Cartwright, "Host candidate gene polymorphisms and clearance of drug-resistant Plasmodium falciparum parasites.," *Malaria journal*, vol. 10, no. 1, p. 250, 2011.

[104] D. P. Mason, F. E. McKenzie, and W. H. Bossert, “The blood-stage dynamics of mixed Plasmodium malariae-Plasmodium falciparum infections.,” *Journal of theoretical biology*, vol. 198, no. 4, pp. 549–566, 1999.

[105] J. T. Bousema, C. J. Drakeley, P. F. Mens, T. Arens, R. Houben, S. a Omar, L. C. Gouagna, H. Schallig, and R. W. Sauerwein, “Increased Plasmodium falciparum gametocyte production in mixed infections with P. malariae.,” *The American journal of tropical medicine and hygiene*, vol. 78, no. 3, pp. 442–448, 2008.

[106] U. A. Meyer, “Pharmacogenetics and adverse drug reactions,” The Lancet, vol. 356, issue 9242, pp. 1667–1671, 2000.

[107] 6. Bouquier M, “No TitleThese sur le titre place de la pharmacogenomique dans les strategies de recherche et de developpement des medicaments: enjeux scientifiques, industriels et ethiques,” 2003.

[108] K. Walker, G. Ginsberg, D. Hattis, D. O. Johns, K. Z. Guyton, and B. Sonawane, “Genetic Polymorphism in N-Acetyltransferase (NAT): Population Distribution of NAT1 and NAT2 Activity,” *Journal of Toxicology and Environmental Health, Part B*, vol. 12, no. 5–6, pp. 440–472, 2009.

[109] S. Longuemaux, C. Deloménie, C. Gallou, A. Me, M. Vincent-viry, R. Bouvier, D. Droz, R. Krishnamoorthy, M. Galteau, and C. Junien, “Candidate Genetic Modifiers of Individual Susceptibility to Renal Cell Carcinoma : A Study of Polymorphic Human Xenobiotic-metabolizing Enzymes Candidate Genetic Modifiers of Individual Susceptibility to Renal Cell Carcinoma : A Study of Polymorphic Human,” Cancer research, vol 59,pp. 2903–2908, 1999.

[110] P. N. Rather, E. Orosz, K. J. Shaw, R. Hare, and G. Miller, “Characterization and Transcriptional Regulation of the 2 ’ -N-Acetyltransferase Gene from Providencia stuartii,” vol. 175, no. 20, pp. 6492–6498, 1993.

[111] L. Urdaneta, a Lal, C. Barnabe, B. Oury, I. Goldman, F. J. Ayala, and M. Tibayrenc, “Evidence for clonal propagation in natural isolates of Plasmodium falciparum from Venezuela.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 12, pp. 6725–6729, 2001.

[112] G. D. Foote SJ Cowman AF, “Amino acids in the dihydrofolate reductase-thymidilate synthase gene of Plasmodium falciparum involved in cycloguanil resistance differ from those involved in pyriméthamine resistance,” *Proc Natl Academy Science*, vol. 87, pp. 3014–3017, 1990.

[113] W. D. Peterson DS Wellems TE, “Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria,” *Proc Natl Acadedemic Science*, vol. 85, pp. 9114–9118, 1988.

[114] M. J. G. Triglia T Wilson C, Cowman AF., “Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in Plasmodium falciparum,” *Proc Natl Acad Sci USA*, no. 94, pp. 13944–13949, 1997.

[115] W. P. Brooks DR Read M, Watkins WM, Sims PFG, Hyde JE, “Sequence variation of the hydroxymethyl dihydroprotein pyrophosphokinase-dihydropteroate synthase gene in lines of the humam malaria parasite Plasmodium falciparum with differing resistance to sulfadoxine,” *Europa Journal of Biochemistry*, vol. 224, pp. 397–405, 1994.

[116] D. F. K. Kublin JG Kamwendo DD, Malkin EM, Cortese JF, Martino LM, Mukadam, R. S. J. RA Lescano AG, Molyneux ME, Winstanley PA, Chimpeni P, Taylor TE, Et, and P. CV., “Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanildapsone treatment of Plasmodium falciparum malaria.,” *Journal of Infection and Disase*, vol. 185, no. 3, pp. 380–388, 2002.

[117] W. P. Triglia T Sims PF, Hyde JE, Cowman AF, “Allelic exchange at the endogenous genomic locus in Plasmodium falciparum proves the role of dihydropteroate synthase in sulfadoxine-resisatnt malaria,” *EMBO Journal*, no. 17, pp. 3807–3815, 1998.

[118] D. A. Plowe CV Bouare M, Doumbo O, Wellems TE, “Pyriméthamine and proguanil resistance conferring mutations in Plasmodium falciparum dihydrofolate reductase: Polymerase chain reaction methods for surveillance in Africa,” *American Journal of Tropical Medecine Hygiene*, vol. 52, pp. 565–568, 1995.

[119] A. Gregson and C. V Plowe, “Mechanisms of Resistance of Malaria Parasites to Antifolates,” vol. 57, no. 1, pp. 117–145, 2005.

[120] G. Y. I. Krogstad DJ Kyle DE, Oduola AMJ, Martin SK, Milhous WK, Schlesinger and PH, “Efflux of chloroquine from Plasmodium falciparum mùechanism of chloroquine resistance,” *Science*, vol. 238, pp. 1238–1285, 1987.

[121] G. D. Cowman AF Thompson JK, “Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of pfmdrI gene and cross-resistance to halofantrine and quinine.,” *Proc Natl Academy Science*, vol. 91, pp. 1143–1147, 1994.

[122] de P. P. E. Basco LK LeBras J, Wilson CM, “Plasmodium falciparum molecular caracterization of multidrug resistant Cambodian isolates,” *Exp Parasitol*, vol. 82, pp. 97–103, 1996.

[123] A. F. Durand R Cojean S, Fontanet A, Ranaivo L, Ranalivelo LA, Vonimpaisomihanta JA and P. V Ménard D LeBras J, Modiano D, “Analysis of circulating populations of Plasmodium falciparum in mild and severe malaria in

two different epidemiological patterns in Madagascar," *Tropical Medicine and International Health2*, vol. 13, pp. 1392–1399, 2008.

[124] D. O. K. Djimdé A Cortese JF, Kayentao K, Dombo Y, Diourte A, Dicko A, Su XZ and F. D. A. Nomura T Wellems TE, Plowe CV, Coulibaly D, "A molecular marker for chloroquine resistant Plasmodium falciparum malaria," *New England Journal Medeicine*, vol. 344, pp. 257–263, 2001.

[125] H. K. et W. T. Su X, "Genetic linkage and association analyses for trait mapping in Plasmodium falciparum.," *Nat Rev Genet*, vol. 8, no. 7, pp. 497–506, 2007.

[126] N. T. Fidock DA Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM and N. B. Sidhu ABS Deitsch K, Su XZ, Wootton JC, Roepe PD, Wellems TE, "Mutations in the Plasmodium falciparum digestive vacuole transmembrane protein Pfcrt and evidence for their role in chloroquine resistant," *Molecular cellular*, vol. 6, pp. 861–871, 2000.

[127] P. S. I. Babiker HA Abdel-Muhsin A, Mackinmon M, Hunt P, Walliker D., "High-level chloroquine resistance in Sudanese isolates of Plasmodium falciparum is associated with mutation in the chloroquine resistance transporter gene pfcrt and the multidrug resistance gene pfmdr1.," *Journal of Infection and Disease*, vol. 183, pp. 1535–1538, 2001.

[128] Q. Chen, M. Schlichtherle, and M. Wahlgren, "Molecular aspects of severe malaria.," *Clinical microbiology reviews*, vol. 13, no. 3, pp. 439–450, 2000.

[129] P. P. Dharmeshkumar N Patel MM Surti, SB Agarwal, "Clinical Manifestations of Complicated Malaria –," vol. 4, no. 4, pp. 323–331, 2003.

[130] D. J. Weatherall, L. H. Miller, D. I. Baruch, K. Marsh, O. K. Doumbo, C. Casals-pascual, and D. J. Roberts, "Malaria and the Red Cell.", Heamatology, vol. 2002 no. 1 35-57

[131] B. Work, A. Society, W. Dc, and T. A. Society, "Microvascular sequestration of Plasmodium falciparum," *Blood*, vol. 117, no. 24, p. 6410, 2011.

[132] P. M. O'Neill, V. E. Barton, and S. a Ward, "The molecular mechanism of action of artemisinin--the debate continues.," *Molecules (Basel, Switzerland)*, vol. 15, no. 3, pp. 1705–1721, 2010.

[133] K. Chotivanich, R. Udomsangpetch, a Dondorp, T. Williams, B. Angus, J. a Simpson, S. Pukrittayakamee, S. Looareesuwan, C. I. Newbold, and N. J. White, "The mechanisms of parasite clearance after antimalarial treatment of Plasmodium falciparum malaria.," *The Journal of infectious diseases*, vol. 182, no. 2, pp. 629–633, 2000.

[134] O. K. Doumbo, O. Traore, A. B. Guindo, A. A. Djimde, K. Kayentao, Y. Diourte, S. Niare-doumbo, D. Coulibaly, A. K. Kone, Y. Cissoko, M. Tekete, B. Fofana, A. Dicko, D. A. Diallo, T. E. Wellems, D. Kwiatkowski, and C. V Plowe, "CLEARANCE OF DRUG-RESISTANT PARASITES AS A MODEL FOR PROTECTIVE," vol. 69, no. 5, pp. 558–563, 2003.

[135] S. PIANTADOSI, *Clinical Trials-A methodologic Perspective*, Second Edi. John Wiley & Sons, I nc, 2005, p. 290.

[136] M. J. Whittingham, P. a Stephens, R. B. Bradbury, and R. P. Freckleton, "Why do we still use stepwise modelling in ecology and behaviour?," *The Journal of animal ecology*, vol. 75, no. 5, pp. 1182–9, Sep. 2006.

[137] C. O. Falade, O. O. Ogunkunle, H. O. Dada-Adegbola, A. G. Falade, P. I. De Palacios, P. Hunt, M. Virtanen, A. M. Oduola, and L. a Salako, "Safety and efficacy of dihydroartemisinin-piperaquine versus artemether-lumefantrine in the treatment of uncomplicated Plasmodium falciparum malaria in Zambian children," *Malaria Journal*, vol. 10, no. 12, p. 246, 2011.

[138] A. M. Kabanywanyi, A. Mwita, D. Sumari, R. Mandike, K. Mugittu, and S. Abdulla, "Efficacy and safety of artemisinin-based antimalarial in the treatment of uncomplicated malaria in children in southern Tanzania," *Malaria Journal*, vol. 6, no. 1, p. 146, 2007.

[139] G. O. Adjei, J. Al Kurtzhals, O. P. Rodrigues, M. Alifrangis, L. C. Hoegberg, E. D. Kitcher, E. V Badoe, R. Lamptey, and B. Q. Goka, "Amodiaquine-artesunate vs artemether-lumefantrine for uncomplicated malaria in Ghanaian children: a randomized efficacy and safety trial with one year follow-up," *Malaria Journal*, vol. 7, no. 1, p. 127, 2008.

[140] B. Anderson and C. Vullo, "Did malaria select for primary adult lactase deficiency ?," Gut, vol 35, pp. 1487–1489, 1994.

[141] L. J. Mangham, B. Cundill, O. a Achonduh, J. N. Ambebila, A. K. Lele, T. N. Metoh, S. N. Ndive, I. C. Ndong, R. L. Nguela, A. M. Nji, B. Orang-Ojong, V. Wiseman, J. Pamen-Ngako, and W. F. Mbacham, "Malaria prevalence and treatment of febrile patients at health facilities and medicine retailers in Cameroon.," *Tropical medicine international health TM IH*, vol. 17, no. 3, pp. 330–342, 2011.

[142] D. Das, A. P. Phyo, J. Tarning, D. Ph, K. M. Lwin, F. Ariey, W. Hanpithakpong, S. J. Lee, P. Ringwald, K. Silamut, T. Herdman, S. S. An, S. Yeung, D. Socheat, and N. J. White, "Artemisinin Resistance in plasomodium falciparium malaria,"New England Journal of Medicine, vol 361, pp. 455–467, 2009.

[143] C. E. Shulman, T. Marshall, E. K. Dorman, J. N. Bulmer, F. Cutts, N. Peshu, and K. Marsh, “Malaria in pregnancy : adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae,” vol. 6, no. 10, pp. 4–12, 2001.

[144] A. M. Kabanywanyi, A. Mwita, D. Sumari, R. Mandike, K. Mugittu, and S. Abdulla, “Efficacy and safety of artemisinin-based antimalarial in the treatment of uncomplicated malaria in children in southern Tanzania.,” *Malaria journal*, vol. 6, p. 146, 2007.

[145] K. Chotivanich, R. Udomsangpetch, K. Pattanapanyasat, W. Chierakul, S. Looareesuwan, and N. White, “Hemoglobin E : a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria Hemoglobin E : a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria,” Blood, vol. 100 no. 4 1172-1176,2002.

[146] M. J. F. NDUBISI A. NNALUE, “No TitleEvidence for a neutrophil-mediated protective response in malaria,” *Parasite Immunology*, vol. 10, no. 1, pp. 47–58.

[147] M. B. Denis, R. Tsuyuoka, Y. Poravuth, T. S. Narann, S. Seila, C. Lim, S. Incardona, P. Lim, R. Sem, D. Socheat, E. M. Christophel, and P. Ringwald, “Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia.,” *Tropical medicine & international health : TM & IH*, vol. 11, no. 9, pp. 1360–1366, 2006.

[148] C. Amaratunga, S. Sreng, S. Suon, E. S. Phelps, K. Stepniewska, P. Lim, C. Zhou, S. Mao, J. M. Anderson, N. Lindegardh, H. Jiang, J. Song, X. Su, N. J. White, A. M. Dondorp, T. J. C. Anderson, M. P. Fay, J. Mu, S. Duong, and R. M. Fairhurst, “Artemisinin-resistant Plasmodium falciparum in Pursat province, western Cambodia: a parasite clearance rate study.,” *The Lancet infectious diseases*, vol. 12, no. 11, pp. 851–858, 2012.

Annex 1: Human NAT-2 haplotypes

***NAT2* Allele (Haplotype) a**	**Nucleotide Change(s) and rs Identifiersb**	**Amino Acid Change(s)b**	**Phenotypec**
NAT2*4	**Reference**	**Reference**	**Rapid**
NAT2*5A	**341T>C**(rs1801280) **481C>T** (rs1799929)	**I114T** **L161L (synonymous)**	**Slow**
NAT2*5B	**341T>C**(rs1801280) **481C>T**(rs1799929) **803A>G** (rs1208)	**I114T L161L (synonymous)** **K268R**	**Slow**
NAT2*5C	**341T>C**(rs1801280) **803A>G** (rs1208)	**I114T K268R**	**Slow**
NAT2*6A	**282C>T(rs1041983)** **590G>A (rs1799930)**	**Y94Y (synonymous)** **R197Q**	**Slow**
NAT2*6B	**590G>A (rs1799930)**	**R197Q**	**Slow**
NAT2*6C	**282C>T(rs1041983)** **590G>A(rs1799930)** **803A>G (rs1208)**	**Y94Y (synonymous)** **R197Q K268R**	**Slow**
NAT2*6D	**111T>C** **282C>T (rs1041983)** **590G>A (rs1799930)**	**F37F (synonymous)** **Y94Y (synonymous)** **R197Q**	**Slow**
NAT2*7A	**857G>A (rs1799931)**	**G286E**	**Slow Substrate dependent?**

NAT2*7B	**282C>T(rs1041983)** **857G>A (rs1799931)**	**Y94Y** **(synonymous)** **G286E**	**Slow** **Substrate** **dependent?**
NAT2*10	**499G>A**	**E167K**	**Slow** **Substrate** **dependent?**
NAT2*11A	**481C>T (rs1799929)**	**L161L** **(synonymous)**	**Rapid**
NAT2*11B	**481C>T (rs1799929)** **859Del**	**L161L** **(synonymous)** **S287 Frame shift**	**Unknown**
NAT2*12A	**803A>G (rs1208)**	**K268R**	**Rapid**
NAT2*12B	**282C>T (rs1041983)** **803A>G (rs1208)**	**Y94Y** **(synonymous)** **K268R**	**Rapid**
NAT2*12C	**481C>T (rs1799929)** **803A>G (rs1208)**	**L161L** **(synonymous)** **K268R**	**Rapid**
NAT2*12D	**364G>A (rs4986996)** **803A>G (rs1208)**	**D122N K268R**	**Slow**
NAT2*13A	**282C>T (rs1041983)**	**Y94Y** **(synonymous)**	**Rapid**
NAT2*13B	**282C>T (rs1041983)** **578C>T**	**Y94Y** **(synonymous)** **T193M**	
NAT2*14A	**191G>A** (rs1801279)	**R64Q**	**Slow**
NAT2*14B	**191G>A** (rs1801279) **282C>T** (rs1041983)	**R64Q** **Y94Y**	**Slow**

		(synonymous)	
NAT2*14C	**191G>A** (rs1801279) **341T>C** (rs1801280) **481C>T** (rs1799929) **803A>G** (rs1208)	**R64Q I114T L161L (synonymous) K268R**	**Slow**
NAT2*17	**434A>C**	**Q145P**	**Slow**
NAT2*18	**845A>C**	**K282T**	**Rapid**
NAT2*19	**190C>T (rs1805158)**	**R64W**	**Slow**
NAT2*20	**600A>G**(rs72466461)	**synonymous**	
NAT2*21	**458C>T**(rs72466460)	**T153I**	

Table 4.2: Possible genotypes and phenotypes combination after RFLP analysis

Bands for BamHI (pb)	**Bands for KpnI (pb)**	**Bands for TaqI (pb)**	**Genotypes**	**Phenotypes**
428 ; 107	483 ; 52	205 ; 170 ; 160	NAT2*4/4	Fast acetylator
428 ; 107	535	205 ; 170 ; 160	NAT2*5/5	Slow acetylator
428 ; 107	483 ; 52	330 ; 205	NAT2*6/6	Slow acetylator
535	483 ; 52	205 ; 170 ; 160	NAT2*7/7	Slow acetylator
428 ; 107	535 ; 483 ; 52	330 ; 205 ; 170 ; 160	NAT2*5/6	Slow acetylator
535 ; 428 ; 107	535 ; 483 ; 52	205 ; 170 ; 160	NAT2*5/7	Slow acetylator
535 ; 428 ; 107	483 ; 52	330 ; 205 ; 170 ; 160	NAT2*6/7	Slow acetylator
428 ; 107	535 ; 483 ; 52	205 ; 170 ; 160	NAT2*4/5	Intermediate acetylator
535 ; 428 ; 107	483 ; 52	205 ; 170 ; 160	NAT2*4/7	Intermediate acetylator
428 ; 107	483 ; 52	330 ; 205 ; 170 ; 160	NAT2*4/6	Intermediate acetylator

Annex 2:Informed Assent

The Biotechnology Centre, Box 8094 Yaoundé University of Yaoundé I, Cameroon.

Project Title: **Research on Anti-Malaria Drug Resistance in Cameroon: Safety and Efficacy of Atemether-lumefantrine, dihydroartemisinin-piperaquine & Artesunate-Amodiaquine**

Principal Investigator: **Dr. Wilfred F. MBACHAM**;

Study Number _____

1. *Purpose*

There are many drugs that can be used to treat malaria.. These old drugs no longer cure well. Many other drugs are being sold. Some others are being newly made and will be sold soon. Some of these new drugs are a combination of more than one anti-malarial drug. In this study, we are trying to compare how well the new drugs such as artesunate-modiaquine (Co-Arsucam®), dihydroartemisinin-piperaquine (Duo-cotecxin®), and artemether-lumefantrine (CoArtem®) cure malarial infection in children.

Your child is invited to participate in this study because your child is suffering from malarial infection. We will ask the permission of parents of 740 children aged less than 5 years with acute malarial infection to take part in this study.

2. *Procedure*

If you agree for your child to be in the trial, you will be asked some questions about how you have treated your child in the past. Your address will be asked so that we can visit your sick child. A study doctor or nurse will examine your child. We will prick your child's finger to take a few drops of blood to look for parasites. We will also take blood from the vein of your child, the tip of a teaspoon, to do some laboratory tests and to find out why some people respond quickly when treated and other people do not.

Your child will be assigned into one of three groups to receive either artesunate-amodiaquine, artemether-lumefantrine or dihydroartemisinin-piperaquine. The assignment will be decided by chance. You and the study staffs will not know which group your child will be in. Your child will be given the drug at the hospital every day for 3 days.

We will ask you to return with your child to the study clinic on days 7, 14, 21, 28, 35 and 42 or at any other time if your child feels unwell. If you and your child do not come to the clinic, we will visit you at your home. On followed-up day (including the they when

clinical sign/symptom of malaria appears), your child will be examined and assessed for clinical symptoms of malaria. We will prick your child's finger to take blood to look for the presence of malaria in your blood and its genetics (how the parasite is made up inside). At some scheduled visits, we will also take blood from your child to do some tests such as blood cell count, sugar level and the levels of some substance in your blood other

3. Risks and discomforts

Most children will have no problems with the drugs given but occasionally they may develop mild itching, rashes or intestinal upsets, headache or blurred vision.

4. Benefits

The drugs may help your child. However, this cannot be guaranteed. Allowing your child to take part in this study will benefit the community by helping to tell the doctors which drugs are good and how to use the new drugs for malaria.

5. Cost

All malaria and additional tests, drugs and hospital fee for staying in hospital during your child's involvement in the study are for free.

6. Payment

We will pay for your transport to bring your child back to the clinic.

7. Alternatives

If you decide not to take part in the study, your child will still receive the standard care in this hospital. This is quinine tablets.

8. Confidentiality

The clinical and laboratory data recorded will be kept confidential and used for this research only. The results of this research may be published in scientific journals or presented at medical meeting, but your identity will not be disclosed.

9. Injury and Compensation

If your child has experienced any research-related illness or injury, you can contact Dr._____ at this clinic. ….(Who)…. will pay any charges required for the treatment of study related illness/injury.

10. New information and result of the study

It is possible that new information becomes available about the medicines used in this study. If this happens, the study doctor will tell you about it and also discuss with you whether you want your child to continue in the study. If you decide not to continue, the study will still arrange for your child's care to continue. If you decide to continue in the study you will be asked to sign an updated parental permission form. The investigator will also inform you about the progress and outcome of the research.

11. Questions

If you feel you have not been properly told as to the discomfort, benefits or your rights, please feel free to take the matter to your local head of the hospital or contact the following people: *Prof Pius Tih, Public Health Officer, Banso Baptist Hospital, NW Province Cameroon(7776-4781 or 7793-6550)*.

12. Voluntary Participation

Your child's participation in this study is purely voluntary and you (your child) will be given sufficient time to decide whether or not to take part. You (and your child) may refuse to participate at any time and still benefit from full treatment for malaria. There will be no injustice, punishment, loss of benefits to which you (or your child) are otherwise entitled at this hospital.

12. Acceptance

My questions concerning this study have been answered by ----------. Or I have read the information sheet concerning this study [or have understood the verbal explanation] and I understand what will be required of me and what will happen to my child if I take part in it. I understand that at any time I may withdraw my child from this study without giving a reason and without affecting normal care and management. I agree that my child should take part in the study.

Name of Interpreter---------- Date ----------

Child's name _____

Parent's/guardian's signature (or thumb print):_____ Date_____

Parent's/guardian's Printed Name:_____

Witness's Signature: _____ Date: _____

Witness's Printed Name:_____

Researcher Recognizance

The subject has been fully informed of the nature and purpose of the procedures described above including any discomfort involved in its performance. The subject has been asked if any questions have arisen regarding the procedures and

Annex 3: R-code for the models

Response to therapy model(logistic model)

```
library(ROCR)

library(MASS)

# To load the file

# load("C:/Users/Akindeh/Desktop/dataphd")

# Creating categories(only 3 are shown here)

newdata$Age_group <- factor(newdata$Age_group, labels=c("<=60",">60") )

newdata$Sitecode <- factor(newdata$Sitecode, labels=c("Garoua","Mutengene") )

newdata$Sex <- factor(newdata$Sex, labels=c("female", "male"))

#Subsetting the variables to be used for model

actualdata <- subset(modelres, select=c( Subjnumber,result_adj,Sex,Age_group,Sitecode,Drug,
Acetelator,                normNeutroD0,normALATD0,Ecotype,Rtype,Weight,Temp,LowHb,
normCreaD0))

  # To get a dataset with complete cases

obsind <- which( complete.cases(actualdata) )  actualdata_cc <- actualdata[obsind,]
write.csv2(file="actualdata_cc.csv",x=actualdata_cc)

#Fitting the full model

ACPR1<-glm(result_adj~ Sex+ Age_group+Sitecode+Drug+Drug*Age_group+ Drug*Sitecode +
Sitecode*Age_group+ Acetelator+ normNeutroD0 +normALATD0+Ecotype+Rtype+s(Weight)+ s(Temp)+
LowHb+normCreaD0,family=binomial(link="logit"), data=actualdata_cc)  print(summary(ACPR1))

# use the step function in R to selected the best model

stepAIC(ACPR1)

# fitting the final selected model using the Akaike criterion

ACPR2 <- glm( result_adj ~ Age_group + Sitecode + Drug + Drug*Age_group,
family=binomial(link="logit"), data=actualdata_cc) bprint(summary(ACPR2))

# fitting the final model, taking out the interaction of Age_group and age because of 0 cell problems

ACPR3 <- glm( result_adj ~ Age_group + Sitecode + Drug, family=binomial(link="logit"),
data=actualdata_cc)

print(summary(ACPR3))
```

R Code for the Discrete survival Model

```
library(ROCR)

library(MASS)

#To load the file

load("C:/Users/Akindeh/Desktop/Defence material_PHD_International Health/Submission
Documents_Thesis_akindeh/Thesis_version3/Logistic and Discretesurvival modelling/discrete.rda")

#load("discrete.rda")

newdata <- discrete

# Creating categories(only 3 are shown here)

newdata$Age_group <- factor(newdata$Age_group, labels=c("<=60",">60") )

newdata$Sitecode <- factor(newdata$Sitecode, labels=c("Garoua","Mutengene") )

newdata$Sex <- factor(newdata$Sex, labels=c("female", "male"))

#giving actual visit days labels to  day variable.

newdata$day <- factor(newdata$day,labels=c("0","1","2","3","7","14","21","28","35","42"))

#Subsetting the variables to be used for model

actualdata <- subset(newdata, select=c( Subjnumber,cleared,day,Sex,Age_group,Sitecode,Drug,
Acetelator,                normNeutroD0,normALATD0,Ecotype,Rtype,Weight,Temp,LowHb,
normCreaD0))

  # To get a dataset with complete cases

obsind <- which( complete.cases(actualdata) )    actualdata_cc <- actualdata[obsind,]

#write.csv2(file="actualdata_cc.csv",x=actualdata_cc)  We first start up with the hazard function
irrespective of heterogeneity therefore we fit a model with time(day)as the only covariate

PCL0 <-glm(cleared~ day, family=binomial(link="logit"),data=actualdata_cc) print(summary(PCL0))

childs1 <- data.frame(day=factor(c(1)))   predday1 <- predict( PCL0, newdata=childs1,type="response")

childs2 <- data.frame(day=factor(c(2))) predday2 <- predict( PCL0, newdata=childs2,type="response")

childs3 <- data.frame(day=factor(c(3))) predday3 <- predict( PCL0, newdata=childs3,type="response")

childs7 <- data.frame(day=factor(c(7)))      predday7 <- predict( PCL0, newdata=childs7,type="response")

childs14 <- data.frame(day=factor(c(14))) predday14 <- predict( PCL0,
newdata=childs14,type="response")
```

```
  # plotting the hazard function for time effect   plotdata0<-
rbind(predday1,predday2,predday3,predday7,predday14)

matplot(plotdata0,xaxt='n',type="l",lwd=2,xlab="Days after treatment", ylab="Conditional Probability of
clearence",main="Hazard conditional Probabilities irrespective of heterogeneity")
axis(1,at=1:5,labels=c(1,2,3,7,14))

#plotting the survival function for time effect

surv1 <- cumprod( (1-plotdata0[,1]) )  matplot(surv1,xaxt='n', type="l",xlab="Days after
treatment",ylab="probability parasite persistence",main='Survival probabilities irrespective of
heterogeneity') axis(1,at=1:5,labels=c(1,2,3,7,14))

# We fit the full Discrete model with all possible covariates  and time

PCL1 <-glm(cleared~ day+Sex+ Age_group+Sitecode+Drug+Drug*Age_group+ Drug*Sitecode +
Sitecode*Age_group+ Acetelator+ normNeutroD0 +normALATD0+Ecotype+Rtype+s(Weight)+ s(Temp)+
LowHb+normCreaD0,family=binomial(link="logit"), data=actualdata_cc)   print(summary(PCL1))

  # checking on well the full model(model 2) classify by using a ROC curve

pred <- prediction(fitted(PCL1), actualdata_cc$cleared)  perf <- performance(pred, measure = "tpr",
x.measure = "fpr") plot(perf, col=rainbow(10))

 # use the step function in R to selected the best model

stepAIC(PCL1)

  # fitting the best model(final model) selected by the step function

PCL2 <-glm(cleared~ day+ Age_group+ Sitecode+ Sitecode*Age_group+normALATD0+LowHb
family=binomial(link="logit"),data=actualdata_cc) print(summary(PCL2))

# Rechecking how the fit is better using the ROC curve

Opred <- prediction(fitted(PCL2), actualdata_cc$cleared) perf <- performance(pred, measure = "tpr",
x.measure = "fpr") plot(perf, col=rainbow(10))

# getting the odds ratio from the final fitted model and the confidence intervals

OR <- exp(coef(PCL2)[-1])  OR.ci <- exp(confint(PCL2)[-1,])

#Investigating the interaction between site and Age group on day 1,2,3,7 and 14

childs1 <- data.frame(day=factor(c(1,1,1,1)),Age_group=factor(c("<=60",">60","<=60",">60")),
Sitecode=factor(c("Mutengene","Mutengene","Garoua","Garoua")), normALATD0=c(0,0,0,0),
normCreaD0=c("abnormal","abnormal","abnormal","abnormal"),
LowHb=factor(c("<=10","<=10","<=10","<=10")) ) predday1 <- predict( PCL2,
newdata=childs1,type="response")

childs2 <- data.frame(day=factor(c(2,2,2,2)),   Age_group=factor(c("<=60",">60","<=60",">60")),
Sitecode=factor(c("Mutengene","Mutengene","Garoua","Garoua")),
normALATD0=c(0,0,0,0),normCreaD0=c("abnormal","abnormal","abnormal","abnormal"),
```

```
LowHb=factor(c("<=10","<=10","<=10","<=10")) )predday2 <- predict(
PCL2,newdata=childs2,type="response")

childs3 <- data.frame(day=factor(c(3,3,3,3)), Age_group=factor(c("<=60",">60","<=60",">60")),
Sitecode=factor(c("Mutengene","Mutengene","Garoua","Garoua")), normALATD0=c(0,0,0,0),
normCreaD0=c("abnormal","abnormal","abnormal","abnormal"),
LowHb=factor(c("<=10","<=10","<=10","<=10")) )  predday3 <- predict( PCL2,
newdata=childs3,type="response")

childs4 <- data.frame(day=factor(c(7,7,7,7)), Age_group=factor(c("<=60",">60","<=60",">60")),
Sitecode=factor(c("Mutengene","Mutengene","Garoua","Garoua")), normALATD0=c(0,0,0,0),
normCreaD0=c("abnormal","abnormal","abnormal","abnormal"),LowHb=factor(c("<=10","<=10","<=10","<=
10")) )  predday4 <- predict( PCL2, newdata=childs4,type="response")

childs5 <- data.frame(day=factor(c(14,14,14,14)),  Age_group=factor(c("<=60",">60","<=60",">60")),
Sitecode=factor(c("Mutengene","Mutengene","Garoua","Garoua")), normALATD0=c(0,0,0,0),
normCreaD0=c("abnormal","abnormal","abnormal","abnormal"),
LowHb=factor(c("<=10","<=10","<=10","<=10")) )  predday5 <- predict( PCL2,
newdata=childs5,type="response")

  # plotting the hazard function for the interaction between age group and site

plotdata1 <- rbind(predday1,predday2,predday3,predday4,predday5)  matplot(plotdata1,xaxt='n',
type="l",lwd=3,xlab="Days after treatement", ylab="Conditional Probability of clearence",main="Hazard
Probabilities by site and age group") leg.txt <- c( "<=5yrs, Mutenegene", " >5yrs, Mutengene", "<=5yrs,
Garoua", ">5yrs, Garoua") legend(1.7,0.5, leg.txt, cex=0.8, col=1:4, pch=21:23, lty=1:4)
axis(1,at=1:5,labels=c(1,2,3,7,14))

#  Plotting of Survival function for the site and age group interaction.

surv1 <- cumprod( (1-plotdata1[,1]) )  surv2 <- cumprod( (1-plotdata1[,2]) ) surv3 <- cumprod( (1-
plotdata1[,3]) )

surv4 <- cumprod( (1-plotdata1[,4]) ) matplot(cbind(surv1,surv2,surv3,surv4),xaxt='n',
type="l",lwd=3,xlab="Days after treatement", ylab="Probability of persistence of
clearence",main="Survivial Probabilities by site and age group") leg.txt <- c( "<=5yrs, Mutenegene", "
>5yrs, Mutengene", "<=5yrs, Garoua", ">5yrs, Garoua") legend(2.1,0.8, leg.txt, cex=0.8, col=1:4,
pch=21:23, lty=1:4) axis(1,at=1:5,labels=c(1,2,3,7,14))

# Plotting conditional Probabilities of parasite clearance by ALAT levels

childs1 <- data.frame(day=factor(c(1,1)), Age_group=factor(c("<=60","<=60")),
Sitecode=factor(c("Mutengene","Mutengene")),normALATD0=c(0,1),
normCreaD0=c("abnormal","abnormal"),LowHb=factor(c("<=10","<=10")) ) predday1 <- predict( PCL2,
newdata=childs1,type="response")

childs2 <- data.frame(day=factor(c(2,2)), Age_group=factor(c("<=60","<=60")),
Sitecode=factor(c("Mutengene","Mutengene")), normALATD0=c(0,1),
normCreaD0=c("abnormal","abnormal"), LowHb=factor(c("<=10","<=10")) ) predday2 <- predict( PCL2,
newdata=childs2,type="response")
```

```
childs3 <- data.frame(day=factor(c(3,3)), Age_group=factor(c("<=60","<=60")),
Sitecode=factor(c("Mutengene","Mutengene")), normALATD0=c(0,1),
normCreaD0=c("abnormal","abnormal" ),LowHb=factor(c("<=10","<=10")) ) predday3 <- predict( PCL2,
newdata=childs3,type="response")

childs4<- data.frame(day=factor(c(7,7)), Age_group=factor(c("<=60","<=60")),
Sitecode=factor(c("Mutengene","Mutengene")),
normALATD0=c(0,1),normCreaD0=c("abnormal","abnormal"), LowHb=factor(c("<=10","<=10")) )
predday7 <- predict( PCL2, newdata=childs4,type="response")

childs5 <- data.frame(day=factor(c(14,14)),  Age_group=factor(c("<=60","<=60")),
Sitecode=factor(c("Mutengene","Mutengene")) normALATD0=c(0,1),
normCreaD0=c("abnormal","abnormal"), LowHb=factor(c("<=10","<=10")) ) predday14 <- predict( PCL2,
newdata=childs5,type="response")

#Plotting hazard function for ALAT levels

plotdata2 <- rbind(predday1,predday2,predday3,predday7,predday14) matplot(plotdata2,xaxt="n",
type="l",lwd=3,xlab="Days after treatement", ylab="Conditional Probability of
clearence",main="Conditional Probabilities of parasite clearance by ALAT level ") leg.txt <- c( "Abnormal
ALAT", " Normal ALAT")  legend(1.7,0.4, leg.txt, cex=0.8, col=1:4, pch=21:23, lty=1:2)
axis(1,at=1:5,labels=c(1,2,3,7,14))

#  Plotting of Survival function for ALAT level w.r.t other covariates

surv1 <- cumprod( (1-plotdata2[,1]) ) surv2 <- cumprod( (1-plotdata2[,2]) )

matplot(cbind(surv1,surv2), xaxt="n",type="l",lwd=3,xlab="Days after treatement", ylab="Conditional
Probability of clearence",main="Survival  Probabilities by ALAT level") leg.txt <- c( "Abnormal ALAT", "
Normal ALAT")   legend(2.1,0.8, leg.txt, cex=0.8, col=1:4, pch=21:23, lty=1:4)
axis(1,at=1:5,labels=c(1,2,3,7,14))

# hazard and survival functions for Creatinine and haemoglobin levels follow the same coding as for
ALAT

# model diagnostics

# GAM model for checking on linearity assumption. the continuous variables are smoothened

library(mgcv)

PCL1gam <-gam(cleared~ day+Sex+ Age_group+Sitecode+Drug+Drug*Age_group+ Drug*Sitecode +
Sitecode*Age_group+ Acetelator+ normNeutroD0 +normALATD0+Ecotype+Rtype+s(Weight)+ s(Temp)+
LowHb+normCreaD0,family=binomial(link="logit"), data=actualdata_cc)   print(summary(PCL1gam))

# Mixed model to check the "no unobserved heterogeneity" assumptionlibrary(lme4)

PCL1glmm <-glmer(cleared~ day+ Age_group+ Sitecode+ Sitecode*Age_group+normALATD0+LowHb +
(1|Subjnumber),family=binomial(link="logit"), data=actualdata_cc)  print(summary(PCL1glmm))
```

Annex 4: Variables used during modeling phase

Variable	Description	Coding
Sitecode	Trial site	0=Garoua; 1= Mutengene
TreatmentD0	Treatment arm into which patient is randomised	ASAQ, DHP and AL
Result	Is patient attained adequate clinical and parasitologic response(ACPR) at day 42	0= Not ACPR; 1=ACPR
Result14	Is patient attained adequate clinical and parasitologic response(ACPR) at day 14	0= Not ACPR; 1=ACPR
Result28	Is patient attained adequate clinical and parasitologic response(ACPR) at day 28	0= Not ACPR; 1=ACPR
Age	Age of patient in months	
Age_group	Variable Age dichotomised into children under 5 years and above 5 years	0= ≤5yrs; 1=>5yrs
Sex	Patient's sex	0=female; 1= Male
Weight	Weight of patient in kgs	
Rtype	Residence type	urban=0; Rural=1
Ecotype	Ecology of patient's	Savannah=0; Forest=1,Sahel=2
HbD0	Haemoglobin level at day 0	
NeutroD0	Neutrophil level at day 0	
AlatD0	Alanine amino transferase level at day 0	
CreaD0	Creatinine Level at day 0	
Acetelator	Metaboliser status	0=Slow metaboliser; 1= fast metaboliser
normHbD0	Haemoglobin level at day 0 compared to cameroonian normal range	0=Hb<10g/dl ; 1=Hb>10g/dl
normNeutroD0	Neutrophil level at day 0 compared to cameroonian normal range	0= neutrophil level<42% and 1= otherwise
normALATD0	Alanine amino transferase level at day 0 compared to cameroonian normal range	0= alanine transferase>61; and 1 =other wise
acpr	Testing ACPR status	0= Not ACPR; 1=ACPR
ParD0	Parasite count at Day 0	

Curriculum Vitae

Akindeh Mbuh Nji

FORMAL EDUCATION

2010 till current: PhD Candidate in international Health at the Centre of International Health(CIH) of Ludwig Maximilian University, Munich, Germany.

2004-2005 Masters of science in applied statistics at the centre for statistics at the universitaire Centrum Limburg, Diepenbeek, Belgium(now U. Hasselt)

1994-1998 Bachelors degree in mathematics at the university of Buea, Cameroon

1991-1993 Advanced level Certificate at Cameroon Protestant College Bali

1986-1991 Ordinary Level Certificate at Cameroon Protestant College Bali

INFORMAL EDUCATION

2008 Two months training with WHO TDR clinical Data Management unit of the University of Thammasat, Bangkok , Thailand.

2008 Clinical Data Management for Vaccine Trials, Bamako, Mali.

2007 Management and quality assurance in Tuberculosis Research, Addis, Ethiopia

2003 European Course on Tropical Epidemiology, at the Nordic School of Public Health (NHV), Gotenborg, Sweden.

Professional Life

2011-current Lecturer at University of Yaounde I, department of biochemistry

2005-current Data manager/Statistician, Laboratory for Public Health Research Biotechnology (LAPHER)

2006-2008 WHO consultant as lead data manager for 4FDC trial(TB fixed dose combination drugs trial) with sites in Nigeria and Ethiopia.

List of Publications

2008 **Limited variation of the 5'cis-control region of the transmission blocking vaccine candidate Pfs25 amid great genetic diversity of *Plasmodium falciparum* in Cameroon**

Wilfred Fon Mbacham1*, Patrice Nsangou Mimche1, Palmer Masumbe Netongo1, Evehe Bebandoue Marie-Solange1, **Akindeh Nji1**, Immaculate Amunom1, Johanna Daily2, ValerieMakoge1, Kayla Laserson2, Songmbe Michael Yong3, Nicoline Lomah3, Peter Enyong4, Vincent V P. Titanji3 and Dyann F. Wirth2.

African Journal of Biotechnology Vol. 7 (5), pp. 523-533, 4 March, 2008

2010 **Efficacy of amodiaquine, sulphadoxine-pyrimethamine and their combination for the treatment of uncomplicated Plasmodium falciparum malaria in children in Cameroon at the time of policy change to artemisinin-based combination therapy**

Wilfred F Mbacham, Marie-Solange B Evehe, Palmer M Netongo, Isabel A Ateh , Patrice N Mimche, Anthony Ajua, **Akindeh M Nji**, Justin B Echouffo-Tcheugui, Bantar Tawe , Rachel Hallett, Cally Roper, Geoffrey Targett and Brian Greenwood

Malaria Journal 2010, 9:34 doi:10.1186/14752875-9-34 Published 27-01-2010

2012 **Malaria prevalence and treatment of febrile patients at health facilities and medicine retailers in Cameroon.**

Mangham LJ, Cundill B, Achonduh OA, Ambebila JN, Lele AK, Metoh TN, Ndive SN, Ndong IC, Nguela RL, **Nji A.M**, Orang-Ojong B, Wiseman V, Pamen-Ngako J, Mbacham WF.

Trop Med Int Health. 2012 Mar;17(3):330-42. doi: 10.1111/j.1365-

2012 **A cost-effectiveness analysis of provider interventions to improve health worker practice in providing treatment for uncomplicated malaria in Cameroon: a study protocol for a cluster randomised controlled trial**

Virginia Wiseman , Lindsay J Mangham, Bonnie Cundill1,Olivia A Achonduh , **Akindeh Mbuh Nji**, Abanda NGU Njei, Clare Chandler,Wilfred F. mbacham

Trials 2012, 13:4 doi:10.1186/1745-6215-13-4

Manuscripts

2013 **Non Inferiority and Safety of Dihydroartemisin-piperaquine and Artesunate amodiaqine versus Artemether-Lumefantrine in the treatment of uncomplicated *Plasmodium falciparum* Malaria in Cameroonian Children**

Akindeh M. Nji, Marcel Moyeh , Eric O.Ngongang, Innocent M. ALI , Valentine N. NDIKUM , Marie S. Evehe, Ekollo Mbange Aristid, Jean Paul Chedjou ,Guenter Froeschl ,Christian Heuman ,Ulrich Mansmann ,Wilfred F. Mbacham

(Submitted to the Malaria Journal April 30, 2013)