

UNIVERSITY OF YAOUNDE 1
UNIVERSITÉ DE YAOUNDÉ 1

FACULTY OF SCIENCE
FACULTÉ DES SCIENCES

DEPARTMENT OF BIOCHEMISTRY
DEPARTEMENT DE BIOCHIMIE

LABORATORY FOR PUBLIC HEALTH RESEARCH BIOTECHNOLOGIES
THE BIOTECHNOLOGY CENTRE, NKOLBISSON

Identification of human immune response factors and molecular correlations with fever and parasite clearance during infection with *Plasmodium falciparum* among children in Cameroon.

Thesis presented and defended in partial fulfilment of the requirements for the award of a Doctorate /PhD in Biochemistry

Specialty: Molecular Parasitology and Immunogenomics

By

Innocent Mbulli ALI
Matricule: 03W525
MSc in Biochemistry

Supervisor

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ACADEMIC YEAR 2014-2015.



REPUBLIQUE DU CAMEROUN
Paix—Travail—Patrie
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REPUBLIC OF CAMEROON
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ATTESTATION OF CORRECTION OF DOCTORAT/PhD THESIS.

We, the undersigned, **Professor ROSE GANA ROMBAN LEKE**, President and Members of the Doctorat/Ph.D thesis defense jury of the candidate **Mr. Innocent MBULLI ATI**, student of the Department of Biochemistry, Registration number 03WS25, born on the 04 January, 1977 in Wum, Menchum Division, authorised through correspondence N° 16-0018/LYI/VREPTIC/DAAC/DEPE/SPD of the RECTOR of the University of Yaounde I, issued on the 07th of January, 2016.

attest that after publicly defending his thesis on the 24th of February, 2016 at the University of Yaounde I, he has proceeded and made all the corrections in conformity with the remarks of the entire jury.

This attestation is issued to serve the purpose for which it is requested.


Yaounde the 10 5 JAN 2021

President of the Jury


Ph.D D.Sc. Honoris Causa

Head of Department, Biochemistry.


Members of Jury

 **prof. rkmr fzu, M.C.**



Professor of Biochemistry



06 JAN 2021


Professor
Enzymology-Toxicology

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 (Par Département et par Grade)

DATE D'ACTUALISATION : 05 Février 2016

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31	SIMO Elie	Chargé de Cours	En poste
32	TABI Conrad Bertrand	Chargé de Cours	En poste
33	TCHOFFO Fidèle	Chargé de Cours	En poste
34	VONDOU DERBETINI Appolinaire	Chargé de Cours	En Poste
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Le Doyen de la Faculté des Sciences

Pr Paul BILONG

DEDICATION

*To the memory of my late father, Papa Ezekiel Ntani ALI and my late sister, Anna Nforshi
ALI; the most remarkable persons in my life.*

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AAK	SP Sensitive haplotype defined at codons 436+437+540 representing respectively single letter amino acid codes for alanine+alanine+lysine at the stated loci.
ABO	Blood group antigens
ACPR	Adequate clinical and parasitological response
ACT	Artemisinin combination therapy
ADCY9	Adenyl cyclase 9
AE	Adverse event
AGK	Haplotype of SP resistance mutation at position 437 representing respectively single letter amino acid codes for alanine+glycine+lysine at the stated loci.
AMA 1	Apical membrane antigen 1
AMP	Adenosine monophosphate
AQ	Amodiaquine
AQSP	Amodiaquine plus sukphadoxine-pyrimethamine combination
ARC	Artemisinin resistance consortium
ASAQ	Artesunate plus amodiaquine combination
bgIII	Restriction enzyme from an E. coli strain Bacillus globigii
BLAST	Basic linked annotated sequence tag
BLASTP	Basic linked aligned sequence tag for protein
CQ	Chloroquine
CRF	Case report form
CVIET	CQ resistant haplotype with mutations at codons 72, 74, 75, 76
CVMNK	CQ resistance haplotype with mutations at codons 72 and 76.

CYP	Cytochrome P-450
DDT	Dichlorodiphenyltrichloroethane
DEAQ	Desethylamodiaquine
<i>PFDHFR</i>	Dihydrofolate reductase
<i>PFDHPS</i>	Dihydroteroate synthase
EBA	Erythrocyte binding antigen
EDTA	Ethylene diamine tetracetate
ELISA	Enzyme linked immunosorbent assay
ETF	Early treatment failure
EXPASY	Protein sequence database
FAE	SP sensitive haplotype of the malaria parasites representing single letter codes for amino acids phenylalanine+alanine+glutamic acid
FAK	SP resistant haplotype, mutation at codon 540 of the malaria parasite representing single letter amino acid codes for phenylalanine, alanine and lysine respectively.
FCT	Fever clearance time
FITC	Fluorescein isothioyanate
GAG	Glycosylaminoglycan
GLURP	Glutamine rich protein
GPI	Glycosylphosphatidyl inositol
GSH	Glutathione synthase
GSSG	Oxidized glutathione synthetase
GWAS	Genome wide association studies
HSP	Heat shock protein
HWE	Hardy Weinberg equilibrium

Ig	Immunoglobulin
IgG	Immunoglobulin type G
IMP	Inosine monophosphate
IPTG	Isopropyl thiogalactosylate
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment in pregnancy
IRN	SP resistant haplotype, representing single letter amino acid codes for isoleucine, arginine and asparagine at codons 51, 59 and 108 of the dihydrofolate reductase gene of <i>P. falciparum</i> .
IRNG	SP resistant haplotype with mutations at codons 51, 59, 108, 437 of the <i>pfdhfr</i> and <i>pfdhps</i> genes respectively. G represents single letter amino acid code for glycine.
<i>kpn</i>	Restriction enzyme defined restriction site, enzyme produced from <i>Klebsiella pneumonia</i>
LAMP	Loop mediated isothermal amplification
LCF	Late clinical failure
LD	Linkage disequilibrium
LPF	Late parasitological failure
LTFU	Lost to follow up
M/Z	Mass to charge ratio
M13	Phage type
MAF	Minor allele frequency
MALDI-TOF	Matrix assisted laser desorption time-of-flight mass spectrometry
MQ	Mefloquine
MRP	Multidrug resistant protein of the ABC family of protein

MSP	Merozoite surface protein
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenin dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCBI	National centre for biomedical information
NCS	SP sensitive haplotype, no mutation at codons 51, 59, 108 on the <i>pfldhfr</i> gene.
NOS	Nitric oxide synthase
OD	Optical density
pABA	Para amino benzoic acid
PCR	Polymerase chain reaction
PCT	Parasite clearance time
PECAM	P type cell adhesion molecule
PEG	Polyethylene glycol
PEP-PCR	Primer extension preamplification-PCR
pfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
<i>pfmdr-1</i>	<i>Plasmodium falciparum</i> multidrug resistant protein1
pfTAPASE6	<i>Plasmodium falciparum</i> atpase 6
pfu	Plaque forming unit
PhD 12	Phage display 12 mer library
PQ	Primaquiune
QBC	Quantitative buffy coat
QC	Quality control
RBC	Red blood cell

RESA	Ring infected surface antigen
RNI	Reactive nitrogen intermediate
ROS	Reactive oxygen species
RQC	Routine quality control
SAK	SP sensitive haplotype with no mutation at codon 108, 437, 540 on the <i>pfdhfr</i> and <i>pfdhps</i> genes
SERCA	Sarcoendoplasmic reticulum calcium atpase
SGE	SP resistant haplotype with mutations at positions 437 and 540 of the <i>pfdhps</i> gene
SGK	SP resistant haplotype with mutations at positions 437 of the <i>pfdhps</i> gene
SNP	Single nucleotide polymorphism
SP	Sulphadoxine-pyrimethamine
SVMNT	<i>Pfcr</i> chloroquine sensitive haplotype around codon 76.
TBE	Tris borate EDTA.
TBST	Tris borate saline
VCAM	V type cell adhesion molecule
VSA	Variant Surface Antigen
WHO	World health organisation
W/H	Weight to height ratio
WGA	Whole genome amplification
Xgal	X galatoside

TABLE OF CONTENTS

LISTE DES ENSEIGNANTS PERMANENTS	ii
DEDICATION	xviii
ACKNOWLEDGEMENT	xviii
LIST OF ABBREVIATIONS	xx
TABLE OF CONTENTS.....	xxv
LIST OF TABLES	xxx
LIST OF FIGURES	xxxii
ABSTRACT.....	xxxii
RESUMÉ	xxxiv
GENERAL INTRODUCTION	1
CHAPTER 1: LITERATURE REVIEW AND RESEARCH PROBLEM	6
1.1. <i>Clinical manifestations and diagnosis of malaria.</i>	6
1.2. Epidemiology of Malaria	8
1.3. The Life cycle of Plasmodium falciparum.....	11
1.3.1. The Intra-erythrocytic cycle of P. falciparum	12
Ring stage:.....	13
Trophozoite:	13
The Schizont.....	13
1.3.2. The extra erythrocytic stage of the life cycle of the malaria parasite.	13
1.4. The Pathophysiology of Malaria	14
1.4.1. Cytokines and malaria severity.....	14
1.4.2. PfEMP1, Cytoadherence, sequestration and severity of infection	15
1.4.3. Rosettes and pathological consequences.....	16
Table 1: major ligands and receptors associated.....	17
1.4.4. Anaemia and infectious dyserythropoiesis.....	17
1.5. The metabolism of the growing intra-erythrocytic parasite.....	18
1.5.1. Haemoglobin processing and heme detoxification.....	19
1.5.2. Metabolism of purines, pyrimidines, folate and amino acids.....	20
1.6. Antimalarial interventions.....	22
1.6.1. Antimalarial Drugs	22
1.6.2. Quinoline antimalarials	22
1.6.3. Sulphadoxine Pyrimethamine.....	23
1.6.4. Artemisinins.	25
1.7. Emergence and spread of resistance to antimalarials	27
1.7.1. Parasite based factors.....	28

1.7.2. Drug based factors	29
Host based factors.....	31
1.8. Mechanisms of drug resistance	32
1.8.1. Sulphadoxine-pyrimethamine (SP).....	32
1.8.2. Aminoquinolines.....	32
1.8.3. Artemisinin combinations	33
1.9. Assessment of drug efficacy	34
1.9.1. Evaluation of clinical and parasitological response in malaria patients.	34
1.9.2. In vitro drug sensitivity assays.	35
1.9.3. Molecular markers of drug resistance.....	35
1.9.4. Markers of resistance to SP.....	36
1.9.5. Markers of resistance to chloroquine and amodiaquine.....	36
1.9.6. Markers of resistance to artemisinins (ACTs).....	36
1.10. Fever as a stress modulator in malaria infection	39
1.10.1. Biological role of fever	40
1.11. Host responses to malaria Infection.....	41
1.11.1. Immune Mechanisms in Malaria	41
1.11.2. Innate Immune Responses to Malaria.....	45
1.11.3. Acquired immune response against malaria infection.....	46
1.11.4. Genetic regulation of host-parasite responses	47
1.12. The haemoglobinopathies.	48
1.12.1. Enzymopathies.....	49
1.12.2. Erythrocyte Polymorphisms	50
1.12.3. Histocompatibility leucocyte antigens (HLA).....	50
1.12.4. Adhesion molecules	51
1.12.5. Nitric Oxide synthase (NOS).....	51
1.13. Genetic regulation of immune response to malaria by cytokines.....	52
1.13.1. Variations in the human genome and approaches to identify host response molecular signatures.	53
1.13.2. Linkage analysis	54
1.13.3. Genome wide association studies (GWAS).	54
1.13.4. Candidate gene association studies.....	55
1.14. Molecular methods associated with genome typing research	55
1.15. Rationale and aims of the study.....	57
1.16. Objectives of the study:.....	58
CHAPTER 2: RESEARCH DESIGN AND METHODOLOGY	59
2.1. Study Area.....	59
2.2. Study Design	59

2.2.1.Design types incorporated in the study.....	59
2.2.2.Patient enrolment, drug administration and follow up procedures.....	59
2.3.Drug administration.....	61
2.4. Treatment follow up procedures.....	62
2.5.Parasite density determination.....	62
2.6.Outcomes Classification.....	62
2.7. Molecular methods.....	64
2.7.1.Genotyping Parasite Single Nucleotide Polymorphisms.....	64
2.7.2.PCR correction of treatment response.....	64
2.7.3.PCR amplification of msp1, msp 2 and glurp genes.....	65
2.7.4.Determination of pfmdr1-86 polymorphism in Ngaoundere.....	65
2.7.5.Determination of single nucleotide polymorphisms in drug metabolizing genes and construction of haplotypes.....	66
PCR and restriction fragment length polymorphism.....	67
2.7.7.Dot Blot Assay and Haplotype Construction.....	67
2.8.Molecular genotyping of candidate human immune genes.....	69
2.8.1.Selection of host candidate immune response genes.....	69
2.8.2.Multiplex genotyping of immune response genes.....	78
2.8.3.Human DNA extraction.....	79
2.8.4.PicoGreen ds DNA quantitation assay.....	79
2.8.5.Amplification of parasite genome to increase DNA template.....	79
2.8.6.Strategy for mass spectrometry based genotyping.....	81
2.8.7.Post PCR product clean-up by Shrimp Alkaline Phosphatase assay.....	83
2.8.8.Primer Extension of PCR products.....	83
2.8.9.Detection of primer extension products by matrix assisted light desorption time-of-flight mass spectrometry.....	86
2.8.10. MALDI-TOF Mass Spectrometry.....	86
2.8.11.SNP and genotype calling.....	86
2.8.12.Genotype and data quality control.....	87
2.9.Data Analysis.....	87
2.9.1.Definition of explanatory variables.....	87
2.9.2. Definition of the dependent or response variable.....	88
2.9.3.Statistical analysis.....	88
2.9.4.Sample size calculations:.....	90
2.10.Ethics Review.....	91
CHAPTER 3: RESULTS AND DISCUSSION.....	92
Results.....	92
3.1.Characteristics of study participants at baseline.....	92

3.2. Baseline characteristics of children included in the study in Ngaoundere:	93
3.3. Baseline characteristics of children in the Bangolan study.....	94
3.4. Distinction between recrudescence and re-infections based on the highly polymorphic msp-2 gene.....	95
3.4.1. Allelic variation in pre-treatment parasites based on msp-2 gene.....	95
3.5. Baseline prevalence of drug resistance mutations in five genes in five ecological sites of Cameroon.....	97
3.6. Comparison of baseline prevalence of resistance conferring mutations to AQ between the northern and southern ecologies.....	97
3.7. Baseline prevalence of resistance conferring mutation and clinical responses.....	99
3.8. Distribution of mutations according to drug response classification.....	102
3.9. Haplotype frequencies for the pfert gene among patients with Day 14 and Day 28 outcome.	103
3.10. Haplotype frequencies of the pfdhfr and pfdhps genes in isolates classified for day 14 and day 28 endpoints.....	104
3.11. Combined resistance conferring haplotypes in adequate clinical and parasitological responses.	106
3.12. Cure rates for amodiaquine, fansidar and the combination of both drugs combined for the three study sites.	108
3.13. Association of drug resistant mutations with response to treatment.	109
3.14. Single Nucleotide Polymorphisms in Host Immune Genes and treatment response.....	112
3.14.1. Verification of the Hardy Weinberg Equilibrium Law	112
3.14.2. Distribution of SNPs across the study population	113
3.14.3. Association of SNPs and pre-treatment patient temperature.....	115
3.14.4. Frequency distribution of alleles in Fulanis and non-Fulanis in the North of Cameroon.	116
3.14.5. Association of SNPs and clearance of drug resistant parasites among the Fulani population in North ecology.....	118
3.14.6. Differences in the distribution of candidate gene polymorphisms and treatment outcomes by geo-ecological regions	121
3.14.7. SNP Association with AQ resistant TYN haplotype by ecology.	124
3.14.8. Differential correlation of SNPs and parasite or fever clearance among the Fulani	125
3.15. Discussion	126
3.15.1. Parasite identity, genetic diversity and resistance in different towns of Cameroon.	127
3.15.2. Single nucleotide polymorphisms in drug resistant genes and association with treatment response.....	128
3.15.3. Differences in drug resistant SNP distribution by study site.....	128
3.15.4. Frequency distribution of wild and mutant alleles by day of efficacy assessment.....	131
3.15.5. Association of selected resistance conferring haplotypes with treatment outcome.....	132
3.15.6. Single nucleotide polymorphisms in immune genes and correlation with treatment response.....	132

3.15.7.SNPs in IL-22, related toIL-10 and mediating Th17 cellular response is possibly associated with treatment response.	133
3.15.8.Complement receptor polymorphisms, less frequent in the North represented risk alleles of adequate response to treatment.	134
3.15.9.Interleukin 4 and IL-4R receptor polymorphisms investigated may have had conflicting roles in influencing response to treatment.	135
3.15.10.TNF α enhancer haplotype (CACGG= -1031T>C, -851G>A, -308G>A, -238G>A) polymorphisms were disproportionately represented among those who cleared parasites in the North.	136
3.15.11.Single nucleotide polymorphisms in the CD36 gene may significantly influence immune response to malaria in children treated in Cameroon.	137
CHAPTER 4: GENERAL CONCLUSIONS AND PERSPECTIVES.	140
4.1.General Conclusions	140
4.2.Perspectives.	141
Bibliographic references	142
Annexes	167
annexe 1: Antimalarial Drug Targets	167
annexe 2: Activation of Artemisinins	168
annexe 4: Resistance Markers, Genotypes and Resistance Status.	169
annexe 5: Resistance Marker Genotypes	171
Annexe 7: Sequence of various primers and PCR Reaction conditions for different resistance conferring pfmdr-1 and Pfprt genes.....	174
Annexe 8: Structural localisation of immune genes and chromosomes considered for genomics analysis.....	176
Annexe 9 : A schematic representation of the effect of a gene polymorphism on the outcome of infection with malaria	187
Annexe 10: Extraction of DNA using Chelex-100 protocol.....	188

LIST OF TABLES

Table 1: Major ligands and receptors associated with severe malaria.....	17
Table 2: mechanisms of chloroquine action in malaria parasite and associated.....	22
Table 3: Blood Disorders and role in malaria protection.....	49
Table 4 Drug administration dose table.....	61
Table 5: Genes, codons of interests and haplotypes of wild and mutant forms of the parasite.....	66
Table 6: Probes and probe sequences and target gene haplotypes in <i>P. Falciparum</i> dhfr and dhps.....	69
Table 7 Selected human immune candidate genes.....	70
Table 8 PCR Amplification and cycling conditions for the Long PCR reaction.....	80
Table 9 Protocol table for master mix preparation for amplification of target loci by PCR.....	82
Table 10 Amplification reaction condition for target loci.....	82
Table 11 Protocol for clean-up of PCR amplification products for downstream analysis.....	83
Table 12 incubation conditions for optimal activity of Shrimp Alkaline Phosphatase.....	83
Table 13 Mastermix preparation protocol for primer extension reaction.....	84
Table 14: PCR condition for primer extension reaction.....	85
Table 15: Baseline characteristics of study participants in Garoua, Yaounde and Mutengene.....	92
Table 16 Baseline characteristics of 150 children enrolled in the Ngaoundere study.....	93
Table 17 Baseline characteristics of children enrolled in the Bangolan study.....	94
Table 18 frequency of resistance conferring mutation to AQ the northern and southern ecologies.....	97
Table 19: Baseline occurrence of resistance conferring mutations and clinical resistance levels to amodiaquine in the five sites.....	99
Table 20: Association between the prevalence of resistant haplotypes in the pfdhfr and pfdhps genes and treatment failure to SP combination.....	109
Table 21: Association between the prevalence of resistant haplotypes in the Pfcrt and pfmdr-1 gene and treatment failure to AQ combination.....	110
Table 22 : Different alleles of the rs7537937 used to verify Hardy Weinberg Equilibrium in our sample population.....	112
Table 23: SNPs represented in our study population showing correlations with treatment outcome.....	113
Table 24: Association between SNPs and late parasitological failure.....	114
Table 25 : SNPS showing correlations with high pre-treatment temperature defined as temperature below or above 39°C for 48 hours.....	116
Table 26: SNPS most frequently encountered among Fulanis clearing resistant parasites.....	117
Table 27: Association analysis of SNPs and Clearance of drug resistant parasites among the Fulanis in North Ecology.....	119
Table 28 : Association between SNPs and treatment response classified as success or failure.....	120
Table 29: Geo-ecological differences in clearance of resistance conferring mutants and SNPs.....	122
Table 30: Geo-ecological differences in clearance of AQ resistant mutants.....	123
Table 31: Association of SNPs with clearance of AQ resistant mutants by ecological site.....	125

LIST OF FIGURES

Figure 1:the Global Burden of malaria.....	10
Figure 2: the life cycle of the malaria parasite.....	12
Figure 3: the effect of adding an artemisinin to a more slow acting drug in the treatment of un-complicated malaria.....	27
Figure 4 the effect of initial parasite density on treatment response.....	29
Figure 5 transect model of the pfcrt Protein showing the Locations where Mutation occur in the Protein.....	33
Figure 6 Principal pathways of immune activation and intracellular pathogen killing.....	42
Figure 7 A presentations of the various cell Surface sensors known as pathogen recognition receptors and molecular mechanisms.....	43
Figure 8 :Targets of immune response in malaria.....	44
Figure 9 : bridging the innate and acquired immune systems.....	46
Figure 10 : Biochemical reaction principles underlying genotyping.....	56
Figure 11: Distinction between recrudescence and reinfection based on genotyping msp-2genes in pre-and failure samples.....	95
Figure 12 :Cross-section of bands patterns of msp-2 in samples used to determine parasite diversity in the study population.....	96
Figure 13: Parasite genetic diversity as a function of the msp-2 genetic profile.....	96
Figure 14: Baseline chloroquine and amodiaquine resistance mutations in five geo-ecological sites in Cameroon.....	100
Figure 15: Frequency of pfdhps haplotypes in five ecological sites in Cameroon.....	101
Figure 16: Key dhfr haplotypes observed in five study regions.....	102
Figure 17Haplotypes frequencies for the pfcrt in isolates from patients with Day 14 clearanc or failures in Mutengene, Yaounde and Garoua.....	103
Figure 18 Haplotypes frequencies for the pfrndr-1 gene among in patien who clered or not comparing Day (left panel) and endpoints in Mutengene Yoaounde and Garoua.....	110
Figure 19 Haplotypes frequencies of the pfdhfr and pfdhfr genes in isolates classified for day 14 and day 28 endpoints.....	104
Figure 20: Haplotype of dhps in different sites were not by isolated in clinical failures.....	105
Figure 21:Resistance conferring haplotypes in adequate clinical and parasitological response.....	107
Figure 22: Combined Cure Calculated for the Three Study Site.....	108

ABSTRACT

Background

This study undertook to characterise drug resistance mutants of *Plasmodium falciparum* infections in five key geo-ecological locations in Cameroon and investigated parasite and molecular immune factors associated with clinical response and clearance of drug resistant *Plasmodium falciparum* among infected children less than 14 years.

Methodology

We conducted clinical efficacy trials of antimalarials in five locations in Cameroon. Total parasite nucleic acid from 750 *P. falciparum* infected samples was used to amplify target sequences by polymerase chain reaction (PCR) and restriction fragment length polymorphism and/or enhanced chemluminescence for point mutations in *Pfprt*, *pfmdr-1*, *Pfprdhfr* and *pfprdhps* genes. Single nucleotide polymorphisms (SNP) in candidate immune genes identified through bioinformatics were detected by matrix-assisted laser desorption mass Spectrometry (MALDI-TOF). The distribution of SNPs was analysed by drug response, by pre-therapy hyperpyrexia, by ethnic/ecological group and also by clearance of drug resistant parasite forms.

Results.

Mutations in the *pfdhfr* (51I, 59R, and 108N), *pfdhps* 437Gly as well as *Pfprt* 76T and *pfmdr-1* 86Y, 184F and 1246Y were detected invariably in all regions. The fansidar resistant SGK haplotype appeared to be associated with the drug response in Mutengene but not in Yaoundé or Garoua; and correlated inversely with adequate clinical and parasitological response. SNPs in IL-22, IL-4R1 and CD36 appeared to have been associated with clearance of resistant parasites [p= 0.017 OR(C allele):1.44 [95% CI (OR) of 1.06-1.95]; [P= 0.014, OR = 1.31, 95% CI (OR):1.07-1.83]; [P= 5.78 x10⁻⁵ OR=0.27, 95% CI(OR):0.13-0.54] respectively; with high fever (> 39°C, 48 hrs) [IL-22, P= 0.01, OR=1.5, (95%CI(OR): 1.8-2.1)] and also in high frequency among the Fulani participants [P= 0.006, OR=1.83, 95% CI (OR):1.11-3.08]. The CD36-1264 null allele was completely absent in the northern population.

Conclusion

No independent associations were found between parasite factors and drug response. Molecular immune signatures in IL-22, IL-4, IL-4/IL-4R, and CD36 could potentially influence drug resistant parasite clearance in Cameroon. Differences in CD36-T1264G mutants may reflect ethnic lipid metabolic differences or an unknown evolutionary genetic phenomenon in West Africa.

Key words: Plasmodium falciparum, Single Nucleotide Polymorphism, Resistance, Immune genes, Fulani, amodiaquine, sulphadoxine-pyrimethamine, Interkeukin 22.

RESUMÉ

La prise en charge de cas, y compris le traitement intermittent préventif avec la sulphadoxine pyriméthamine forme un millier dans la lutte contre le paludisme au Cameroun. La résistance parasitaire aux antipaludiques reste peu caractérisée au Cameroun. Cette étude a entrepris de caractériser la résistance génétique de *Plasmodium falciparum* aux antipaludiques dans cinq sites géo-écologiques différents du Cameroun. En plus, elle a étudié l'influence des facteurs immunitaires moléculaires associés à la réponse clinique et à la clairance parasitaire pendant le traitement chez les enfants.

Méthodologie

Nous avons mené des essais cliniques d'efficacité des antipaludiques dans cinq localités du Cameroun. L'acide nucléique total du parasite provenant de 750 échantillons infectés par *P. falciparum* a été utilisé pour amplifier les fragments des gènes cibles des antipaludiques par réaction en chaîne par polymérase (PCR) et polymorphisme de longueur des fragments de restriction et / ou chimioluminescence améliorée pour les mutations ponctuelles dans les gènes *pfprt*, *pfmdr-1*, *pfdhfr* et *pfdhps*. Les polymorphismes mononucléotidiques (SNP) dans les gènes immunitaires candidats identifiés par la bio-informatique ont été détectés par spectrométrie de masse à désorption laser assistée par matrice (MALDI-TOF). La distribution des SNP a été analysée par type de réponse médicamenteuse, par pyrexie pré-thérapeutique, par groupe ethnique / écologique et également par la capacité à éliminer des formes parasitaires résistantes aux médicaments.

Résultats.

Des mutations dans le *pfdhfr* (51I, 59R et 108N), *pfdhps* 437Gly ainsi que *pfprt* 76T et *pfmdr-1* 86Y, 184F et 1246Y ont été détectées invariablement dans toutes les régions étudiées. L'haplo-type SGK résistant au fansidar semble être associé à la réponse médicamenteuse à Mutengene mais pas à Yaoundé ou Garoua; et ce dernier a été corrélé inversement avec une réponse clinique et parasitologique adéquate. Les polymorphismes par changement d'un nucléotide dans le gène *se* IL-22, IL-4R1 et CD36 semblaient avoir été associés à la clairance des parasites résistants [$P = 0,017$ OR (allèle C): 1,44 [IC à 95% (OR) de 1,06 à 1,95]; [$P = 0,014$, OR = 1,31, IC à 95% (OR): 1,07-1,83]; [$P = 5,78 \times 10^{-5}$ OR = 0,27, IC à 95% (OR): 0,13-0,54] respectivement; avec l'hyperpyrexie ($> 39^\circ \text{C}$, 48 h) [IL-22, $P = 0,01$, OR = 1,5, (IC à 95% (OR): 1,8-2,1)] et on a également observé en fréquence élevée chez les participants peuls [$P = 0,006$, OR = 1,83, IC à 95% (OR): 1,11-3,08]. L'allèle nul CD36-1264 était complètement absent dans la population du nord.

Conclusion

Aucune association indépendante n'a été trouvée entre les facteurs parasitaires et la réponse aux médicaments. Les signatures immunitaires moléculaires dans l'IL-22, l'IL-4, l'IL-4R et le CD36 pourraient potentiellement influencer la clairance des parasites résistants aux médicaments au Cameroun. Les différences entre les mutants CD36-T1264G peuvent refléter des différences de métabolisme lipidiques entre les ethnies ou à un phénomène génétique évolutif en Afrique de l'Ouest encore peu connu et qui mérite d'être investigué en profondeur.

Mots clés: Plasmodium falciparum, polymorphisme, chimiorésistance, gènes immuns, Foulani, amodiaquine, sulphadoxine-pyriméthamine, interleukin 22.

GENERAL INTRODUCTION

Malaria is caused by parasitic infections of the genus *Plasmodium* and it is an important disease of the tropics and sub tropics especially Sub-Saharan Africa. The death toll caused by malaria in Africa alone, estimated to up two million per year mostly children. The associated debility and cognitive inhibition makes malaria one of the most challenging public health problems in recent times (Greenwood 2010) and a significant contributor to societal underdevelopment.

The development of strategies to curb malaria in the past has relied on the use of chemotherapy, vector control, environmental management and most recently, the much desired vaccination is being considered; with the most advanced vaccine RTS'S being considered to be deployed in malaria infested countries by WHO after considering final efficacy results of the study(Rts 2015). The sequencing of the genome of the parasite, the host and the vector and the development of high throughput methodologies have thrown more insights into the understanding of the biology of this parasite and why the parasite or the vector is refractory to most current tools used for control (Gardner 2002). Of importance, genetic variation in the parasite causes it to escape immune targeting and survive drug therapy challenge to the most widely used cheap and affordable medications. However, recent vaccines have proven to reduce the risk of infection with the parasite by as much as 50% (4). However, this report may be over-estimating the effects of the vaccine as in the same areas where the study occurred, there was concomitant implementation of vector control strategies notably bed net use.

Attributable drug resistant malaria deters further control of infection. This is because high rates of resistance renders clinically effective drug useless and jeopardizes malaria case management and public health efforts including the potential for epidemics of mutant strains. Furthermore, persistence of circulating mutants may be more pathogenic, causing uncomplicated infections to progress to severe forms and subsequent death. One way to mitigate the effects of drug resistant malaria therefore is to detect and monitor circulating drug resistant parasites to describe their distribution and pattern of spread during drug use. This informs policy on better strategies to implement in order to curb potential malaria disasters in communities. To this effect, the search for markers of drug resistance has been the object of research in malaria. Examples of drugs for which molecular markers exist are chloroquine (CQ), fansidar (SP), amodiaquine (AQ). Another way is to withdraw drug pressure and reintroduce when sensitive clones of the parasites appear as have been observed previously for chloroquine (Laufer 2010). For the artemisinins, molecular markers are yet to be discovered. At the present time, the most reliable markers include delay in parasite clearance time, which has been shown to be heritable and therefore should have a genetic

basis (Anderson T. J. 2010). In Cameroon, studies of different molecular markers of resistance to a host of antimalarials have been done. Basco and co-workers have used a number of molecular approaches including gene sequencing, restriction fragment length polymorphism, etc. to identify new and existing mutations that are used and have the potential to be used as markers of resistance (Basco 1998, Basco L. K 1995, Basco 2002, Basco 2002, Basco 2002). The importance of the presence of the markers in the isolate has in most cases been deduced by investigating the sensitivity of the isolates carrying the markers identified *in vitro*. This type of analysis provides information on the potential developed by the parasite to render treatment useless. One important limitation however, is that potential factors influencing response to therapy, namely immunity and human genetic background are not taken into consideration in this *in vitro* condition. In addition, ecological diversity affects parasite population diversity, an effect that is translated in to phenotypic outcomes including drug sensitivity and parasite fitness is not understood. Furthermore, the dynamics of the spread of resistance alleles cannot be explored which can further limit the breath of important public health control measures. It is one objective of this thesis to investigate both ecological differences in the prevalence and distribution of these markers and to explore their *in vivo* drug sensitivity.

People treated for drug resistant malaria sometimes recover after treatment. The ability to recover and the risk of treatment failure have been linked to the age of the patient, the host genetics, and the genetic characteristics of the parasite and the transmission intensity of the parasite (Greenhouse 2009). This highlights the effect of host immunity in assisting clearance of drug resistant parasites. Furthermore, variations in haemoglobin molecules such as HbS have been associated with increased efficacy of fansidar treatment (Terlouw 2002). Therefore dissecting the role of the host component in clearance of drug efficacy is complicated as it requires both innate resistance and adaptive responses. Some studies have shown the additional effects of antibodies to ring stage antigen (anti-RESA), variant surface antigen (anti VSA) and glutamine rich protein antibodies (anti-GLURP to clearance of parasites resistant for SP, AQ and CQ while others have not found any significance (Lim 2010, Pinder 2006, Feng 2009). These variable findings may be accounted for by differences in intensity of transmission, levels of drug resistant markers, the patient age, host genetics and the innate resistance factors (Greenhouse 2009, Francis 2006).

In Cameroon, SP and AQ are still being used for the treatment of uncomplicated malaria but only if any of them is in combination with an artemisinin derivative. In addition, in the absence of a better alternative to SP due to rising drug resistance, SP is used in intermittent preventive treatment in pregnancy, even though a prevailing argument is that such declining efficacy observed with SP has been investigated in children with significant physiological differences compared to

pregnant women. A combination of AQ+SP was used in Cameroon as interim measure to manage uncomplicated resistant malaria at the time when artemisinin based combination therapy was not yet available in public outlets. It is in this regard that in attempting to dissect the biological factors associated with malaria treatment response in Cameroon, we set out to study the efficacy and safety of this combination used in treatment of uncomplicated malaria in three epidemiological zones in Cameroon. We therefore in the context of this present study evaluate parasite and human genetic factors influencing the outcome of treatment with SP and AQ. Single nucleotide polymorphisms in major genes encoding drug targets as well as, in genes encoding endogenous immune related molecules involved in the pathophysiology of malaria are all investigated. The prevalence and distribution of these various categories of markers were determined and correlated with treatment response.

In Cameroon, artemisinin based combination therapy came into use after a transitory use of fansidar/amodiaquine combination to manage clinical malaria. Today, artemisinin based combination using amodiaquine is the first line therapy for malaria with the possibility to switch to artemisinin/fansidar combination. While molecular markers of drug resistance in fansidar and amodiaquine are fairly established, molecular signatures of resistance to artemisinin are still in the discovery/validation phase. Sporadic studies on molecular epidemiology drug resistance to some antimalarial drugs have been done in Cameroon (Basco 2000, Basco 2003). However, no detailed study had examined the distribution of these markers in different ecological zones notably between the northern and southern parts of the country within the same period under the same in vivo treatment conditions within a well-designed randomized double blind and placebo controlled trial. In addition, previous studies have not examined the effects of variations in human immunogenetics, and ecologic and associated influence on treatment response variables such as fever and parasite clearance.

Having these considerations in mind, the objectives of the present study are outlined as follows:

1. Describe and determine the prevalence of major *Plasmodium falciparum* drug resistant mutations in Pfert-codon 76, pfmdr-1-codon 89, pfdhfr-codons 51, 59 and 108 and pfdhps codons 437 and 540 among circulating parasites in five geographically distinct malaria endemic sites in Cameroon.
2. Assess the association between the molecular markers of *Plasmodium falciparum* drug resistance and treatment response in a trial evaluating the clinical efficacy of AQ and SP in Cameroon.

3. Quantify the molecular correlations between host candidate gene polymorphisms associated with malaria pathophysiology and treatment response in children with AQ or SP resistance conferring *Plasmodium falciparum* mutants.
4. Decipher potential candidate immune genes that may be implicated in ecologic or treatment related responses to clearance of circulating *Plasmodium falciparum* drug resistant mutants.

This work is presented in several chapters. Chapter one presents the introduction, the review of literature with regards to the current understanding of the epidemiology, diagnosis and treatment of malaria as well as resistance to antimalarial treatment; the pathophysiology, the immune mechanisms in malaria as well as the human genetic basis of the outcome of the malaria infection, an overview of antioxidant changes in malaria oxidative and environmental stress as well as their potential as new antimalarial target. This chapter ends with a rationale for the research investigations.

Chapter two provides with an overview of the research design, its implementation in the field sites as well as the methods used for field research and sample collection. Besides a description of the clinical trial designs for two major trials in which the analysis depend as well as the geographic description of trial sites, this section also contains a description of the study participants, enrolment procedures, participant randomization, drug administration and observance, follow up procedures and evaluation of treatment response. This chapter ends with the methodologies used to determine the sample size of the studies, the molecular analyses of parasite genome and the approach, selection, design and molecular analysis of candidate human genes contributing to different treatment outcomes.

Chapter 3 is a more detailed analysis of the different aspects of the study contributing to the thesis. This chapter on the whole shows the body of different results and the interpretation thereof. This chapter is divided in to three sections. The first section corresponding to the first objective deals with an analysis of the prevalence and distribution of single nucleotide polymorphisms (SNPs) in some *Plasmodium falciparum* drug metabolizing genes associated with resistance to fansidar, amodiaquine, and artemisinins; as well as the association between these SNPs and treatment response. Section two on the other hand deals with the genetic variations in genes implicated in the pathophysiology of malaria and their association with the response variables of treatment in the same study and by verbal ethnicity. Section three corresponding to the third objective deals with the molecular correlations and parasite clearance. The last section ends with an analysis of the influence of molecular variations in immune genes on the rates of parasite clearance during treatment in the two major divisions of our sample population.

Chapter 4 broadly presents a general interpretation of the findings as well as a detailed discussion of the results in the light of current literature and the findings of other research groups. This also includes analysis of genetic association evidence pointing to potential mechanisms for natural immune response and clearance during drug therapy in Cameroonian children as well as emerging themes in the subject.

CHAPTER 1: LITERATURE REVIEW AND RESEARCH PROBLEM

1.1. Clinical manifestations and diagnosis of malaria.

Malaria is a parasitic infection considered as a syndrome. This is a disease which covers a wide spectrum of clinical presentations that depend on a number of factors. These factors are related to the nature of the parasite, to the host's genetic, physical, and even economic condition. The key characteristic symptoms of malaria infection include periodic fevers, shivering, muscle weakness, enlarged spleen, varying severity of anaemia, headaches, poor appetite etc. The fever paroxysms in malaria correspond to the synchronized maturation of erythrocytic schizonts and their subsequent release of merozoites and toxic substances into the bloodstream (Miller 1994). The paroxysms continue for up to six hours and end with profuse sweating. These paroxysms can return after some days, thus giving physicians the original definition of the different types of malaria infection. Tertian or quartan fever caused by *P. vivax* and *P. ovale* have a period of 48 hrs so the fever is tertian, in *P. malariae* it is 72 hrs resulting in quartan malaria, whereas *P. falciparum* is considered apart with its 48 hour or daily period). This periodicity also defines a prepatent period that is the minimal time elapsing between the initial sporozoite infection and the first appearance of the parasite in the erythrocytes, and the incubation period, defined as the time elapsing between infection and the first clinical manifestations. The prepatent period is characteristic of each species and is thus used as a criterion for the clinical diagnosis of the infecting parasite. For the human malarias they are: 5 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale*, and 15 days for *P. malariae*. A relapse occurs when the infection returns anew after an interval greater than the incubation period. Relapse is attributed to reactivation of a hibernating form of *P. vivax*, *P. ovale* and *P. cynomolgi* known as hypnozoites from hepatic parenchyma (Krotoski 1989, Mayxay 2004).

Increasingly, the limitations of clinical diagnosis of malaria are being recognized (Tangpukdee 2009). It is now mandatory for most national malaria control strategies to combine clinical diagnosis with laboratory findings to effectively diagnosis and treat malaria (W.H.O 2010). Malaria diagnosis involves identifying the entire malaria parasite or antigens/products liberated during parasite metabolism in the patient's blood. Although this may seem simple, the diagnostic efficacy is subject to many factors. The different forms of the five malaria species; the different stages of erythrocytic schizogony, the endemicity of different species, the interrelation between levels of transmission, population movement, parasitemia, immunity, and signs and symptoms; drug resistance, the problems of recurrent malaria, persisting viable or non-viable parasitemia, and sequestration of the parasites in the deeper tissues, and the use of chemoprophylaxis or even

presumptive treatment on the basis of clinical diagnosis, can all influence the identification and interpretation of malaria parasitemia in a diagnostic test (Tangpukdee 2009).

A range of laboratory strategies exist for the laboratory diagnosis of malaria. These strategies rely on morphological, immunological, molecular characteristics of the parasites.

Malaria diagnosis by light microscopy remains the gold standard for laboratory confirmation of malaria. The parasites are identified by examining a drop of the patient's blood under the microscope, spread out as a "blood smear" on a microscope slide. Prior to examination, the specimen is stained (most often with the Giemsa stain) to give to the parasites a distinctive appearance. The quality of the results depends on the quality of the reagents, of the microscope, and on the experience of the laboratory staff (Wongsrichanalai 2007). Though with these limitations, this method of diagnosis remains the appropriate method for monitoring drug and vaccine efficacy studies in malaria (Wongsrichanalai C 2007).

Besides conventional microscopic observations, another method utilises the quantitative buffy coat (QBC). This technique basically uses microhematocrit centrifugation to concentrate malaria parasites prior to direct examination after staining with fluorescence material. It employs a precisely constructed capillary tube which is internally coated with EDTA and acridine orange. The use of the dye is based on the premise that infected red cells appear to be less dense than uninfected ones, and concentrate primarily within the zone at the interface - a small 1-2 mm region near the top of the RBC column. These parasites fluoresce green and orange objects because of the uptake of the dye by parasite DNA (Rickman 1989). This technique showed comparable sensitivity with microscopy.

Early diagnosis of malaria infection is one of the measures implemented for the prompt treatment of malaria infections and to mitigate the downstream consequences of treatment delay (S. 1998). To this effect, rapid diagnostic tests based on immunological techniques exist for the rapid diagnosis of malaria. These techniques are based on the capture of malaria derived antigens immobilized in a test pad assembly or cassette format. Many different tests exist based on the immobilized antigen incorporated. In this regard, malaria rapid diagnostic tests are valuable though in recent times, efficacy of the tests, defined by its sensitivity and specificity still needs to be improved and evaluated in many settings (W.H.O 2010). Of note this test finds utility in quick clinical decision making in high transmission areas. In this setting, the presence of a fever may not necessarily mean malaria. In addition, a negative test may not rule out an infection and a positive test does not greatly improve diagnosis (Graz). Therefore, besides using rapid diagnostic tests in high transmission settings, it is important to judge on a case by case basis taking into con-

sideration the local epidemiology of other infections with similar symptoms. The relationship between antigen concentration and parasite density can vary with the degree of sequestration of parasites, the stage of parasite growth, and the persistence of antigen after reduction or elimination of the parasite population (Baker 2005). Furthermore, variation in the structure of some parasite antigens affects binding to antibody. Besides their high cost, RDT methods are pretty easy and require no expertise, but training is mandatory. In addition, they can be effectively applied to rural settings where electricity supply is limited or inexistent. Other immunological tests indirectly demonstrate parasite exposure. They are based on the immunofluorimetric and immunoenzymatic systems developed in an enzyme linked immunosorbent assay (ELISA) that detects and quantitates malaria antigens (Noedl 2002). These methods are expensive and are not generally amenable to district laboratories.

Molecular biology based techniques, principally based on the amplification of genes specific to the malaria parasite have emerged to be the most reliable method for parasite detection (Berry 2008). Essentially, genes defined to be parasite specific are amplified by the polymerase chain reaction to yield products that can be detected on agarose gel electrophoresis using a UV transilluminator. Some of the genes targeted include the 18s subunit of the ribosomal RNA, dihydropteroate synthase, pf155 Ring infected erythrocyte surface antigen, P126 antigen and the STEVOR gene, etc. In this way, parasite speciation is possible as compared to immunological methods. The method is expensive and requires specialized training. Other variants of the classical PCR based method exist. The Realtime PCR principally based on real time observation and quantitation of amplified products. Such sophisticated variant is employed for research purposes and rarely for routine diagnosis. Overall, PCR based technologies are clinically not relevant for the reasons given earlier. However, recent technological advances seek to make DNA based tests simple enough to be used in routine district labs. For this reason, another variant called the Loop Amplified isothermal Amplification (LAMP) is in development. This technique employs the use of high efficiency DNA polymerase and a set of three primers that, on binding to parasite DNA produces a hairpin structure acting as a template now for high efficiency amplification and concatenation. This method has been shown to be comparable to RDT and microscopy but will clearly require further optimisation (Paris 2007). Molecular biology based techniques are mostly applied in research settings or in reference laboratories.

1.2.Epidemiology of Malaria

The distribution of malaria worldwide follows a simple and understandable pattern. About 91% of all malaria deaths in the world today occur in Africa south of the Sahara (W.H.O 2013). This is because the majority of infections in Africa are caused by *Plasmodium falciparum*, the

most dangerous of the four human malaria parasites. The climate of this region also is very suitable for the thriving of the *Anopheles gambiae*, the most effective malaria vector. Up to 40 % of the world's population lives in areas with malaria risk. In these regions, *Plasmodium falciparum* is directly responsible for 207 million clinical malaria cases in 2012(W.H.O 2013), and a death toll of 627000 million causing the greatest suffering and impoverishment among the poor people (Sachs 2002, D. 2001). Pregnant women and children less than five years remain the most vulnerable due to reduced or inadequate immune competence (McGregor 1984). The disease is less dangerous in areas of stable transmission than in areas of unstable transmission due to the acquisition of incomplete immunity to the disease (Greenwood 2002). A review of past studies by Breden et al. (Bremen 2007) applying disease rates with current demographic profiles showed that close to 600,000 people contract cerebral malaria yearly, with a case fatality rate of about 20 per cent . Neurologic complications lasting longer than six months may occur in up to 19,000 of these patients. Severe anaemia due to malaria occurs in between 1.5–6.0 million African children; with a case fatality rate of nearly 15 percent, up to one million children may die every year from malaria-induced anaemia. Respiratory distress, hypoglycemia, and overlapping conditions contribute another one to two million cases and, with a mortality nearing 20 per cent, well over 200,000 deaths. In Africa, there may be up to one million malaria associated low birth weight babies born each year and approximately 400,000 of these children will die (Fernando 2010, Bremen 2001). All of these “gaps” in the burden contribute up to 1.7 million deaths in African children yearly, with more than 50 percent due to anaemia, a pathophysiological consequence of the infection. The accuracy of the above figures is impeded by the facts that most malarial deaths occur at home, many cases are misdiagnosed and functional microscopes are not available to most clinics in the many areas (Greenwood 2002). Figure 1 provides a representative map of the global burden of malaria as reported by Hay et al. in 2007.

The 2013 World Malaria Report (W.H.O 2013) shows that 71% of the total population in Cameroon live in high transmission zones, with *Anopheles gambiae* and *Anopheles funestus* being the most efficient transmission vectors. An estimated increase of 1000 malaria attributed deaths were reported in 2008 alone reducing to slightly less than 1000 in 2010. Hospital admissions due to malaria far exceed this number, making malaria the first cause for hospital visits among the rural poor in Cameroon. Despite the implementation of artemisinin based combinations for malaria in 2004, the use of non-artemisinin based malaria drugs remained high (about 100%) until 2009, probably due to massive roll out of artemisinin based combinations (ACTs) subsidized by the Global Fund. It is estimated that with the recent large scale distribution of long lasting insecticide treated bednets, malaria transmission, as well as malaria attributed deaths will

drop and Cameroon will hopefully pass on from control to elimination of the disease within the next few years.

From a development point of view, malaria is a significant contributor to impaired cognitive development (Fernando 2010) with 13% to 50 % school absenteeism due to ill health. It has been shown that 5 to 20% of those who survive severe malaria experience neurological sequelae including behavioural disorders and impairment in the ability to carry out executive functions (Sachs 2002). This, as well as other consequences results in approximately \$12 billion of GDP loss due to the illness for African countries (Sachs 2002). Though this figure is debated as arbitrary, arguing that high quality data on malaria incidence or prevalence in the most severely affected countries is limited, it provides an estimation of the toll that malaria has on the economies of poor and developing nations. A review carried out to explore the link between poverty and malaria did not conclude on a direct relationship between the two parameters (Worrall 2005), mainly due to the lack of comparability between measures of poverty and socioeconomic status. However, the authors could note a clear link between the uptake of malaria interventions and poverty indicators. This at least enable us to assert that where poverty predominates, malaria predominates and because both disease and poverty maps overlap in many points, this makes malaria a both a cause and a consequence of under-development.

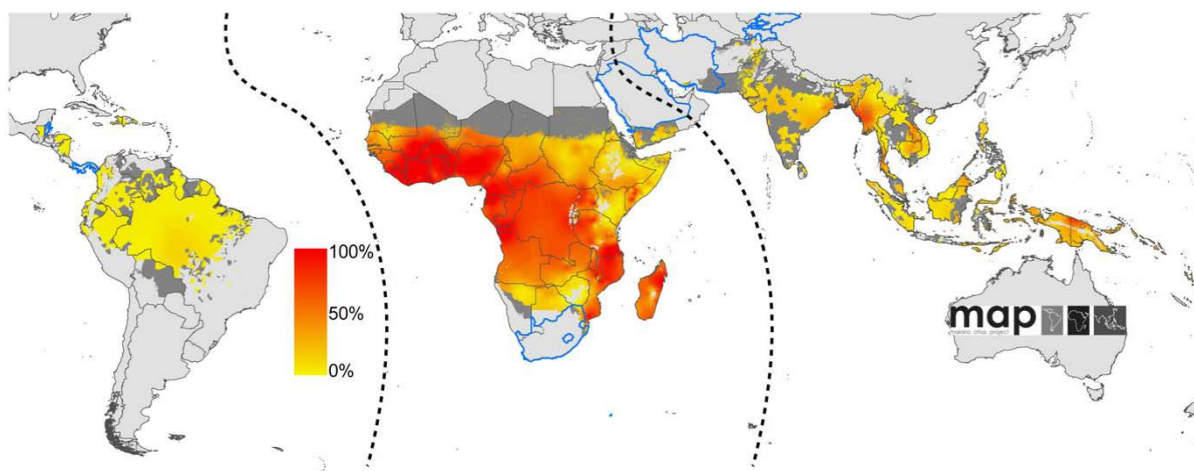


Figure 1:the Global Burden of malaria

The land area was defined as no risk (Tunisia, Morocco, Algeria, Egypt, Chile, Uruguay, Paraguay, Australia), unstable risk (Niger, Guinea Conakry,), and stable risk. The dashed lines separate the Americas, Africa+, and the CSE Asia region, respectively, from left to right. The countries with thick blue borders South Africa, Iran, Mexico, Kazakhstan etc. have very low P falcipara-

rum burden and reliable national health information systems. Countries in Yellow mainly in the Equatorial belt do suffer greatest burden of Malaria. (Hay S. I. 2009).

1.3.The Life cycle of Plasmodium falciparum

To understand the challenges faced in combating malaria and the strategies for vaccine and treatment development, it is essential to understand the basic life cycle of this parasite. Plasmodium parasites are protozoa of the phylum Apicomplexa (often referred to as sporozoans). These are animal parasites which exist in two hosts, have sexual and asexual stages, and alternate between haploid and diploid genetic phases. In addition, they must be able to survive inside both hosts to complete their life cycle. There are five species of Plasmodium that infect human beings: *P. vivax*, *P. falciparum*, *P. malariae*, *P. knowlesi* and *P. ovale*. Of these, *P. falciparum* causes the most severe and often fatal form of malaria. The life cycle of the parasite as presented below starts with infection of the human host. Briefly, when an infected female mosquito bites a human being, sporozoites are injected into the subcutaneous tissue and occasionally directly into the bloodstream. Within an hour they disappear from the circulation and invade the hepatocytes of the liver initiating the pre-erythrocytic or exo-erythrocytic stage of the life cycle. Injection of sporozoites into the tissues is associated with heat shock and the parasite secretes chaperone proteins to cope with the stress. Within the hepatocyte, the parasite rapidly develops within a membrane bound vacuolar compartment and matures giving rise to thousands of another form known as merozoites through repeated invaginations of the parasite plasma membrane. This process involves expansion of the parasite plasma membrane requiring endogenous production of fatty acids through the type II fatty acid synthesis pathway (Tarun 2008). These small merozoites break through the hepatocytes containing them through degradation of vacuolar membrane by cysteine proteases (Sturm 2006) and they later traverse the sinusoids each inside a merozoite vesicle. The merozoites gain entrance into the blood stream and invade erythrocytes in the blood, thereby initiating the intra-erythrocytic cycle of the parasite. The intra-erythrocytic cycle of *P. falciparum* takes 48 h. It begins with invasion of a red blood cell (RBC) by the merozoite binding via merozoite surface protein 1(MSP 1) or apical membrane protein 1(AMA 1) to receptors on the erythrocyte membrane followed by reorientation aided by exocytosis of erythrocyte binding proteins (EBA) like EBA 175 and formation of a vacuole derived from the RBC membrane called the parasitophorous vacuole (Chitnis 2000). The parasite develops in the RBC within the parasitophorous vacuole through a series of stages called ring, trophozoite and schizont. The ring stage parasite feeds on the host cell cytoplasm and develops into the metabolically active trophozoite stage. DNA synthesis occurs during the trophozoite stage and nuclear division during the schizont stage giving rise to a number of merozoites, typically 8-24 per infected erythrocyte.

Schizont rupture releases the merozoites which can reinvade fresh erythrocytes to begin another intra-erythrocytic cycle. Alternatively, some merozoites develop within erythrocytes into sexual forms called macrogametocytes and microgametocytes. When erythrocytes containing gametocytes are ingested by a female Anopheles mosquito during a blood meal, the gametocytes develop into gametes within the mosquito gut (Figure 2).

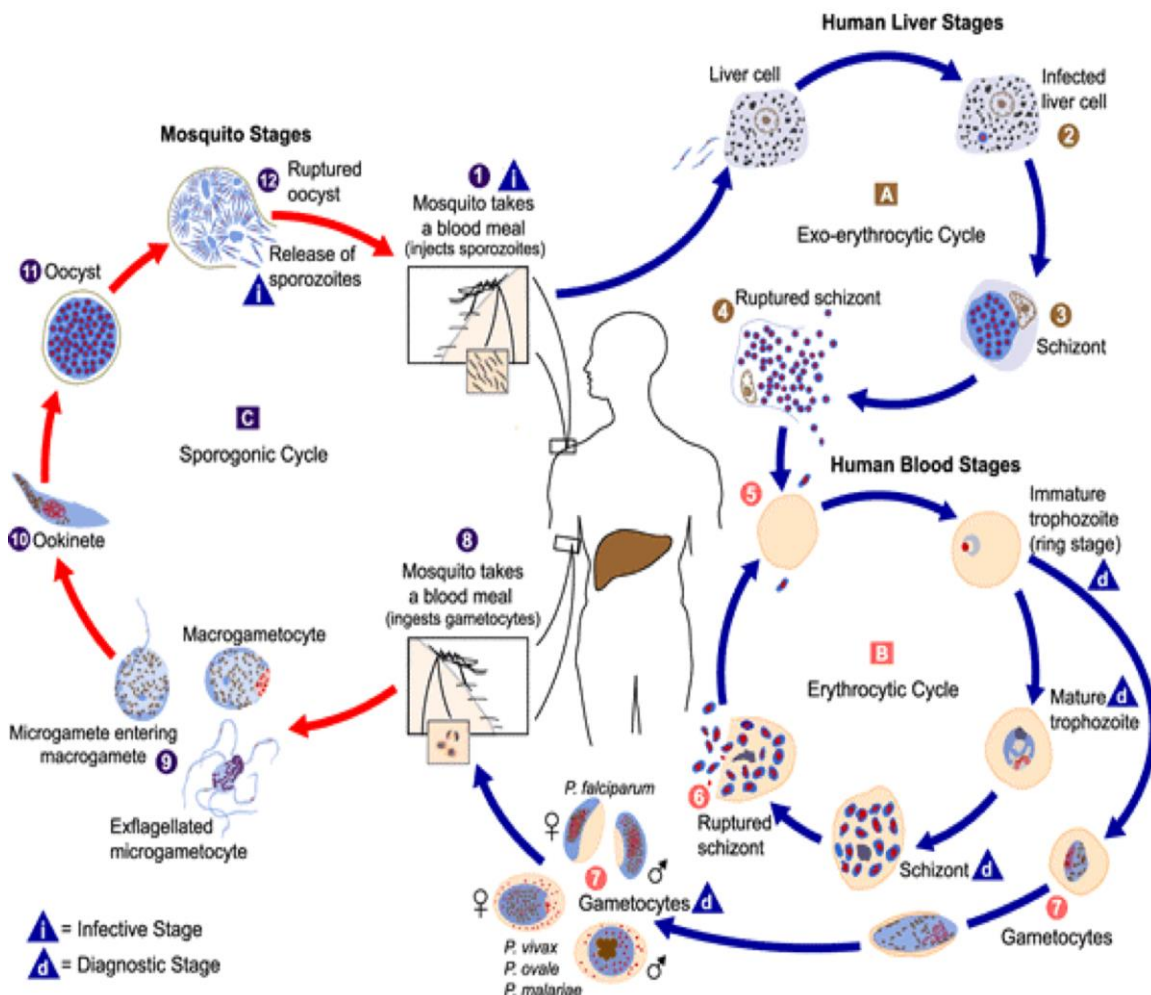


Figure 2: the life cycle of the malaria parasite

The life cycle involves three sub-cycles (sporogonic (c), exo-erythrocytic (a) and erythrocytic (b) and two hosts (mosquito and man). Source: <http://www.cdc.gov/dpdx/malaria/index.html> (accessed 12 October, 2014).

1.3.1. The Intra-erythrocytic cycle of *P. falciparum*

The intra-erythrocytic cycle is responsible for the symptoms of human malaria and hence known as the disease causing stage. Disease begins when the parasite begins to multiply asexual-ly within the red blood cell. During the asexual intra-erythrocytic cycle, the malaria parasite progresses through the ring, trophozoite and schizont stages (Figure 2).

Ring stage:

The form of the parasite immediately after invasion is called the ring stage. This is due to the fact that ingestion of host cell cytoplasm gives the parasite the appearance of a ring of cytoplasm with the nucleus conspicuously displayed at one edge- the so-called “signet ring stage”. Traditionally, the stages have been distinguished based on the morphology of the infected erythrocyte as observed by nuclear staining (usually Giemsa staining) followed by light microscopy. As the ring stage parasite grows by ingesting host haemoglobin, it develops into the trophozoite stage.

Trophozoite:

This is the metabolically active stage of the parasite. The parasite actively modifies the host compartment (47, 48) and new structures are formed in the erythrocyte including the Maurer’s cleft, an assembly of flat membranous structures thought to function as a protein sorting compartment between the parasite and the erythrocyte membrane (49). Some of the exported proteins form electron dense structures on the erythrocyte plasma membrane known as “knobs”.

The Schizont.

Nuclear division occurs during the schizont stage resulting in the formation of individual merozoites in the parasite periphery. The stage at which the cytoplasm is coalescing around the individual nuclei before cytokinesis is called the segmented stage. Once the merozoites are fully formed, the RBC membrane and the parasitophorous vacuolar membrane rupture by a protease dependent process (Salmon 2001) releasing the merozoites.

1.3.2. The extra erythrocytic stage of the life cycle of the malaria parasite.

The extra erythrocytic stages of *Plasmodium falciparum* involve both the sporogonic cycle in the mosquito host and the exo-erythrocytic cycle in the vertebrate liver. The entire process takes place within 14-21 days.

The sporogonic cycle in the mosquito starts during a blood meal when parasites are taken up by the anophelid mosquito as gametocytes. They transform into male exflagellated microgametocytes or female macrogametocytes which are released from the erythrocytes in the midgut of the insect. Here, fertilization occurs between the micro and macrogametocyte forming the ‘zygote’ within 18h of the blood meal intake. The zygote within the midgut then elongates into a motile ookinete which enters the midgut epithelium and comes to rest beneath the basal lamina where it forms an oocyst 24-72h after the blood meal. In *Plasmodium bergeri*, the ookinete invasion through the midgut epithelium elicits a potent innate response in the mosquito (Dimopoulos 1998, 1998), which may modulate the transmission of the parasite. While in the midgut epithelium, oocyst undergo mitosis 7-15 days after to produce thousands of sporozoites which migrate through pores into haemocoel and binds specific receptors on the acinar cells of the mosquito

salivary glands. Anti-sporozoite monoclonal antibodies have been found to block salivary gland invasion (52). While in the salivary gland, sporozoites that survive mosquito immunity will mature into infective forms that penetrate the vertebrate host during a subsequent blood meal to begin the exo-erythrocytic cycle.

The exo-erythrocytic stage of the parasite also known as the pre-erythrocytic stage involves an invasion of the hepatic parenchymal cells by infective sporozoites. They bind to specific receptors on hepatocytes. On the surface of the sporozoite, there are a number of ligands one of which is the co-receptor complex circumsporozoite protein and thrombospondin related adhesive protein (TRAP) (Usynin 2007) . It is the latter domain, specifically the thrombospondin repeat motif which mediates binding to the glycosaminoglycan (GAG) chain of the heparan sulphate proteoglycans of hepatocytes (45, 54). The sporozoites undergo multiple rounds of asexual divisions after internalization to produce merozoites within a containment called the schizont in a process otherwise known as merogony. This takes place during a period of about five days but depends on the species of infecting parasites. In *P. falciparum* for example, it takes a minimum of five and half days for the merogony to complete. Meanwhile, in *P. vivax*, *P. Ovale* or *P. cynomolgi*, there is formation of a hibernating form known as the hypnozoite which when activated release thousands of merozoites into the bloodstream through the liver sinusoids. While at this stage, the schizont containing cells are targets for cell mediated immune response. Principally CD8+ cytotoxic cells produce IFN- γ that mediates nitric oxide dependent killing of infected hepatocytes(McCall). Liberation of merozoites after merogony ushers the process of erythrocyte invasion and begins the intra-erythrocytic stage, also known as the disease causing stage of malaria parasite (Figure 2).

1.4. The Pathophysiology of Malaria

Plasmodium falciparum is the causative agent of the most malignant, severe form of malaria. Although the vast majority of malaria cases are mild febrile illnesses, the risk of progression to severe disease with life-threatening complications is high (56). Different immunological events involving factors are associated with the pathophysiology observed in malaria infecti

1.4.1. Cytokines and malaria severity.

Illness in malaria is caused by the erythrocytic stage of the parasite. There are no symptoms associated with the exo-erythrocytic stage developing in the liver as well as the release of merozoites from the liver, or gametocytes. The first symptoms and signs of malaria are associated with the rupture of erythrocytes when erythrocytic stage schizonts mature. The release of parasite products likes glycosylphosphatidylinositol (GPI) moieties, the malaria pigment and plasmodi-

um-derived nitric oxide synthase (NOS)-inducing soluble factors (57) will trigger a host immune response. This response is associated with oxidative stress on human cells. The simultaneous rupture of the schizont infected erythrocytes and the concomitant release of parasite antigens and waste products account for the intermittent fever paroxysms associated with malaria (Wijesekera 1996). These by-products stimulate human mononuclear cells to release pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF- α , interleukin 6, interleukin 1 and other pyrogenic cytokines (Clark I. A. 1988). Cytokines, reactive oxygen intermediates, and other cellular products released during the immune response against parasite antigens play a prominent role in pathogenesis, and are considered responsible for the fevers, chills, sweats, weakness, and other systemic symptoms associated with malaria. Pro-inflammatory cytokines such as TNF α suppress haematopoiesis, and by so doing contribute to anaemia (Clark 2000). Plasmodium falciparum has other unique characteristics that help to explain its distinct potential to cause severe or fatal disease. The parasite has a short generation time due to prolific nucleic acid replication (48hr replication cycle and up to 32 trophozoites develop from a single infected erythrocyte). In blood, they occur in higher numbers compared to other Plasmodium species. It is generally agreed that the haploid genome of the intra-erythrocytic parasite gives it a unique opportunity for antigenic variation enabling evasion of immune clearance (Chen 2000). This property promotes persistence of infection and disease.

1.4.2 PfEMP1, Cytoadherence, sequestration and severity of infection

As P falciparum parasites mature within red blood cells, they induce the formation of knob-like protrusions on the surface of infected erythrocytes. These sticky knobs contain proteins or ligands of which the principal protein is the erythrocyte membrane protein 1 (pfEMP1) that bind to receptors like heparin sulphate, CD36, ICAM 1, vascular cell adhesion molecule 1 (VCAM), and thrombospondin, chondroitin sulphate A on endothelial cells in capillaries and venules in multiple vital organs in the body including the placenta, brain etc. (Britta 2001), (63). The binding to these receptors lead to cytoadherence and sequestration of malaria parasites in deep vasculature (Clark I. A. 2003). This protein coded by the var multigene family made of 50 members with just one of the polymorphic genes switched on at each infection, thereby giving rise to just a single expressed phenotype clone. The ability of this gene family to show both clonal variation and antigen switching within and between infections allows the propensity of great antigenic variation and immune evasion (Blomqvist 2011). This greatly favours parasite persistence and persistent cytoadherence. The cytoadherence and sequestration of red cells within small vessels leads to obstruction of microvascular and obstruction to blood flow. Besides these effects, binding of infected RBCs to endothelial surfaces has been shown to modulate signal transduction with

variable consequences on disease outcome. Ho and White (Ho 1999) reviewed the mechanisms involved in cytoadherence highlighting the involvement of critical signal pathways leading to the formation of superoxide ion and activation of T cell receptors. This effect can greatly modify disease progression through overproduction of cytokines with pathological consequences. Furthermore, the presence of Duffy antigen receptor (DARC) on infected red blood cells suggests that these cells respond to gradients of chemokines of the C-X-C and C-C families, thus it is possible that these chemokines play a role in cytoadherence as well. High plasma chemokine levels have been observed in malaria infection (Burgmann 1995) . It is important to note that erythrocytes parasitized by *P. vivax* do not readily bind to endothelium as opposed to *P. Falciparum*. Thus, despite very high plasma concentrations of TNF α that may occur in vivax malaria, this infection does not lead to cerebral disease (Karunaweera 1992).

1.4.3. Rosettes and pathological consequences

An additional phenomenon causing severe pathology in malaria is that of uninfected red cells sticking to infected red cells and form what is known as rosettes (Chen 2000). From a molecular point of view, binding in rosette formation is mediated by carbohydrate moieties present in blood groups A or B, CR1, CD36 and heparan sulphate (HS)-like glycosaminoglycans (GAGs) which are parasite isolate specific. This clogging of uninfected erythrocytes around the infected cell blocks microcirculation causing respiratory distress, cellular stress and severe disease including cerebral malaria although this has not been formally demonstrated (Miller 1994). A study from Thailand failed to show any correlation between rosettes and cerebral malaria.

Ultimate to cytoadherence and rosette formation is secondary organ dysfunction and severe complications in the host can occur. In the brain this causes cerebral malaria; in the kidneys it may cause acute tubular necrosis and renal failure; and in the intestines it can cause ischemia and ulceration, leading to gastrointestinal bleeding and to bacterial septicaemia secondary to the entry of intestinal bacteria into the systemic circulation. The table below shows the various ligands and receptors involved in endothelial cell adhesion in falciparum malaria (Chen 2000).

Table 1: Major ligands and receptors associated with severe malaria.

<i>P. falciparum</i> ligand	Receptor	Adhesion phenotype
PfEMP1, CIDR domain	CD36	Cytoadherence, rosetting
PfEMP1 with DBL β -C2 domains	ICAM1(CD54)	Cytoadherence
	P-selectin (CD62P)	Cytoadherence
Red cell derived phosphatidyleserine or Band 3 protein or some PfEMP1 variants	Thrombospondin	Cytoadherence
PfEMP1 variant (CIDR α and DBL2 δ domains of specific variants)	PECAM1 (CD31)	Cytoadherence
Unclear	E-selectin (CD62E)	Cytoadherence
Unclear	VCAM1(CD106)	Cytoadherence
PfEMP1 (DBL α domain of specific variants)	Heparan sulphate	Cytoadherence
PfEMP1-N-terminal domain of PfEMP1	CR1(CD35)	Rosetting
PfEMP (DBL α domain)	A and B blood group sugars	Rosetting

1.4.4. Anaemia and infectious dyserythropoiesis

The outcome of *P. falciparum* malaria infection is not solely determined by the sequestration ability of mature forms, and there is a wide spectrum of manifestations of severe malaria, in addition to cerebral malaria. In particular, severe malarial anaemia is prominent. The most important mechanism of anaemia in malaria is massive hemolysis due to destruction of infected and uninfected RBCs in the spleen. In malaria infection, infected RBCs are deformed following interaction between the parasite and uninfected RBC during invasion (Awah 2009). In addition, uninfected RBCs are also 'decorated' with parasite intra-erythrocytic bodies thereby deforming them. This RBC deformity does not pass mechanical quality control imposed by the spleen through forced microcirculation within its inter-endothelial microcirculatory beds. The result is retention

of the deformed RBC and subsequent clearance from the system through macrophage mediated phagocytosis or complement mediated lysis in the presence of antibodies (Awah 2009) exacerbating anaemia in infected patients. Secondly, dyserythropoeisis is common in people who have chronic infections due to inhibition of erythropoiesis in rather than decreased folate or cobalamin (Menendez C 2000) . Dyserythropoeisis can also contribute to malaria anaemia through cytokine mediated mechanisms as described above. However, this is different in naive and immune patients. In patients with low grade falciparum malaria (as can be observed in drug resistance), immune mechanisms favouring inhibition of erythropoiesis prevail as opposed to patients with acute infections where massive hemolysis and splenic clearance is predominant. Therefore, antibody mediated hemolysis and dyserythropoeisis are the major contributors to anaemia in chronic infections (Buffet P.A. 2011). However, it should be stressed that some hemolysis occur because of the ingestion of some antimalarial drugs especially primaquine in patients with glucose-6-phosphate dehydrogenase deficiency. In artemisinin combination therapy, rising resistance may be partially explained by a slower transformation of ring parasites into undeformed bodies delaying pitting in the spleen. This is translated into delayed parasite clearance times in the plasma (Buffet P.A. 2011).

In summary, the pathophysiology of malaria essentially associated with the intra-erythrocytic cycle of the parasite involves a series of molecular events linked to the progress in the parasite's lifecycle and host response to these changes and associated pathology. Exacerbated host responses lead to significant disease affecting different organs and systems culminating in death in some cases. For example, maternal anaemia contributes to low birth weight which is a determinant factor of neonatal death. The survival of patients in endemic areas reveals essentially an evolutionary adaptation in host parasite relationships shown to be promoted by genetic changes in innate factors and other mechanisms as will be explored later in this thesis.

1.5. The metabolism of the growing intra-erythrocytic parasite.

The development of antimalarial agents against Plasmodium requires that the differences between its metabolic pathways and those of the host be exploited. With the availability of the Plasmodium falciparum genome sequence, it is possible to map all the metabolic pathways that operate in the parasite. Of the 5268 predicted proteins, only 733 have been identified as enzymes (Gardner M. J. 2002). The apparent paucity of enzymes could reflect the difficulty in identifying enzymes by similarity searches due in part to the great evolutionary distance between the malaria parasite and other well studied organisms (Gardner M. J. 2002) and to the complexity of aligning lengthy and highly variable sequences. However, advances in alignment technology including 2D

and 3D alignment methods will provide better insights in the near future. The identification of genes coding for all enzymes of certain metabolic pathways suggests that this approach of similarity searches performed to identify new enzymes was often successful and the dearth of enzymes probably reflects the parasitic life style of *Plasmodium falciparum*. The intra-erythrocytic developing parasite is multi-compartmented with a cytoplasm, a nucleus, an apicoplast, a mitochondrion, endoplasmic reticulum and Golgi body, a digestive vacuole and a parasitophorous vacuole. Besides the host cell cytoplasm and exo-membranous structures like the Maurer's cleft, the parasite also produces other compartments such as the dense granules, micronemes and the rhoptries during the schizogony and merozoite stages (Kats 2008). Carbohydrate Metabolism

The rapidly growing asexual stages of the malaria parasite mainly depend on anaerobic glycolysis for energy, with a net yield of two molecules each of ATP and pyruvate for every molecule of glucose utilized. All enzymes of the glycolytic pathway have been identified, and several key enzymes occur as isoenzymes. The enzymes, hexokinase, lactate dehydrogenase, , aldolase and triosephosphate isomerase have been studied in considerable detail . The enzyme, diphosphoglycerate mutase, an enzyme prominent in the red blood cell for regulation of hemoglobin oxygen affinity through 2,3-diphosphoglycerate, is absent in the malaria parasite (Roth 1988). Glucose-6-phosphate is also metabolized by the pentose phosphate pathway enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to ribose-5-phosphate and NADPH. Other phosphorylated sugars are also produced by the activity of transketolase. Genes for gluconeogenesis, synthesis of trehalose, glycogen and other carbohydrate stores are absent (Gardner M. J. 2002). Though genes for all the enzymes of the citric acid cycle have been demonstrated and isolated mitochondria from *Plasmodium yoelii* and *Plasmodium falciparum* were able to oxidize alpha glycerophosphate, succinate, proline and dihydroorotate by what appeared to be a classical electron transport system (MacRae 2013), there is no conclusive evidence of the role of a functional citric acid cycle in the malaria parasite. Some believe that the cycle exist to produce reducing equivalents feeding the respiratory chain necessary for parasite viability (Olszewski 2010). There is insufficient evidence for the presence of an oxidative energy-yielding pathway, and it is unlikely that in the malaria parasite, oxygen utilization is coupled with energy generation.

1.5.1. Haemoglobin processing and heme detoxification

The malaria parasite feeds voraciously on host hemoglobin, hydrolyzing globin to small peptides and detoxifying haem to hemozoin (Goldberg 1991). In fact, hemoglobin digestion is the major source of amino acids in the malaria parasite, an observation reflected in the similarity of the amino acid composition of parasite proteins on the whole to that of hemoglobin. Hemoglobin

is devoured by the parasite by simple endocytosis of the host cell stroma in the early stages, and later, through a specialized mouth-like cytostome. Endocytosed hemoglobin is acted upon by a host of proteases in the food vacuole – a specialized lysosome-like organelle (Goldberg 1991). The malaria parasite employs several plasmepsins (aspartate proteases), falcipains (cysteine proteases), falcilysin and aminopeptidases to accomplish the task of digesting host hemoglobin (Mungthin 1998). Free haem produced on digesting haemoglobin is detoxified by a non-enzymatic, lipid catalysed and physiochemical polymerization step to hemozoin, assisted by several histidine rich proteins (Pandey 2012). Oxidative stress, produced by haem-generated free radicals, is buffered by endocytosed host superoxide dismutase (Olliaro 1999) as well as by parasite reducing power – NADPH generated by the hexose monophosphate shunt pathway, superoxide dismutase, catalase, glutathione reductase and thioredoxin reductase (Agnandji 2012). The antimalarial chloroquine is thought to act by interfering with haemoglobin processing and detoxification as will be seen in the subsections.

1.5.2. Metabolism of purines, pyrimidines, folate and amino acids

The intra-erythrocytic asexual stages of *Plasmodium* just like red blood cells are not capable of de novo purine synthesis, and hence are completely dependent on salvage pathways for their purine requirements. However, purine transporters and enzymes for interconversion of purine bases and nucleosides are present in the parasite. Though uninfected red blood cells take up preformed purines, uptake into parasitized red blood cells is much more rapid (Quashie 2010). The purine salvage pathway enzymes present in the malaria parasite include adenine phosphoribosyltransferase, adenosine kinase, adenosine deaminase, purine nucleoside phosphorylase, hypoxanthine guanine phosphoribosyltransferase, Adenosine monophosphate (AMP) dehydrogenase, adenylosuccinate synthetase and adenylosuccinate lyase (Jayalakshmi 2002, Bulusu 2011). The major source of purine in vivo is hypoxanthine, which is generated from erythrocytic Inositol monophosphate (IMP) by the consecutive action of adenylosuccinate synthetase and adenylosuccinate lyase, (Bulusu 2011) giving rise to AMP and Fumarate. It is possible for *Plasmodium falciparum* to be cultured in vitro in the absence of purines suggesting that some of the purine requirement is supplied by the host red cell. Parasite growth is enhanced by the addition of hypoxanthine, and exogenously supplied hypoxanthine is incorporated into parasite DNA, thus making hypoxanthine a key molecule to monitor the growth of the malaria parasite (Downie 2008). Unlike mature erythrocytes which cannot synthesize pyrimidines de novo, the malaria parasite can synthesize pyrimidines de novo from glutamine, bicarbonate and aspartate, and genes coding for all the enzymes of the pathway are present in the genome (Gardner 2002). One of the key steps in pyrimidine synthesis, the conversion of dihydroorotate to orotate is catalysed by dihydroorotate

dehydrogenase and is unique in that it involves electron transport through ubiquinone (coenzyme Q) and cytochrome to molecular oxygen. Deoxyribonucleotides are formed via an anaerobic ribonucleoside diphosphate reductase which is linked to thioredoxin reductase via thioredoxin. Although the nucleic acid biosynthesis pathways have been studied in considerable detail and significant knowledge is available on several of the unique features no antimalarial of commercial use has so far emerged. Interestingly, however, an analogue of ubiquinone, atovaquone, has found application as an antimalarial. The site of action of this drug appears to be the cytochrome bc1 complex, the inhibition of which leads to the ultimate blockage of nucleic acid synthesis (Fry 1992). The de novo biosynthesis of pyrimidines also involves para-aminobenzoic acid and folate cofactors. While para-aminobenzoic acid (pABA) may be obtained from the host diet or synthesized from chorismate by pABA synthase, folate is synthesized from guanosine triphosphate (GTP) by the malaria parasite, and genes for all but one enzyme of this pathway have been identified (72, 86). The major drug targets in the folate pathway are dihydropteroate synthetase (PFDHPS) and dihydrofolate reductase (PFDHFR), as pABA is not indispensable for host cell viability (Salcedo-Sora 2011). The sulfa drugs such as sulphadoxine and dapsone used as antimalarials are analogues of pABA. Pyrimethamine and cycloguanil are potent inhibitors of PFDHFR. Though originally speculated to be an apicoplast process, chorismate is synthesized in the cytosol from erythrose-4-phosphate and phosphoenolpyruvate by the shikimate pathway, and parasite growth is inhibited by glyphosate, an inhibitor of 5-enopyruvyl shikimate 3-phosphate synthase (Ralph 2001). However, the shikimate pathway does not seem to play any role in the synthesis of the aromatic amino acids – tryptophan, tyrosine and phenylalanine in the malaria parasite, and pABA is probably the only molecule derived from chorismate. The only genes identified for enzymes involved in amino acid synthesis in the parasite are for those involved in interconversions of glycine-serine, proline-ornithine, aspartate-asparagine, glutamate-glutamine and cysteine-alanine (3) in accordance with the theory that the malaria parasite fulfils all its amino acid requirements through digestion of host hemoglobin.

The subcellular location of parasite metabolic products and proteins provides a context for the organisation of the intracellular parasite and possible protein-protein interactions during the parasite's development. An understanding of such a landscape can provide insights about regulatory mechanisms involved in protein trafficking, signal transduction, post-translational modifications and biosynthetic cooperation between organelles (Ginsburg H 2011). An update of the organisation of data from the parasite genome project put together into assemblies which cooperate to fulfil different cellular functions is provided in the following website: <http://sites.huji.ac.il/malaria/> (Ginsburg H 2011).

1.6. Antimalarial interventions

1.6.1. Antimalarial Drugs

Prevention of malaria was once the mainstay for the control of this disease. Prevention efforts were based on extensive use of the insecticide dihydrodiethyltrichloroethane (DDT) to control the vector larvae. Even recently, the WHO has not ruled out completely the use of DDT in controlling malaria in epidemic situations (van den Berg 2009). However, decreasing interest in this preventive strategy has due to increasing concerns about the persistence of DDT in the environment and its potential deleterious effects on humans consuming crops exposed to DDT due to its high liposolubility (Sadasivaiah 2007). Other alternatives, including pyrethroids have been used to impregnate bednets and in indoor residual spraying for malaria prevention (van den Berg 2009). Global attention turned to the use of drugs with antiparasitic properties.

1.6.2. Quinoline antimalarials

One of the first drugs to be used for malaria treatment is chloroquine (CQ). Chloroquine was introduced in 1944 -1945 and soon became the mainstay of therapy and prevention, since this drug was cheap, non-toxic, and active against all strains of malaria parasites. In 1994, CQ was the third most widely consumed drug in the world after aspirin and paracetamol (Foster 1994). The precise mode of action of the quinoline antimalarials is still not completely understood. The following hypotheses (as presented in Table 2 and their limitations have been proposed to explain the mode of action of CQ (Foley 1998):

Table 2: mechanisms of chloroquine action in malaria parasite and associated experimental support.

Mechanism	Experimental support	Limitation
Direct heme binding and inhibition of oxidative stress defense processes and free radical damage causing parasite death.	(94)	Does not explain reversal of CQ resistance.

Inhibition of vacuolar phospholipase thereby preventing degradation of endocytic vesicles in food vacuole and subsequent haemoglobin degradation.	(95)	CQ inhibition is irreversible as opposed to the inhibition of vacuolar phospholipase.
Inhibition of protein synthesis by inhibiting proteases involved in haemoglobin degradation	(78)	Protease inhibition is reversible process meanwhile CQ action is irreversible.
interaction with parasite DNA	(96)	The concentration required to produce inactivation of DNA is of several orders of magnitude greater than the inhibitory concentration. In addition, this model does not explain the selective toxicity of CQ to Plasmodium parasites.

From the review of evidence, it is likely that chloroquine inhibits parasite growth by a number of additive or synergistic effects that are difficult to reproduce in studies using parasite components. Nonetheless, it seems very likely that heme-chloroquine interactions play a very important role in the mechanism of chloroquine inhibition of the malaria parasite (Pukrittayakamee 2004, Sullivan 1996).

1.6.3. Sulphadoxine Pyrimethamine.

Sulphadoxine-pyrimethamine (S/P) is a fixed dose combination of two antifolates with the advantage of a single dose treatment. Sulfa drug-pyrimethamine combinations are highly active blood schizonticides against *P. falciparum* but are less effective against other Plasmodium species. This combination of antifolate drugs for the treatment of malaria is a synergistic mixture that acts against the parasite-specific enzymes, dihydropteroate synthetase (PFDHPS) and dihydrofolate reductase (PFDHFR). Sulphonamide drugs work by inhibiting para-aminobenzoic acid (pABA) formation, which is needed for the synthesis of tetrahydrofolate. Parasites that become

resistant to sulphonamides must bypass the metabolic step at which PABA is incorporated into dihydropterate. Tetrahydrofolate derivatives serve as donors of one-carbon compounds in a variety of essential biosynthetic pathways. Both the drug components are completely absorbed after oral administration, are highly protein bound, and reach peak plasma concentrations after 2–6 hr. The mean elimination half-life for sulfadoxine ranges between 123 and 195 hrs, and for pyrimethamine from 80-95hrs.

Adverse reactions are infrequent and mild during malaria treatment, mainly gastrointestinal upset, headache, and rarely itching. Severe adverse effects such as Erythema multiforme, Stevens Johnson syndrome and toxic epidermal necrolysis have been documented with SP used in prophylaxis (Roujeau 1995). The risk of severe skin reactions following treatments is unknown, but apparently substantially lower than during prophylaxis (100). Hepatic toxicity, haematological reactions and hypersensitivity reactions have been reported as well. The list of the possible sulphonamide related adverse effects are long (Taylor 2004).

Unfortunately, resistance is widespread in Asia, India, and now in Africa. It is cheap, practicable (only one dose is needed because of the slow elimination from the body), and currently efficient in many parts of Africa. However, it is poorly active against highly CQ-resistant strains (Bell 2004). In Africa, this drug is still presently in use for intermittent preventive treatment (IPTp) in pregnant women despite its resistance profile. One of the reasons is because so far, declining efficacy of this drug has been defined for children less than five years only and little is known about the effect of its usage in IPTp. Secondly, there appear to be no alternatives with the advantages associated with SP use namely; affordability, single dose treatment, tolerability, long prophylactic effect etc. Currently, there is increasing evidence for the usefulness of intermittent preventive use of SP (IPTi) in controlling malaria and malaria anaemia in healthy children (Griffin J. T. 2010). Lastly, the prophylactic effect of pyrimethamine induces some immunity to subsequent parasite challenge seems to make this drug valuable in preventive therapy(Friesen 2011). In high transmission endemic areas, the development of antitoxic immunity precedes the development of antiparasitic immunity, explaining the persistence of parasitemia without clinical symptoms. This residual parasites mass is responsible for continuous stimulation of the immune system to regulate the parasite load, but might significantly contribute to clinical anaemia and reduced cognitive ability (Sachs 2002). This appears to provide a rationale for the proposed use of SP in IPTi. The argument therefore is that IPTi will contribute greatly in reducing subclinical anaemia due to sub microscopic parasitemia.

In most parts of Africa and South East Asia, SP is replaced by more fast acting drugs for malaria treatment (Greenwood 2010). This is because of the development and rapid spread of

resistance against this drug. The most recent replacement include drug combinations with artemisinin to which we now turn.

1.6.4.Artemisinins.

Derived from the sweet wormwood plant called *Artemisia annua*, Artemisinin is found in parts of Asia including China. Its cultivation, harvesting and extraction are time consuming and labour-intensive, hence the formulation of its derivatives has been developed. This compound has been used extensively in China for the treatment of fevers. It is now recommended as the drug of choice in combination therapies for acute malaria infections worldwide (W.H.O 2010).

Artemisinin action has widely been attributed to the destruction of parasite proteins and lipids essential for its function (Meshnick 2002). This destruction is thought to be brought about by alkylation of parasite proteins by carbon centred free radicals liberated through the heme mediated oxidative cleavage of the endoperoxide bridge of the artemisinin molecule(Asawamahasakda 1994 1994). Formation of haem-artemisinin adducts has been found in experimental models (Robert) and has been proposed to form in vivo from the low transient concentration of free heme from haemoglobin degradation, although they may not be responsible for parasite killing (Rosenthal 1996). Artemisinins are also thought to be activated through reductive cleavage of the peroxide bond by intracellular iron-sulphur redox centres (Eastman R. T. 2009) common to plasmodium enzymes. This results in the production of alkylated proteins that cause parasite death. In support of this, artemisinin has been identified in many interacting proteins after incubating parasite lysates with radiolabelled artemisinin, and among the specific proteins thought to be targeted by artemisinin are the cysteine proteases (Fidock 2008), the translationally controlled tumour protein, the proteins of the electron transport chain and the SERCA Atpase6. Evidence for the latter comes from studies by Krishna and Co (Eckstein-Ludwig 2003) where they showed that Ca^{2+} -ATPase activity of PfATPase6 is inhibited by artemisinin with similar potency to thapsigargin (another sesquiterpene lactone and highly specific SERCA inhibitor), but not by quinine or CQ in a study with the enzyme's orthologue in *Xenopus leavis* oocytes. However, another report suggested a different mode of action of artemisinin, providing evidence of an early disruption of the parasite digestive vacuole with no effect on the mitochondria where pfatpase6 is located (Eckstein-Ludwig 2003). In both processes, iron seems to be a key element in the process of bio-activation. Its chelation by desferrioxamine abrogates the antiparasitic activity of artemisinins and correspondingly attenuates inhibition of PfATPase6 (Eckstein-Ludwig 2003). It appears therefore, that artemisinins seem to mediate parasite death by a variety of mechanisms involving significant pro-oxidative effects on diverse parasite functions essential for growth, some of which are unknown and need further investigation (Fidock 2008).

The artemisinins act very rapidly, reducing parasitemia by a factor of 10^4 with each replication cycle. Thus, for a parasite burden in the range of 10^{12} , only three cycles are required to abolish parasitemia (Baird 2005). Its broad spectrum of action on lifecycle stages including significant reduction in transmission stages makes the artemisinins a drug class of choice in malaria public health control policy (112 2008). However, due to its rapid elimination from the body, a complete daily treatment for seven days is required for complete cure. Few serious adverse effects have been reported in humans, mainly neutropenia, reduced reticulocyte count, neurotoxicity and anaemia (Meshnick 2002). The artemisinin derivatives appear therefore to be well tolerated. However, the challenge of treatment compliance makes reduced sensitivity and recrudescence a common occurrence (Ittarat 2003, Menard 2005), and hence it is strongly recommended to protect the drug by using it in a combination therapy with more slowly eliminating drug (Nosten 2007). Combination therapy entails the use of two drugs that have separate mechanisms of action on the same stage of the malaria parasite. Thus, the use of sulphadoxine and pyrimethamine cannot be considered as a drug combination. Combination therapy employs the use of two old drugs, an old drug and a new one, or two new drugs against the same form of the parasite. ACT is a combination of a rapidly eliminated artemisinin derivative and a slowly eliminated partner drug, typically an aminoquinoline. Artemisinin and its derivatives artesunate, artemether and dihydroartemisinin are very active drugs that are capable of reducing parasite biomass up to 10 000 folds per asexual cycle. This is faster than any other antimalaria drug. In addition, they are gametocidal, resulting in reduction of transmission potential (Price 1996). Because of their rapid activity and short half-life, they are less prone to favour the development of resistance. They have often been referred to as the magic bullet for malaria control in recent days. In combination therapy, they act by reducing the bulk of parasite biomass leaving relatively few parasites for the partner drug to eliminate (Figure 2). Consequently, the residual parasites meet a high concentration of the partner drug than if it were used in a monotherapy. In this way, parasites less sensitive to the partner drug are eliminated (White 1999). Likewise, the partner drug protects the artemisinin from resistance development by clearing the rest of the parasites and preventing sub-therapeutic exposure. Consequently, ACTs can be used in areas where resistance to partner drug has already developed. However, the development of drug resistance to the partner drug can be expected to be greater in areas of high transmission than in areas of low transmission where re-infecting parasites are more exposed to waning concentrations of the partner drug still lingering in the system when the artemisinin drug is eliminated. Five artemisinin-based combination therapies are recommended by WHO at present: artesunate-mefloquine, artesunate-sulfadoxine-

pyrimethamine, artesunate-amodiaquine, artemether and lumefantrine dihydroartemisinin-piperaquine (W.H.O 2010).

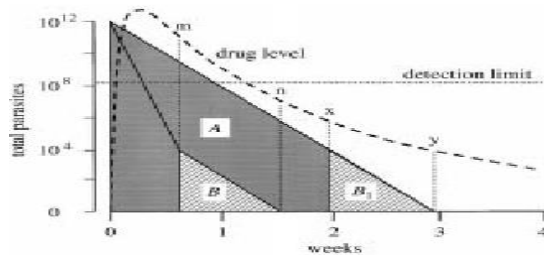


Figure 3: the effect of adding an artemisinin to a more slow acting drug in the treatment of uncomplicated malaria

The grey triangle represents parasites normally exposed to monotherapy. When the fast acting artemisinin is added, the number of parasites exposed decreases is greatly reduced leaving only a small proportion of total parasites to be eliminated by a high concentration of the slow acting partner drug. In this way, the chances of sub-therapeutic exposure is limited, preventing the development of resistance. Source (White 1999).

1.7. Emergence and spread of resistance to antimalarials

Drug resistance is a recurrent theme in the history of infectious disease control. In the case of malaria, resistance to all but one of the five major classes of drugs is widespread. Attributable drug resistant malaria is a major deterrent to the successful control of malaria, and its spread accounts for increased infant mortality and morbidity in endemic areas recently (Roper 2003). Antimalarial drug resistance is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject. This drug must be able to gain full entrance into its site of action for the duration of time necessary for its action (Wernsdorfer 1991).

Many factors explain the development and spread of drug resistance. These factors relate to the parasite, the vector, the drug, the environment and the host. The interplay between these factors makes the interpretation of the in vivo drug resistance picture a complex one, and assessment of drug resistance a complicated subject requiring consideration of many parameters some of which are still not yet fully understood (H. Wernsdorfer 1991). In brief, the spread of resistance would depend on the following:

- Relative fitness and transmission potential of resistant forms compared to sensitive ones
- The intensity of transmission
- The level of immunity of the population
- The pattern of antimalarial drug use.

In the following paragraphs, factors relating to the parasite, the drug and the host shall be considered.

1.7.1.Parasite based factors.

Natural populations of *P. falciparum* consist of mixtures of parasite strains with different characteristics, one of which is their differences in drug response. Random mutations in natural parasite populations occur at a very low frequency of the order of 10^{-8} to 10^{-10} . They are also lost at a similar frequency, showing a fair degree of stability and sensitivity in the absence of drug pressure. Sub-critical drug pressure will favour the selective disappearance of some parasite strains to the advantage of others (Wernsdorfer 1994). Given that drug resistance in parasites is genetically based, strong drug selection pressure will favour the emergence of resistant forms. Many determinants of this selection pressure exist among which are poor treatment adherence, long half-life of the drug (parasite populations are more exposed to lingering sub lethal drug concentrations of slowly eliminated drugs), counterfeit medication, treatment dosing, malabsorption etc. Drug resistance in the malaria parasite is genetically determined and so point mutations in drug metabolizing genes as primarily responsible for resistance mechanisms leading to drug failures. The parasite exists in the blood stage as a haploid organism, having just a single copy of each gene. One consequence of this is that there is genomic plasticity. In this stage of the parasite, no corresponding gene copy exists to compensate for genetic changes that may occur in the other copy of the gene to maintain stability of the phenotype resulting from change on one copy of the gene. As such, mutations in drug metabolizing genes easily translate into the resistance phenotype. Depending on the rate of mutation, these mutations can rapidly accumulate and contribute to the development of full blown drug resistance. White (White 1999) showed that at very high initial parasite biomass, and assuming a random distribution of mutant parasites, a non-immune person with high parasite density is more likely to harbour resistant parasites than another with a low density (Figure 3) This model explaining the influence of initial parasite density and selection of resistant parasites was demonstrated in one study (Ittarat W 2003). The authors evaluated the risk of development of resistance to artesunate in Thailand and demonstrated that patients with greater than 10,000parasites/ μ had a 9-fold risk of developing recrudescence to ar-

tesunate compared to those with low parasitemia. Initial parasite biomass, therefore critically determines the sensitivity of the parasites and hence favour resistance development (Ittarat W 2003).

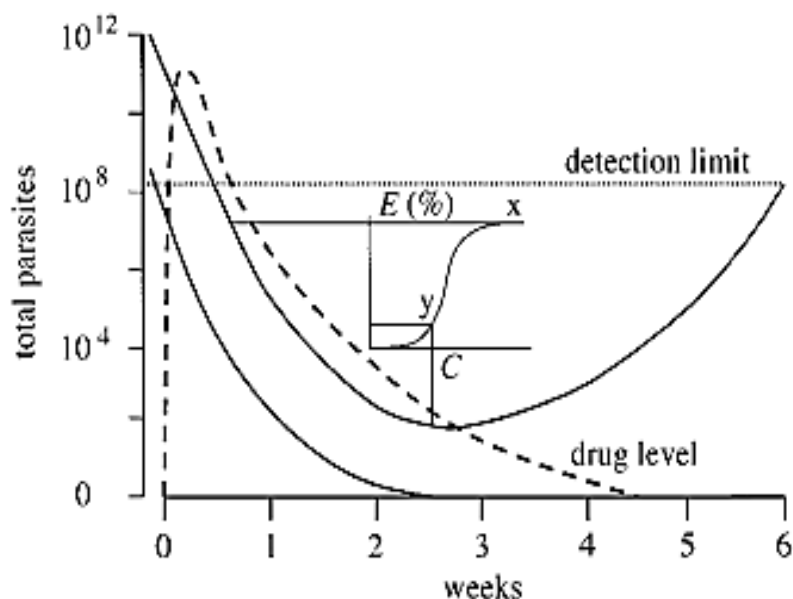


Figure 4 the effect of initial parasite density on treatment response

Figure 4: The effect of initial parasite density on treatment response.

In the upper panel starting with a high initial parasite load in an adult, the drug level, shown by dotted lines fall below the concentration required to provide maximum inhibition. As the days go by, the rate of reduction of parasites reduces with reducing drug concentration until such a point where this rate begins to increase gain in the negative direction. At this point, the drug concentration is sub-therapeutic favouring the development of resistant forms. On the other hand, at a lower starting parasite load, the same drug level is sufficient to clear the residuum. Source: (White 1999)

The existence of multiple parasite clones in a single infection may result in different drug resistance scenarios. In a single clone infection, gametes resulting from mosquito ingestion will self-fertilise giving rise to genetically identical clones and therefore no genetic reassortment. On the other hand if multiple clones, there is the possibility of reassortment following fertilisation between clones which might create, breakdown or reinforce gene combinations required for drug resistance to occur (Hastings 2000). Therefore, genetic diversity of parasites is a key epidemiological parameter in the understanding of the dynamics of drug resistance.

1.7.2. Drug based factors

Increasing attention is now given to the role of pharmacokinetics in determining antimalarial efficacy and in promoting the emergence and spread of drug resistance leading to the creation

of a network on monitoring antimalarial drug resistance on a world wide scale (Sibley 2008). In the past, drug levels were rarely measured, so that all episodes of clinical treatment failure were thought to be due to inherent parasite resistance. In fact, if therapeutic drug levels are not achieved, clinical outcomes are an inaccurate reflection of drug efficacy and parasite susceptibility. This has become especially clear with the use of lumefantrine, a compound that has highly variable absorption which is determined by fat consumption. Low lumefantrine level, given in a seven days therapy regime, was shown to be an independent risk factor for recurrent parasitemia (Travassos 2009). Because pharmacokinetic analysis of drugs is beyond the scope of standard drug efficacy trials, assessment of the drug level at day seven has been proposed as a key data point to determine if therapeutic concentrations were achieved in the blood (Djimdé 2009). This will offer important information about the contribution of pharmacokinetic factors to the observed clinical efficacy of a drug. In dose finding studies, the dose usually selected is the lowest dose that achieves pharmacological efficacy while minimizing toxicity. Often, such therapeutic doses have little effect on moderately susceptible parasites compared to fully susceptible ones and can thus contribute to the development and spread of resistance. Chloroquine resistance was found to be rare in Guinea Bissau between 1990 and 2005 for this reason (Ursing 2009) despite that the therapeutic dose in use is twice the dose administered against malaria in neighbouring countries. While this seems to be a natural experiment, there has been no evaluation of the adverse effects linked to the high dose treatment regimen; or the causal relationship between high and low dose CQ administration and spread of CQ resistance. Counterfeit drugs may also play a role in drug resistance. These drugs may contain much less than the labelled amount of active drug or none at all. In a recent study on the quality of artemisinin drugs in Laos (Sengaloundeth 2009), the contents of tablets labelled as artesunate and other artemisinin derivatives included antimicrobials, non-artemisinin antimalarials, antipyretics and even carcinogenic compounds. The potential for the contribution of fake drugs in the development of drug resistance cannot be overestimated. While artemisinin based combinations (ACT) are being advocated for by international health organizations for the effective fight against drug resistant malaria, one research on the quality control assessment of artemisinin drugs in Africa from China reports a daunting 50 million sub-therapeutic doses of artemisinin drugs. Against such a background, it is to the interest of African nations to develop and implement appropriate functional regulatory mechanisms and institute malaria drug efficacy surveillance for drugs destined for public consumption. This will contribute to curb the most feared clinical resistance to artemisinin combinations.

Host based factors.

Host based factors affecting the emergence and spread of drug resistance in malaria can be classified in to two broad categories namely: Genetic determinants and immunological determinants. A drug's pharmacokinetic profile (i.e., absorption, distribution, metabolism, and excretion) can differ substantially between individuals. Drug-metabolising enzymes (e.g., cytochrome P450 enzymes [CYPs]) and transport proteins (e.g., P-glycoprotein) play an important role in breakdown, intestinal absorption, distribution, and renal or hepatic excretion of drugs (Gil 2007). Genetic polymorphisms in the genes coding for these enzymes affect the activity of the enzymes in the metabolism of drugs. These alterations translate into individual differences in the way they metabolise drugs in their system. Individuals have thus been grouped in to three main phenotypes viz. fast metabolisers, slow metabolisers, and intermediate metabolisers. Patients that are fast metabolisers will metabolise the drug more extensively, resulting in lower plasma concentration and, consequently, inadequate drug effect. Patients that are poor metabolisers, by contrast, will metabolise the drug less extensively, resulting in higher plasma concentrations that might lead to drug toxicity. In addition, poor metabolisers might experience treatment failure when given pro-drugs that need bioactivation.

Genetic polymorphisms observed in the CYPs and transport proteins are responsible for these idiosyncratic responses to therapy. A common example is the metabolism of the antimalarial amodiaquine (AQ). This drug is used as one component in ACTs recommended against uncomplicated malaria in Africa. It is metabolized principally to N-desethylamodiaquine (DEAQ). Though not as potent as the parent drug, its clearance from the plasma is slower. Incubating amodiaquine with human liver microsomes and recombinant cells expressing the most common CYP2C8 gene polymorphisms (Li 2002), CYP2C8*2 and CYP2C8*3, have shown a 50% decrease in the metabolic activity of CYP2C8*2, and an 85% decrease in the activity of CYP2C8*3, compared with CYP2C8 wild type (CYP2C8*1). The consequence of this decreased metabolism is the lingering of sub-therapeutic concentration of DEAQ and increased exposure to residual parasites especially in high transmission areas where reinfection is frequent. Two clinical studies involving 275 patients from Burkina Faso and 103 children from Ghana, however, found no evidence of a CYP2C8 genotype influencing the efficacy of amodiaquine (Parikh 2007) (Adjei 2008). This lack of association can be attributed at the present time to high efficacy of artemisinin based combinations. It is difficult to observe the influence of these CYP2C8 genotypes on clinical outcomes. Increased exposure genotypes might also predispose some individuals to adverse drug events (Gil 2007).

1.8.Mechanisms of drug resistance

1.8.1.Sulphadoxine-pyrimethamine (SP).

SP is particularly prone to rapid emergence of resistance. The mechanism of resistance has been shown to be due to mutations in the genes of *pfdhfr* (Cowman 1995) (Cowman 2012) and *pfdhps*. De novo folate synthesis is essential to parasite survival. The antifolate medications interrupt this process by targeting two enzymes: pyrimethamine and proguanil target dihydrofolate reductase (*pfdhfr*) and sulfa drugs such as sulfadoxine target dihydropteroate synthase (*pfdhps*) (Nzila A. M. 2000). Resistance to antifolate drugs is the result of the accumulation of mutations in *pfdhfr* and *pfdhps*. Non-synonymous nucleotide mutations occur in a step-wise fashion leading to increasing parasite resistance to antifolate drugs (Foote 1994) commencing with the codon108N mutation in *pfdhfr* followed by subsequent mutations at 50R, 51I, 59R and 164L. The order in which mutations occur is likely due to changes in enzymatic activity with each additional mutation, although the data are not clear. The highest levels of antifolate resistance are found in Southeast Asia and South America. In these two regions, a polymorphism at *pfdhfr* residue 164 is almost always found. In contrast, *pfdhfr* I164L has not spread through sub-Saharan Africa, despite extensive use of the drug (Cortese J. F. 2002, Contreras 2002, Travassos 2009). A report indicated the emergence of this mutant in Kenya, occurring in parasites that already possessed mutations at codons 51, 59 and 208 of the *pfdhfr* gene (Kiara). As indicated above, the accrued mutations in the dihydrofolate reductase (*pfdhfr*) gene (triple mutant 51I, 59R, 108N) reduces the efficacy of pyrimethamine and two mutations in the dihydropteroate synthase (*pfdhps*) gene (double mutant 437G + 540E) decreasing the efficacy of sulfadoxine in sub-Saharan Africa (140). Acquisition of a quintuple mutant parasite with all five mutations is associated with an increased risk of failure after treatment with SP (Dorsey 2004). However, as will be seen later, resistance profile of parasites to SP is not uniform across Africa. In Central and West Africa, the 540E mutation is rarely found as opposed to Eastern and southern Africa. However, the association between the prevalence of in vivo treatment failure and the prevalence of the accrued mutations is difficult to establish due to interferences from host and environmental factors (Francis 2006).

1.8.2.Aminoquinolines

- Chloroquine and Amodiaquine:

The production of a genetic cross between a chloroquine sensitive *Plasmodium falciparum* clone from Honduras (HB3) and a chloroquine resistant clone from Indochina (Dd2) provided a genetic approach to study the resistance mechanism. The determinant of CQ sensitivity was shown to reside in the *Pfcr* gene, a 13 exon gene mapped to the 36kb segment on chromosome 7

of the parasite genome. Mutations in the *Pfcr*t gene and CQR phenotype are perfectly associated in the genetic cross (Fidock 2000). Of note, the mutation (Lysine to Threonine) at position 76 is diagnostic and has been shown to be a marker for chloroquine resistance in field isolates (Djimde 2001). This mutation is thought to deplete a positive charge from the first transmembrane segment of the *Pfcr*t protein (Figure 5). The result is the elimination of the electropotential across the membrane and the efflux of CQ from the digestive vacuole (Cooper 2002) thereby conferring a survival advantage to the parasite.

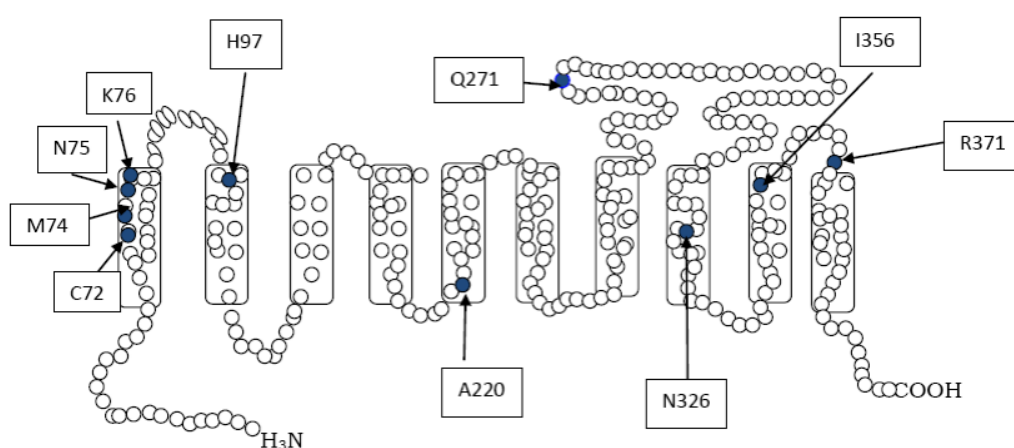


Figure 5 transect model of the *pfcr*t Protein showing the Locations where Mutation occur in the Protein

The arrows show where mutations have been observed with this protein. Of note, the K76 mutation is particularly important as it has been shown to widely affect sensitivity to chloroquine.

1.8.3. Artemisinin combinations

Resistance to ACTs was once believed to be impossible, given the short half-life of the drug and its rapid action in clearing parasites. In the African setting where malaria is a major problem, the efficacy of ACTs remains high (Greenwood 2010). However, selection of reinfections after treatment with Artemether lumefantrine (Sisowath 2009, Hapji 2009) and artesunate-amodiaquine (Holmgren 2007) has been reported indicating tolerance is developing to the partner drug. As early as in 2004, reinfections and recrudescence have been reported with artesunate-mefloquine, indicating resistance and/or tolerance to both drugs is developing (Ittarat 2003). The decreasing efficacy was thought to be linked to the already decreased sensitivity to mefloquine in the region. Other studies have reported declining efficacies of artesunate-mefloquine in the Thai-Cambodian border and Thai-Myanmar border considered hotspots of drug resistance development (Fidock 2008). Although treatment failures in these cases are attributed to declining meflo-

quine sensitivity, artemisinin based component is believed to have a role in this waning efficacy as shown in increased parasite clearance times. A number of recent observations support this role. Elevated 50% inhibitory concentration (IC₅₀) values were recorded with drug assays from French Guiana (Jambou 2005). Stable artemisinin resistance was acquired by selection under drug pressure (Afonso 2006). Recrudescence parasites from a clinical efficacy study with artesunate monotherapy in Central African Republic were shown to have elevated IC₅₀ values to dihydroartemisinin (Menard 2005). After Artesunate monotherapy in Cambodia, some patients were suggested to harbour resistant parasites. In this study, resistant parasites were defined as parasites in the presence of adequate plasma drug concentration, reduced in vitro sensitivity to dihydroartemisinin, and prolonged time to parasite clearance (Noedl 2008). Summarily, these studies show that resistance to ACTs is possible and will develop upon selection of resistant parasites under artemisinin drug pressure. With these observations, an understanding of the molecular mechanisms of resistance as well as the antioxidant defense machinery of the parasite is important to design ways of circumventing resistance to this important class of antimalarials. Without this and in the event of full-blown resistance with ACTs, the public health consequences would be dramatic especially in Africa. Such an understanding will also lead to the development of new ways of targeting the parasite.

1.9. Assessment of drug efficacy

The understanding of the phenomenon of drug resistance in malaria is an important phenomenon in malaria control. This is because a high rate of resistance renders the drug clinically useless and jeopardizes malaria case management and public health efforts including the potential for epidemics of mutant strains. Therefore, it is important to be able to detect circulating drug resistant parasites, to describe their distribution and pattern of spread. This informs policy on better strategies to implement to curb potential malaria disasters in communities. To this effect, the search for markers of drug resistance has been one serious object of research in malaria. Different approaches are usually used to describe the epidemiology of drug resistant malaria. Each approach is based on some defined marker allowing the description of resistance status of circulating parasites. The approaches currently used include the evaluation of clinical and parasitological response to drug treatment in malaria patients, drug sensitivity assays and molecular markers.

1.9.1. Evaluation of clinical and parasitological response in malaria patients.

This approach, also known as in vivo drug tests involves the follow up of patients in a drug trial for 14 to 28 days after drug intake and to measure the effects of the drug clinically and parasitologically (W.H.O 2003). The treatment response measured as clearance of parasites and fever

without recurrence of same parasites within the study period gives an evaluation of the clinical efficacy of the drug under test. However, though this is a method of choice as it reveals the actual drug efficacy in the host, it suffers from a number of drawbacks. Firstly, therapeutic success or failure does not necessarily correspond to resistance or sensitivity to the drug. This is because many confounding factors may interfere with the assessment of drug response. These include the host immunity which may play an important role in fever and parasite clearance, previous drug intake, pharmacokinetic variations, unreported self-medication during trial period etc. Furthermore, the use of combination therapy renders the assessment of drug resistance to each component of the drug difficult if recrudescing parasites are detected during trial.

1.9.2. In vitro drug sensitivity assays.

As its name implies, this approach consist of assessing the sensitivity of the parasite isolated from infected patients in vitro. The susceptibility of the parasites to the drug is studied quantitatively. There is the potential to study individual drugs within a drug combination as well as the possibilities to study cross resistance and synergy/antagonism between different drugs. This sensitivity assay also provides a way of studying drugs that are already failing and so provides an early warning sign on the usefulness of the drug in the long term. However, in vitro sensitivity tests do not represent the actual host conditions. Furthermore variations in parasite strain genetics during laboratory adaptation are possible making the parasites not to be representative of the actual parasite population. There is no standard protocol for field evaluations and the inexistence of clear universally accepted thresholds defining resistance and sensitive parasites makes this approach particularly challenging and of limited utility. The World Wide Antimalarial Drug Resistance Network is developing a series of standard tools that can be used to evaluate such factors that contribute to the emergence and spread of drug resistance (140).

1.9.3. Molecular markers of drug resistance.

Changes in the DNA of parasites in response to stress imposed by the drug can be assessed. Since the sexual parasite is haploid in the blood of malaria patients, these genetic changes that occur easily manifests in the phenotype. As stated above, most of the changes, notably genetic polymorphisms are associated with the development of strategies of survival. Molecular markers for drug resistant malaria are based on genetic changes that confer parasite resistance to drugs used to treat and prevent malaria. They therefore involve genes that are implicated in drug uptake, transport or metabolism. These markers were first identified by inspecting homologues of genes that confer drug resistance to other organisms and analysing the progeny of genetic crosses between sensitive and resistant parasites (143). Sequence variations, single nucleotide polymorphisms, copy number variations etc. were described from these analyses as well as their relation-

ship with the drug resistance phenotype. A few of these markers and their effect on parasite sensitivity to drugs shall be summarised below.

1.9.4. Markers of resistance to SP.

The dihydrofolate reductase and the dihydropteroate synthase genes are principally involved in SP resistance. Resistance in SP is accrued in a step-wise fashion with three mutations in the dihydrofolate reductase (*pf dhfr*) gene (triple mutant 108N + 51I + 59R) reducing the efficacy of pyrimethamine and two mutations in the dihydropteroate synthase (*pf dhps*) gene (double mutant 437G + 540E) decreasing the efficacy of sulfadoxine. Acquisition of the quintuple mutant parasite with all five mutations is associated with an increased risk of failure after treatment with SP. Therefore, tracking of the pattern of spread of various combinations of the mutations at different areas and correlation with treatment efficacy gives reliable information on the drug resistance status of circulating parasites in the region. Other markers exist which have not been shown to particularly predict sensitivity to fansidar. They can be found expressed as either mutations or haplotypes in Annexe 6.

1.9.5. Markers of resistance to chloroquine and amodiaquine.

Genetic polymorphisms in the *Pf crt* and *pf mdr 1* genes are associated with resistance to CQ or AQ. Of note, the K76T mutation in the *Pf crt* gene modifies the coded protein and modulates transport of the drug across the parasite vacuolar membrane. The mutated gene has been associated with reduced susceptibility of the parasites to CQ and increased prevalence of this marker has been observed where CQ sensitivity is reduced (144). This marker however, is thought to be enough to confer *in vivo* resistance to CQ. Mutations in the P-glycoprotein homologue *pgh* and multidrug resistance gene *pf mdr 1* (codons 86, 1034, 1042, 1246), have also been associated with reduced sensitivity of the parasite to Amodiaquine (143). It appears that a combination of these mutations/distinct mechanisms contribute to modulation of AQ resistance *in vivo* (Echeverry 2007). Polymorphisms in *pf mdr 1* and amplification of the gene (copy number variations) has also been implicated in drug resistance in AQ and cross resistance to structurally unrelated anti-malarials like lumefantrine, mefloquine and artesunate. Other markers of resistance include the gene coding for the multidrug resistance associated protein (*pf mpr* codons 1466, 1876). Also located in the plasma membrane of parasites, this protein has been shown to be involved with extrusion of drugs from the parasite contributing to resistance. Therefore tracking mutations in this gene can be useful in resistance monitoring.

1.9.6. Markers of resistance to artemisinin (ACTs).

Currently, the use and high efficacy of artemisinin based combination therapy for management of malaria does not downplay the rationale for the search for molecular markers in tracking

drug resistance in the ACTs. ACTs were designed to kill parasites by selectively targeting two different mechanisms. Currently, the WHO treatment guidelines for malaria recommend the use of ACTs containing SP, AQ and Mefloquine and Piperaquine. Different ACT combinations are adopted for use depending on the level of resistance to these partner drugs in different countries (W.H.O 2010). Therefore, the drug pressure with ACTs will still select for resistance to partner drugs, thereby jeopardising the integrity of the ACT in simultaneously clearing parasites. The current search for molecular markers of artemisinin resistance has led to the determination of a number of genes putatively implicated in the mechanism of action of Artemisinins. These mutations (codons 263, 769, 431, 623, 630, 683) are located in the *pfatpase 6*, an orthologue of the mammalian sarcoendoplasmic reticulum calcium dependent Atpase, and great genetic diversity in parasites carrying these mutations have been observed in field isolates from Africa and Asia (Dahlstrom S 2008). The protein coded by this gene is thought to be located in the vacuole membrane involved in transport of artemisinin into the vacuole of the parasite. However, most of the polymorphisms identified have not been independently confirmed to be associated with reduced susceptibility of the parasites to artemisinins in Africa or in Asia, (155) except codons 769 associated with artemether sensitivity and codon 263 (149, 156). Both wild type alleles (codons 263, 623, 769) and mutant (431K) parasites have been observed in Cameroonian parasite isolates in 2005 but none of them was associated with decreased sensitivity to artesunate or to impaired clinical response (157). On the contrary, gene amplifications in the *pfmdr-1* gene appear to be linked to artemisinin sensitivity. In one study, the authors found that paired isolates obtained at recrudescence during treatment with artesunate-mefloquine showed significant increase in *pfmdr-1* gene copy number and increased fever and parasite clearance times (155). More recently, increased parasite clearance time has been the most informative marker of resistance development in artemisinin therapy(O' Brien 2011). Treatment of *P. falciparum* parasites within the Thai Cambodian border has increasingly been associated with increased tolerance of the drugs by the parasites, and more interestingly when these parasites show no significant decrease in in vitro drug susceptibility (Dondorp 2009), (13, 160). Furthermore, it was realised that parasite clearance times is highly heritable. The heritability of this clearance time was parasite dependent and independent of the host or their relatedness (Anderson T. J. 2010). This finding lends support to the genetic basis of parasite clearance times in *P. falciparum* susceptibility to artemisinins. In addition, dormancy was suggested as one other mechanism for parasite survival following toxic concentrations of artemisinins drugs (LaCrue), suggesting genetic implications in drug metabolism associated with parasite survival. Indeed, Mok et al., (Mok 2011) employed whole genome analysis through DNA microarrays to profile the physiological gene expression patterns of isolates of

P. falciparum from patients failing therapy. They observed a general downregulation of metabolism in the ring stage, an increased protein expression during the schizont stage, which may counteract oxidative damage due to drug action. Among the genes found and currently explored for relationship with resistance to artemisinin is the heat shock protein 70, a key binding partner of the heat shock protein 86 of *P. falciparum*. Variations in this gene as well as in other potential candidate genes were found to be associated with reduced parasite clearance rates in patients treated with artemisinin combinations in the Thai-Cambodia and Thai-Burmese border (Takala-Harrison 2013). Recently, fine resolution mapping and genetic studies using isolates from Thai-Cambodia border have led to the identification of the most reliable gene associated with resistance to artemisinins known as PF3D7_1343700 Kelch propeller domain (K-13 propeller) (164). Indeed, evidence has been generated from in vitro studies, in vivo clearance rate studies and from the field. Of note, mutant K-propeller alleles cluster in areas in Cambodia where resistance has been defined, and the presence of dominant mutant alleles of the K-propeller gene has shown strong correlations with the spread of resistance in West Cambodia (164).

Tracking the geographic spread as well as changes in the distribution of drug resistant parasites over time is one of the best advantages of the use of molecular markers (Roper 2014). Though many protocols for polymerase chain reaction exist, consensus in the interpretation of molecular data in relation to drug resistance has been reached in certain cases. Two examples of the use of molecular markers in drug resistant studies are worth mentioning. The recovery of CQ sensitivity after it was discontinued following high prevalence of treatment failure associated with K76T mutation in Malawi was demonstrated recently; and shown to be a re-expansion of wild type parasites (5). Twelve years down the line, the prevalence of this mutation was very low, indicating that resistant parasites were replaced with sensitive parasites, but which disappeared because of its less fitness cost compared to wild type parasites. This finding points to the possibility of re-introducing of CQ for the treatment of malaria in Malawi. Indeed, the paper shows high in vivo efficacy with CQ and low prevalence of K76T mutant parasites. A second application of the use of molecular markers is illustrated in one study (166) in which authors described the geographic dispersal and evolutionary origins of *pfdhps* mutations by examining the diversity at microsatellite markers flanking the *pfdhps* gene. The rapid spread of drug resistant mutations influences the frequency of flanking microsatellite sequences. This is possible because recombination events for the spread of mutations occur when microsatellites are far apart, such that close microsatellites remain in linkage disequilibrium with the resistant allele, and thus can be transmitted and spread from particular regions. The microsatellites flanking resistant loci were used to characterise lineages of common ancestry that have been subject to recent selection. The

outcome of this haplotype variation study clearly demonstrated that resistant pfdhps haplotypes in Africa have independent origins with major differences between East and West African parasite types translating into differing drug sensitivities. Of note, the K540E mutation, defining the SGE haplotype, prevalent in East Africa is absent in West Africa and Cameroonian isolates as opposed to the mutant 437G defining the SGK haplotype. This means that monitoring the K540E mutation or the quintuple mutant parasites may not be important in tracking or predicting drug resistant malaria in Cameroon at the present time, but presents a simple and effective way of demonstrating the spread of high grade SP resistance across Africa from its East African focus (Naidoo 2010). In addition, evaluating its development would provide information on the pressure of SP within the community and give indication about the continuous viability in both treatment and chemoprophylaxis. The use of molecular markers in these two studies provides examples of the importance of molecular makers in malaria control (Talisuna 2012).

1.10. Fever as a stress modulator in malaria infection

Fever is an evolutionarily conserved response within the animal kingdom. With few exceptions, most fishes, amphibians, reptiles and mammals respond to challenge with microorganisms or pyrogens with fever, leading to the suggestion that fever is an adaptive response (Romanovsky 1998). On the other hand, the febrile response has also been associated with adverse effects on the host. The pyrogenic cytokines involved in the induction of fever are central mediators of the clinical manifestations of gram-negative bacterial sepsis and have generated intense interest in the clinical application of antagonists of these cytokines. Thus, the febrile response can be both beneficial as well as harmful. Fever is the most striking clinical feature of malaria and an invariable feature of infection in non-immune individuals. Fever may be accompanied by other symptoms such as nausea, headache, vomiting and joint and abdominal pain. However, the malarial paroxysm of chills, shivering and high fever followed by sweating are the clinical hallmarks of malaria infection. Several studies have investigated the effect of febrile temperatures on the parasite. Exposure of synchronized parasite cultures to heat shock at 39°C or 41°C has been shown to inhibit parasite growth (Oakley 2007). Interestingly, schizonts are most vulnerable to heat shock while trophozoites, though not as severely affected as schizonts, are also killed. Rings, on the other hand, are less affected by heat shock (Kumar 2003). It has also been demonstrated that initially asynchronous cultures exposed to heat shock at 40°C on alternate days become synchronized, presumably due to the disruption of the latter (trophozoite and schizont) half of the intra-erythrocytic cycle (Kwiatkowski 1997). This study also showed that once synchronized, the parasites grew well in spite of periodic exposure to febrile temperatures.

1.10.1. Biological role of fever

Although several studies have examined the effect of fever on the parasite as well as host, the biological role of fever remains unclear. It is known that malarial fever is linked to the intra-erythrocytic growth of the parasite (Oakley M. S. 2011). The idea that fever is associated with the release of parasite toxins during schizont rupture is not new (Kwiatkowski 1997). It was later proposed that the fever and other clinical symptoms of malaria may be a result of a host response rather than the parasite toxins themselves. The rupture of the schizont during merozoite release results in the release of a large amount of parasite antigens and erythrocyte debris that causes fever by inducing secretion of endogenous pyrogens. Some of these pyrogens are IL-1 β , TNF α , complement factor 5a, IL-6 etc. These pyrogens especially TNF α , are produced by host monocytes and macrophages through a toll like receptor (TLR2, TLR9) dependent activation of myd88 mediated pathway. Interestingly, the clinical features of acute malaria and bacterial endotoxaemia are similar. Both involve induction of cytokines and other soluble mediators by bacterial or malarial toxins (Clark I. A. 2003). Of the endogenous pyrogens, TNF α , has been identified as a key player in the induction of malarial fever (Kwiatkowski 1997). In addition, Gambian children who received anti-TNF α antibody in addition to conventional anti-malarial treatment show faster fever clearance than control children who did not receive the anti-TNF α , antibody (174, 175). These results suggest that TNF α , is a critical mediator of malarial fever. This study also showed that once synchronized, the parasites grew well in spite of periodic exposure to febrile temperatures. A mathematical model based on the above findings suggests that synchronization resulting from febrile temperatures causes the periodic fever characteristic of malaria (Kwiatkowski 1990). There is evidence also that suggests that fever, along with other TNF α , induced mechanisms, may function to stabilize parasite population density within the host (Kwiatkowski 1997). However, it cannot be concluded that fever exerts a solely deleterious effect on parasite growth. Analysis of the effect of febrile temperatures on the cytoadherence properties of infected erythrocytes suggests that fever accelerates and increases cytoadherence of infected erythrocytes to CD36 and ICAM-1, accompanied by an increase in temperature (Udomsangpetch 2002). Thus, fever may also prove to be beneficial to parasite growth and development. Although the pharmacological value of inhibiting fever in the treatment of malaria has not been demonstrated, lowering of body temperature has become a common method of treating malaria (112). Anti-pyretic drugs, tepid sponging and cooling blankets are commonly used to reduce fever. Paracetamol has been shown to slow parasite clearance in a randomized trial with falciparum malaria afflicted children (Brandts 1997). This effect has been attributed to impairment of host defense mechanisms resulting in decreased production of TNF α and oxygen radicals and has therefore been considered

harmful. However, for children with little immunity to malaria or people living in non-endemic areas, fevers have been shown to have an additional beneficial effect, to the extent that high temperatures lower the level of parasite density before the host develops immunity to the infection (Oakley M S 2011). To conclude, the biological effects of fever on the parasite as well as host responses are an exciting area of research with the potential to contribute to understanding of host parasite relationships and novel approaches to control of infection. At present, the effect of fever on parasite growth and antimalarial treatment outcome remains unclear because both host genetic components and level of anti-malarial immunity are potential confounders. In this thesis, attention shall be given to host factors that may influence infection and treatment outcome in an attempt to correlate the role of this factor and immune responses in the clearance of infections with *Plasmodium falciparum*.

1.11.Host responses to malaria Infection.

1.11.1.Immune Mechanisms in Malaria

The evolution of the immune system consists of factors that provide innate and acquired immunity, and has evolved to become more specific, complex, efficient, and regulated. One of the principal functions of the human immune system is to defend against infecting and other foreign agents by distinguishing self from non-self (foreign antigens) and to organize other protective responses from leukocytes. A deregulated immune system can react to self-antigens resulting into autoimmune diseases or failure to defend against infections (Riley 2013). Much of the pathology associated with parasitic infections such as malaria, is immune-mediated. The immune mediators (e.g. cytokines) of protection can also cause disease, and the outcome of infection hinges on a delicate balance between appropriate and inappropriate induction of these mediators. Immune cells such as dendritic cells and macrophages act as antigen presenting cells in malaria infection sensing and internalizing and processing antigen peptides for presentation to specifically T lymphocyte helper (T_H) cells. The outer membrane of dendritic cells contain innate receptors, known as toll like receptors(TLRs) that sense microbes and microbial products or pathogen associated molecular patterns(PAMPs) and trigger dendritic cell maturation and production of cytokines through signal transduction pathways (Figure 7). Thus, they effectively bridge the innate and adaptive immune responses. Binding of PAMPs to these pathogen recognition receptors (PRRs) cause the activation of a nuclear factor κB (NF- κB) and subsequent gene expression and production of proinflammatory cytokines such as IL-12. The main actors therefore are the T cells, which not only perform functions directly related to parasite killing but activate other cells, notable B cells to produce antibodies essential for parasite clearance. They also form a panel of memory cells which are necessary during subsequent challenge with the disease. Figure 6 pre-

sents the major events leading to immune activation mechanism associated with killing of intracellular pathogens. Interaction of specific T cell receptors with parasite derived peptides associated with MHC class 1 molecules on antigen presenting cells will fully activate T cells in the presence of co-stimulatory molecules like CD28 and members of the B7 family. Depending on the cytokine microenvironment, T cells can polarize into TH1 or TH2 response profiles. In malaria infection, TH1 responses appears to come in early acute infection while TH2 response develops slowly (Luty 1999); to activate mechanisms related to parasite clearance through antibody dependent cellular cytotoxicity (Jafarshad 2007). TH1 responses are essentially pro-inflammatory with the production of IL-12 and IFN- γ which has a direct effect on parasitized cells. Besides this, TH1 response protects by inducing the production of nitric oxide which generates stress within the infected erythrocyte leading to parasite death. Nitric Oxide has also been shown to favour the proliferation of CD8+ cells required for clearance of liver stage parasites (Hafalla 2006). IFN- γ has an activating effect on macrophages, leading to the production of reactive oxygen and nitrogen species, nitric oxide and tumour necrosis factor alpha.

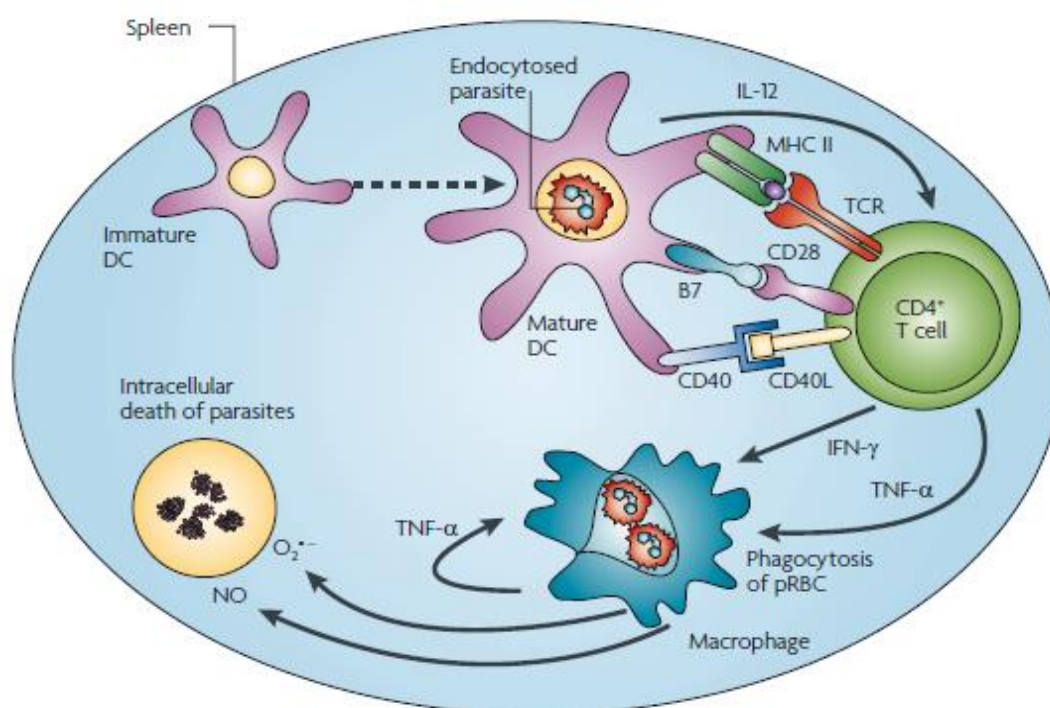


Figure 6 Principal pathways of immune activation and intracellular pathogen killing.

Tumour necrosis factor= alpha on the other hand can induce the production of reactive nitrogen intermediates by neutrophils and kupffer cells and hepatocytes(Gyan 1994). These reactive species are potent stressors causing not just parasite death but also pathology to the host. Hence, immune responses must be tightly regulated for a favourable outcome after a malaria in-

fection. To this regard, a subset of T cells known as regulatory T cells (Tregs) characterized by expression of $CD4+CD25+FOXP3^+$ on the cell surface plays an important regulatory function. In a study assessing the role of Tregs in development of severe malaria in mice, $CD4+$ producing T cells in which CD25 was found to be depleted significantly reversed the production of Th1 type responses in infected mice, and was significantly associated with higher levels of IL-10; indicating that regulatory T cells may affect the level of Th1 response in malaria and influence the pathophysiology of the disease (Wu 2010). This is thought to represent a parasite specific virulence factor (Walther 2005). Therefore, the expression of pro-inflammatory responses will contribute to the clearance of parasites, while unrestricted pro-inflammatory responses will lead to severe immunopathology and IL-10 and TGF- β are associated with this regulatory process.

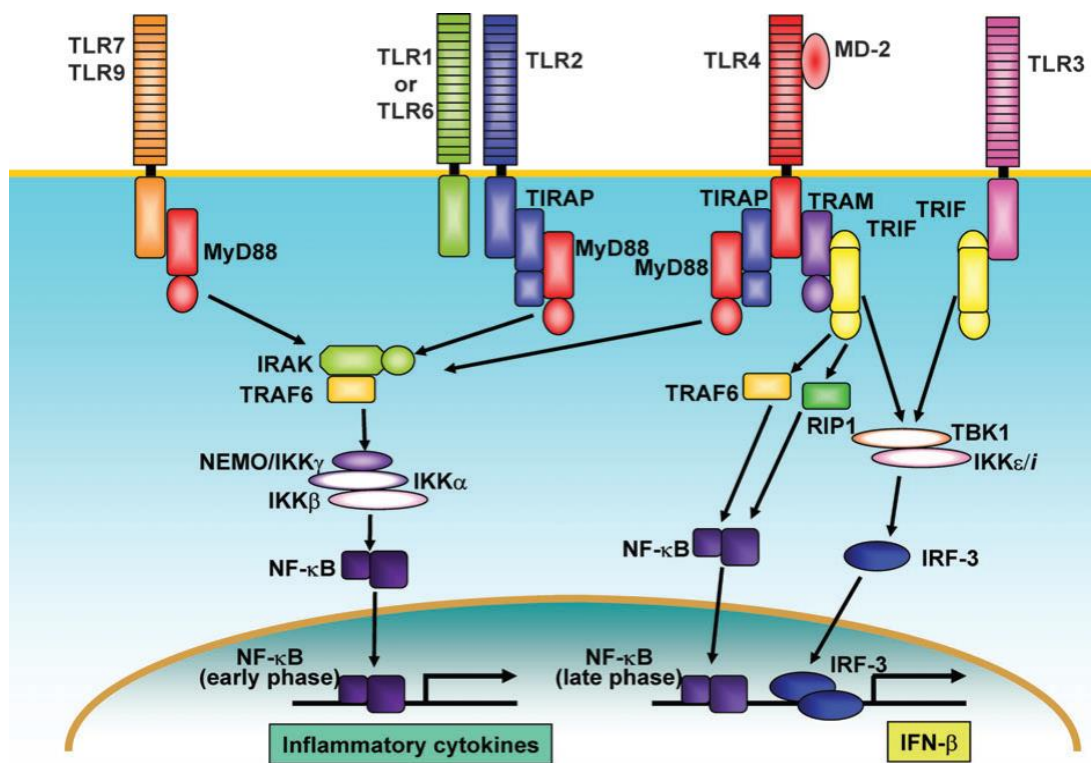


Figure 7 A presentations of the various cell Surface sensors known as pathogen recognition receptors and molecular mechanisms.

Here, the pathogen recognition receptors otherwise here known as the toll like receptors and the downstream mechanisms each toll like receptor provokes are outlined in the above figure. Nuclear Factor kappa B, a nuclear transcription factor seems to be a major intermediate in the expression of immune genes. The binding of adaptors (MyD88, TIRAP, TRAM and TRIF) to cytoplasmic domains of receptors recruits factors like IRAK, TRAF6, leading to the

activation of kinases like IKK which now phosphorylates NFκ-B. Translocation of this phosphorylated NFκ-B to the nucleus induces expression of immune genes associated with the inflammatory process. One such cytokine so expressed is TNF-α (188).

Besides the effect of this cytokine in the activation of DCs, it also effectively polarizes undifferentiated T-helper cell (T_{H0}) repertoire into T_{H1} development. This highlights the protective effects of IFN-γ and IL-12 in clearance of blood stage infections. IFN-γ on the other hand has been shown to mediate class switching to the protective cytophilic immunoglobulin subclass G1 and G3. IFN-γ is also implicated in the modulation of the function of the macrophage to mediate parasite development through antibody dependent cellular inhibition and the production of anti-parasitic molecules like nitric oxide with direct anti-parasitic activity. Also present in the dendritic cell surface is a fatty acid translocase known as CD36, which is a receptor that binds to the Plasmodium falciparum ligand erythrocyte membrane protein 1 (PfEMP1) present in infected erythrocyte surface. Thus, CD36 mediates opsonin-independent phagocytosis of parasite infected erythrocytes (Patel 2004). In humans, immune response to malaria is complex and directed against several targets that span the whole cycle of the malaria parasite as represented in Figure 8 below.

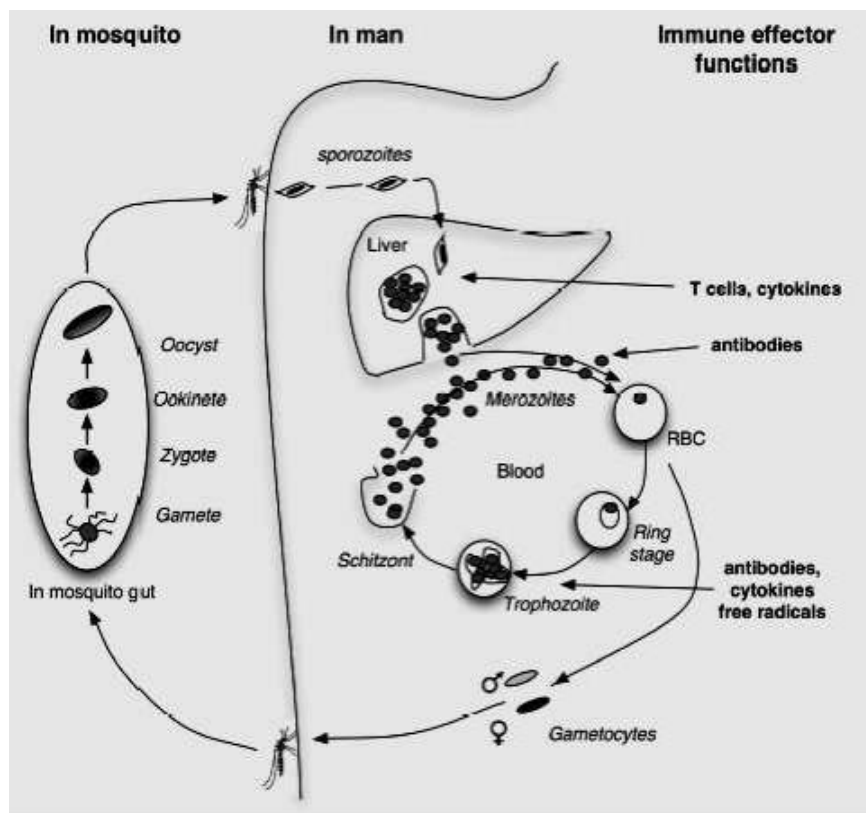


Figure 8 Targets of immune response in malaria.

The malaria parasite enters the human body as a sporozoite that lodges in the liver cells. Here, they are targets of CD8+ T-cells and cytokines IFN- γ . When merozoites are released from liver schizont rupture into the blood stream, they encounter an immune defense made of antibodies against antigens like the merozoite surface proteins. When they infect red blood cells, infected cells are targets of both CD4+ and CD8+ cytotoxic cells, cytokines and free radicals produced from oxidative stress response. These targets are shown in the above figure as straight arrows from right to left beginning from the effector mechanisms also indicated.

1.11.2. Innate Immune Responses to Malaria.

Although acquired immune responses to malaria are principally involved in immunity to malaria, the critical role of innate immune responses is increasingly being recognized. As far back as the Second World War, malaria parasites were used to experimentally treat people with neurosyphilis (Chernin 1984). This study showed that parasitemia at which parasite growth was controlled was predictable between the first and second infections and between different species of parasite. This observation was supported by a longitudinal study carried out in Papua New Guinea among children who were semi immune. The observation of a density-dependent regulation of parasitemia around a threshold independent of genotyping suggested the involvement of host specific mechanisms controlling parasite density (Bruce 2003). The fact that severe malaria is rare among people over ten years in malaria endemic countries and the steady decrease in prevalence of parasitemia after two years old is indicative of a protective immune response that developed much faster than immunity against pyrogens and parasites themselves. With the plasmodium parasite developing within the host hepatocyte being the major target of protective immunity at the exo-erythrocytic stage, the body's immune defence mechanism develops against several liver-stage specific antigens, which along with those brought in with the invading sporozoite, are rapidly processed by the host cell and presented on the surface of infected hepatocytes in combination with MHC class 1. Macrophages, including Kupffer cells, are antigen-presenting cells and antigen presentation leads to recognition by cytotoxic T lymphocytes (CTLs) and killing of the infected cell or stimulation of NK and CD4+ T cells to produce IFN- γ , which can trigger a cascade of immune reactions, eventually leading to death of intracellular parasite. On the other hand, the CTLs may directly be cytolytic to malaria-infected hepatocytes by releasing perforin and granzyme or by binding to apoptosis-inducing receptors on the infected cell. Possible mechanisms involve the production of pro-inflammatory factors acting directly to limit parasite growth. Among these pro-inflammatory cytokines we note IFN- γ , TNF- α , IL-18 etc. produced by PBMCs as observed in naïve individuals (Kwiatkowski 1997). Among the innate cells responsible for the production of these powerful inflammatory molecules are the NK-cells, and gamma

delta T-cells as shown by Artavanis-Tsakonas and Riley in an in vitro study investigating the induction of pro-inflammatory cytokines in PBMCs after challenge with live parasites (192). Gamma delta T-cells ($\gamma\delta$ T) cells are thought to participate by their ability to produce regulatory cytokines but also IFN- γ . However, other effector mechanisms involved in this response remain to be deciphered as well as the factors that affect their efficiency if we have to exploit this to design rational immune based interventions against malaria.

1.11.3. Acquired immune response against malaria infection

Many adults living in endemic zones carry the malaria parasites without accompanying clinical symptoms (193). This situation seems to be a trade-off between the pressure exerted by the parasite on the host immune system and vice versa. The existence of such asymptomatic malaria defines the phenomenon of non-sterile immunity to malaria. This immunity is principally acquired with repeated infections. Acquired immune response against malaria involves the implication of both arms of the immune system through a variety of mechanisms as shown in Figure 9.

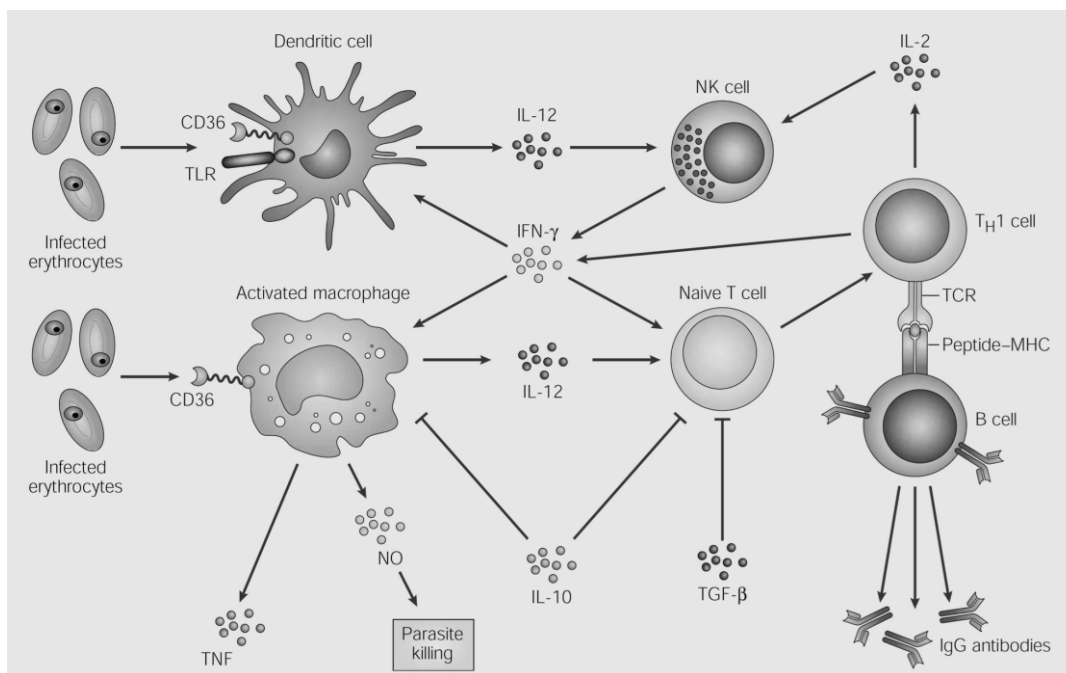


Figure 9 bridging the innate and acquired immune systems.

In the figure above, infected erythrocytes activate the immune system through binding with CD36 or TLRs on macrophage or dendritic cell surface. Among the plethora of molecular immune responses after migration of DCs to the spleen is the production of IL-12 which activates NK cells to produce IFN- γ , which in turn induces differentiation of naive T cells and enhance parasite derived maturation signal. This results in clonal expansion of CD4+ T cells specific for the parasite antigen. IL-2 produced by Th1 cells activates the production of IFN γ by NK cells activating DCs and macrophages. The latter cells produce NO and TNF which can directly kill parasite cells. IL-12 thus mediates the binding of Th1 differentiated cell surface via

CD28 and other co-stimulatory molecules to B cells which can result in the production of antigen-specific antibodies. Thus, the bridge between the innate and the adaptive immune systems is enabled by the DCs and the cytokines they produce notably IL-12(Stevenson M M 2004).

The passive transfer of sera from adults to naïve children to treat malaria was among the first indications of the involvement of humoral immunity in natural protection against malaria. That antibodies primarily mediate immunity to malaria is based on the principle that if antibodies prevent merozoites from invading red blood cells, this would prevent ensuing pathology or clinical symptoms at least. The initial attachment of the merozoite to the erythrocyte is mediated by antigens known as merozoite surface proteins (MSP) and there are nine of them. To successfully invade, the MSP 1 molecule needs to be extensively processed into a 19kDa carboxyl terminus attached to the merozoite. This is followed by re-orientation of the merozoite until the apical end is attached to the erythrocyte. This is aided by the apical membrane protein (AMA-1) after undergoing proteolysis (Tonkin 2006). The formation of a tight junction between the merozoite and the erythrocyte is facilitated by the release of a battery of parasite proteins including the erythrocyte binding antigen (Chitnis 2012) . All these invasion related proteins are targets for antibody mediated effector mechanisms given the relatively short time of exposure to the immune system. This effector mechanism could involve opsonisation and subsequent phagocytosis; complement mediated damage or blocking of critical antigen binding sites on erythrocytes.

In principle, antibody responses against the plethora of parasite antigens should result in protection against clinical malaria. For example, high levels of cytophilic antibodies (IgG1 & IgG3) are present in the serum of malaria exposed individuals and correlate with protection against the disease (Tangteerawatana 2007). However, this is not usually the case, as some antibody production may not always be associated with protection (Fowkes 2010). The presence of lingering non- protective antibodies could only represent an indicator of malaria exposure history rather than a protective immune response (Corran 2007). The conflicting results from such seroepidemiological studies highlight the difficulties in understanding correlates of protective immunity mediated by antibodies in natural settings. It also highlights a genetic regulatory component in development of immunity to malaria.

1.11.4.Genetic regulation of host-parasite responses

In areas endemic with malaria therefore, a co-evolution between the host and malaria parasite has contributed on the one hand in the extensive parasite genetic variability resulting from selective host immune pressure; and on the other hand in the parasite selection and expression of genetic polymorphisms at high frequency in immune genes associated with natural susceptibility or resistance to malaria (Modiano 2001). These host genetic polymorphisms are likely to predis-

pose distinct populations to unique malaria immune response patterns. Among the host genetic factors contributing to this phenomenon are genetic disorders of the blood like the haemoglobinopathies including HbS, HbC, alpha and beta thalassaemia; metabolic disorders like Glucose-6-phosphate dehydrogenase deficiency etc.; immune response genes affecting different lifecycle stages of the malaria parasite including TNF α , Mannose Binding Lectin (MBL), Fc receptors, induced nitric oxide synthase 2 (iNOS2) complement receptor 1(CR1), MHC products, adhesion molecules etc. This thesis shall deal with a review of some of the important factors and dwell a trifle on specific gene polymorphisms on immune regulatory factors and their influence on malaria outcome.

1.12. The haemoglobinopathies.

The haemoglobin molecule, made up of two alpha and two beta globin chains in the adult. Haemoglobinopathies result from structural changes encoded by amino acid polymorphisms as follows: Hb C (Glu/Lys change at position 6 in the β -globin chain), HbS Glu/Val change at position 6 of the β -globin chain), HbE (Glu/Lys change at position 26 of the β -globin chain). These disorders could result in defects in the shape of the red blood cell (Ovalocytosis) or the structure of the haemoglobin molecule (HbS, HbE and HbC) in the red blood cell or in the rate at which this haemoglobin molecule is synthesized (The Thalassaemias). Disorders in the blood coded by genetic factors have been observed to confer resistance to malaria. The frequency of occurrence of the various genotypic variants differ in different settings and prevalent in Africa where malaria is a day to day problem. Frequencies can reach up to 15 % for carriers of HbS while for HbC, it can be up to 13% (Mockenhaupt 2004). These conditions create a microenvironment difficult for the thriving of malaria parasites conferring a degree of resistance to malaria. In this context, the genetic disease in the homozygotes caused by these disorders is thought to be the price of acquisition of resistance to malaria in individuals (Verra 2007). Different mechanisms have been proposed by which these genetic variants confer immunity to malaria. In a large case control study in Burkina Faso in 2001, both HbC and HbS affected the early development of naturally acquired immunity against malaria than HbC or HbS (Verra 2007). The authors found higher immune responses against variant surface antigen and various malaria antigens in a low transmission urban area whereas no differences were detected in a high transmission rural area. In both contexts the response against tetanus toxoid was not influenced by the β -globin genotype. The enhanced immune reactivity in both HbC and HbS carriers supports the hypothesis that the protection against malaria of these genotypes might be at least partially mediated by acquired immunity against malaria. This mediation may involve reduced cytoadhesion and rosetting resulting from a reduced cytoadherence of the PfEMP 1 on endothelial vasculature. This is thought to be due to an

abnormal display of the PfEMP 1 on the surface of erythrocytes of HbS and HbC carriers (204). A second hypothesis relates to a complementary mechanism that involves inhibition of parasite development. Evidence for this hypothesis came from an in vitro study by Freidman et al., (1979) showing it may not accurately represent the situation in vivo where other factors might play a significant role. Evidence of enhanced phagocytosis of ring infected erythrocytes has been reported (Ayi 2004) highlighting that a definite mechanism by which the two haemoglobin variants contribute to enhanced immunity against malaria is still wanting despite epidemiological evidence of protection. A recent evidence points to the observation that the abnormal display of parasite adhesive molecules on the surface of HbS and HbC infected erythrocytes alters their binding properties, forcing them to the surface accelerating splenic clearance (Mangano 2015). Table 3 below gives a summary of the other blood disorders and corresponding resistance it confers to malaria.

Table 3: Blood Disorders and role in malaria protection

Trait	Protection	Reference
HbAS	50% protection from mild malaria, 75% protection against admission to hospital with malaria and 90% protection against severe malaria disease	(207)
Hb C	29% reduction in incidence of clinical malaria in heterozygotes	(200)
Alpha Thalassaemia	60% protection from severe malaria for homozygote and 34% for heterozygotes	(201)

1.12.1. Enzymopathies

Defects in metabolic enzymes in the erythrocytes coded by genetic factors have also been implicated in resistance to malaria. The classical case is that of Glucose-6-phosphate dehydrogenase (G6PD) deficiency. G6PD is the first and key enzyme required in the pentose phosphate pathway. This metabolic pathway is largely responsible for the production of reducing power (NADPH) necessary for the generation of reduced glutathione, a critical factor in the control of cellular oxidative stress. Two mutations in the A-variant (A376G Asn/Asp and G202A Val/Met)

of G6PD implicated in this variation process leads to 10-50% reduction in enzyme activity. Evidence for the association of this enzyme deficiency and protection to severe forms of malaria comes from a large epidemiological study carried out in East and West Africa where haemizygous males (deficient for enzyme) and heterozygous females (mosaic alleles) were protected from developing severe malaria (Ruwende 1995). In a more recent study in Mali the authors found an association between the haemizygous males and protection to severe malaria but not heterozygous females (209). The geographical distribution of this X-linked trait in malarious areas suggests further association with malaria resistance. Studies in G6PD deficient erythrocytes have shown that mechanism of protection is mediated by increased oxidative stress in cells leading to inhibition of parasite growth and enhanced clearance of parasitic erythrocytes by macrophages (Lopez 2010). One such explanation is that accelerated oxidative membrane damage resulting from impaired antioxidant defence renders the cell prone to early phagocytosis.

1.12.2.Erythrocyte Polymorphisms

Even though studies on the interaction between the malaria parasite and the red blood cell has led to the discovery of genetic polymorphisms underlying the phenotypes observed, other genetic events in different systems are also important in defining the resistance phenotype observed or possibly the outcome of infections due to drug resistant malaria. Studies on the influence of the ABO blood grouping and susceptibility to malaria have shown that there is a predominance of blood group O individuals in malaria endemic regions, and that the presence of this blood group is associated with enhanced phagocytic clearance of parasitized erythrocytes by macrophages (Wolofsky 2012), as opposed to blood group A or B. By contrast, increased binding between PfEMP1 adhesins and blood group A and B erythrocytes as opposed to blood group O have been observed; a phenomenon which facilitates resetting, a feature associated with severe malaria (Vigan-Womas 2012).

1.12.3.Histocompatibility leucocyte antigens (HLA)

A human leucocyte class I antigen (HLA-Bw53) and an HLA class II haplotype (DRB1*1302-DQB1*0501), common in West Africans but rare elsewhere, were found to be independently associated with protection from severe malaria (Hill 1992). However, variable findings on the association of disease outcome and genetic polymorphisms on this histocompatibility leucocyte antigen exist. These findings generally relate to the geographical setting. For example reduced risk of malarial anaemia is observed in The Gambia in association with DRB1*1302 by Hill (Hill 2006), and protection against severe malaria is associated with a different MHC class II allele, DRB1*0101, in Kenya. These differences may reflect parasite variations or selection involving other diseases acting in the same population (215). The observation that no protective

single gene or allele has been found suggests that protection or susceptibility to infection and disease might be polyallelic with some alleles acting dominantly while others having only a modifier effect (Plebanski 2002).

1.12.4. Adhesion molecules

The presence of variants of genes encoding adhesion molecules, such as ICAM-1 and CD36, affect the outcome of malarial infections. These molecules affect endothelial binding, and thus vascular occlusion, by infected erythrocytes. These molecules are also involved in the regulation of immunity; for example ICAM-1 is expressed on activated endothelial cells, dendritic cells, and lymphocytes and is preferentially upregulated on memory T cells. ICAM-1 binding to infected erythrocytes exhibits parasite strain specificity, and one host variant, ICAM-1^{Kilifi}, is associated with susceptibility to cerebral malaria in West Africa (Fernandez-Reyes 1997). An SNP in exon 6 of this gene was shown recently to be associated with the risk of severe malaria in a combined analysis in endemic and non-endemic populations Sinha (Sinha 2008). A polymorphism in exon 10 of the CD36 gene was shown to confer protection against severe anaemia in heterozygotes in Kenya, by reducing parasite sequestration (Pain 2001). Other evidence suggests that interaction of CD36 on dendritic cells with specific *P. falciparum* strains leads to defects in dendritic cell maturation (Britta 2001). It appears, therefore, that polymorphisms in CD36 and ICAM 1 affecting the priming of immune responses during infection, as well as parasite sequestration, may influence the outcome of malaria.

1.12.5. Nitric Oxide synthase (NOS).

Although the protective immune responses against malaria parasite are multifactorial, and the final effector molecules that mediate parasite death are not known, NOS and Nitric Oxide (NO) are implicated in this process. NO is induced by IFN- γ and TNF α , which are themselves part of the TH1 response profile and induced by NK cells (192). Nitric Oxide also seems to inhibit both the liver and blood forms of malaria parasites and it has been suggested to play an important role in the early non-specific responses to infection (Hobbs 2002), (220). Furthermore, its antiproliferative role at high concentration on TH1 cells, suggests that this biological substance is able to regulate its production in vivo by preventing over-expression of Th1 and CD8+ T-cells. Polymorphisms in the inducible nitric oxide synthase gene (NOS2) promoter region have been associated with the clinical outcome of malaria. Hobbs (Hobbs 2002) established the protective association between the -1173 C \rightarrow T NOS2 promoter polymorphism in two cohorts of populations living in areas with different malaria endemicity and distinct patterns of severe malaria infection. By examining the distribution of the SNP and [CCTTT]_n repeat in the population, they concluded on the influence of these polymorphisms on the severity of malaria. They proposed a

mechanism for the effect of the -1173 and -954 polymorphism and malaria outcome that is described in annexe 9.

1.13. Genetic regulation of immune response to malaria by cytokines.

Cytokines are mediators of cell communication in immune response. There is some evidence to show that the random distribution of allele frequencies throughout the human genome follows diverse ethnic and/or racial trends (Martin 2003). The frequency of sequence variations can differ by race and ethnicity and this variation may be associated with a difference in risk for disease between these groups. Inter-racial studies of immune function suggest that there are differences between African Americans and Caucasians in leukocyte subsets (222) as well as in the expression of co-stimulatory molecules on the surface of lymphocytes. Furthermore, up to 30% of healthy Caucasians have a constitutively low natural killer cell count, which is likely a result of polymorphic genetic variants.

That susceptibility to malaria is further controlled by unknown genetic factors resulted from large epidemiological surveys conducted in Burkina Faso and Mali. In the study in Burkina Faso, (Modiano 1995) showed that the Fulani have higher antibody titres against several malaria antigens than their neighbours, the Mossi and the Rimaibé, despite similar malaria infection exposure indices including socio-cultural circumstances. Furthermore, they observed that the Fulani have lower parasite rates and densities and fewer malaria fever attacks compared to the Mossi and the Rimaibé. In this study, the authors concluded that this observation should be most likely due to a more efficient immune response (224, 225) as they excluded the involvement of classical malaria resistance genes present at lower frequency among the Fulanis. The study in Mali further compound these observations where higher levels of IgG and IgE against crude malaria antigens, higher spleen rate, lower parasite rate, and lower prevalence of clinical malaria were observed among the Fulani compared to the Dogon, a genetically distinct tribe living in sympatry with the Fulani in this country (Dolo 2005). Thus the series of observations lead to the conclusion that humans have developed ways of coping with the selective pressure exerted by the malaria parasites differentially.

A further step to decipher the signatures of immune protection led to the identification of other regulatory regions. Polymorphisms in cytokine genes within the 5q31-q33 region could be playing a regulatory role in defining susceptibility to malaria (227, 228). As shown in the Malian study above, one functional basis for this difference in susceptibilities is the differences in antibody response. Th2 responses resulting in the production and class switching of IgM/IgG to IgE and antigen presentation is regulated by serum levels of the anti-inflammatory cytokine IL-4 (Tangteerawatana 2007). An SNP upstream the transcription start site (IL-4-524) of the IL-4 gene

affected promoter activity as reported in one study (Borish 1996) and activation of IL-4 producing T cell subsets was associated with high levels of total serum IgE. Typing this SNP among the Fulani in Burkina Faso demonstrated an association with serum anti-CSP and anti-PF332 antibodies in the Fulani but not among the Rimaibé (231). Although the IL-4-254T allele was twice as frequent among the Fulani indicating a protective haplotype, further studies with larger sample size and in other settings are needed to confirm these results and draw a more conclusive statement on the functional role of this allelic variant on susceptibility to malaria.

1.13.1. Variations in the human genome and approaches to identify host response molecular signatures.

Variation in the genes associated with metabolism, transport, targets, target partners, and immunological pathways has a very important role to play in the outcome of malaria infection or treatment as we have started showing above. Many approaches have been developed to discover and test the association and the functional influence of these variations in the outcome of malaria infection. There are many strategies for exploiting DNA markers in mapping and characterizing disease susceptibility loci that influence variation in quantitative traits. These methods depend on the design of the study and the proposed disease transmission model. However, there are a few basic concepts that are common to all disease mapping analysis strategies. These fundamental concepts bear on the need to correlate some measure of genotypic similarity at a particular locus or loci with a measure of phenotypic similarity among related or population-based individuals. If such a correlation exists, then it is possible that variation at the said locus, or another locus nearby, influences susceptibility to disease or variation in the phenotype under study. One of the major tasks in human genome analysis is the identification and typing of DNA sequence variations. Different types of sequence variations in the human genome exist. One type comprises sequences with variations in the number of repeat units such as short tandem repeat polymorphisms in the form of di-, tri-, and tetranucleotide repeats; more complex sequence repeats such as variable number tandem repeats; or variations in the lengths of mononucleotide tracks such as A- or T-tracks in the genome. The other major type of variation in the genome arises from discrete changes in a specific DNA sequence such as small but unique base insertions or deletions, or more frequently as single nucleotide substitutions, also known as single nucleotide polymorphisms (SNPs). SNPs are the most abundant form of DNA sequence variation in the human genome. Based on their natural frequency and presence in both coding and non-coding regions, single nucleotide substitutions are probably the underlying cause of most phenotypic differences among humans (232).

1.13.2.Linkage analysis

Although linkage tests for co-segregation of disease or trait with a locus assuming a model that explains the inheritance pattern between related individuals. Linkage is the method of choice for simple Mendelian traits. Among the approaches examined in linkage studies are gene mapping by linkage, Allelic Association analysis, Family based association analysis, etc.

1.13.3.Genome wide association studies (GWAS).

Of potential interest in the identification of novel gene signals associated with disease outcomes, genome wide association studies are an unbiased scanning of the genome to identify susceptibility factors that enable a better biological understanding of phenotypes. They build directly on the need to map patterns of inheritance to the most common form of genomic variation, known as the single nucleotide polymorphism. It is known that SNPs are transmitted across generations in blocks, allowing a few tagged SNPs to capture a great majority of SNPs within each block. Genome wide scanning can be used in many designs as reviewed by (Manolio 2010). There exist many advantages of GWAS among which we can cite the following

1. Multiple response phenotypes are often collected within the same study, such as efficacy and adverse events, allowing a broader dissection of trait genetics in a single study
2. GWAS provide a context for understanding the relative importance of genetic factors contributing to particular outcomes. This may be unavailable through other approaches.

These advantages are based on the unique property of GWAS to identify novel variation signatures that may have significant effects on the evolution of a particular phenotype. However, the approach is not as simplistic as it is put due to some challenges. Firstly, GWAS studies analyses thousands of SNPs and tests of associations increasing the possibility of spurious associations. To avoid this, GWAS thresholds of statistical significance are stringent enabling large sample sizes, making the process also expensive to run. Another approach used is to utilize a tiered design, where a subset of significant SNPs identified in a discovery study is analysed in a larger sample population (replication set). This helps to exclude false positive associations. Even then, it will be important to evaluate the effect of population sub-structuring to more clearly see effects in population clusters where deviations from Hardy –Weinberg Equilibrium exist (Eid 2010). The limitations of GWAs include the availability of low density sequencing platforms excluding the detection of minority variants contributing to phenotype. Furthermore, structural variations like copy number variations, epigenetic mechanisms, gene-gene interactions, gene-environment interactions that all confound signal or signal strength cannot be assessed.

1.13.4.Candidate gene association studies.

The Candidate gene approach as opposed to genome wide scanning selects relevant genes on the basis of their function and biological plausibility that allows a priori hypothesis about their etiologic role in disease. Evidence must be supported from in vitro models or in vivo studies on humans presenting the conditions. These studies could be expression studies, gene mapping exercise, or protein assays that describe links with the phenotype. The principal advantage of this approach is that it increases the a priori probability of finding a biologically relevant association. Several examples exist of the use of candidate gene approach. One limitation of this approach is the sample size considerations required to demonstrate strong associations (Sedgwick 2012). It has been suggested that the conservative Bonferroni principle be used to overcome such limitations to avoid reporting spurious associations. In addition, because multiple input variables are compared with response variables in genomic analysis studies, statistical models such as traditional regression analysis and multidimensionality reduction(MDR) are used to handle such associations (Mahachie John 2011). The advantage of MDR over regression is the limited power of the latter in variable selection due to magnitude of combinatorial variable selection and detection of interaction within the multiple testing platforms.

1.14.Molecular methods associated with genome typing research

The identification of SNPs in human genes will play an increasingly important role in analysing genotype-phenotype correlations within and among human populations. Amplification of genomic DNA by the polymerase chain reaction (PCR) has greatly simplified the identification of SNPs by eliminating the need to clone and isolate regions of the genome from multiple individuals. Many approaches to find SNPs rely on first amplifying a specific region of the genome from several different individuals using PCR, and then comparing the properties or sequences of the amplified products to identify SNPs.

Given their relative importance in medicine, many different methods have been developed to identify these SNPs. Among them we have the hybridization to oligonucleotide arrays, heteroduplex analysis, denaturing gradient analysis, direct sequencing, etc. Among them, we have automated sequencing platforms based on mass spectrometry time-of-flights (Marks 2004) of different allele variants improved today to platforms like the Sequenom iPlex genotyping platform. The following figure summarises some of the principles of different methods currently used in SNP typing studies to assess genome wide variations and their association with disease susceptibility.

The panel below illustrates the detection of the A allele of an A to G transition. Upper left panel shows allele specific primer extension. Two primers anneal to their target sequence adja-

cent to the SNP. If a primer is complementary to the 3' end of the allelic variant, the DNA polymerase extends the primer. If there is no match, the primer is not extended. The upper right panel shows single nucleotide primer extension. A primer annealing to its target sequence is immediately extended by one nucleotide that is complementary to the nucleotide at the site of the SNP. The identity of the nucleotide by which the primer becomes extended defines the genotype. In the panel below (left), short allele specific oligonucleotides are used, usually complementary to the SNP containing allele in the middle position. The polymerization is carried out under conditions where only perfectly matched probe-target are stable.

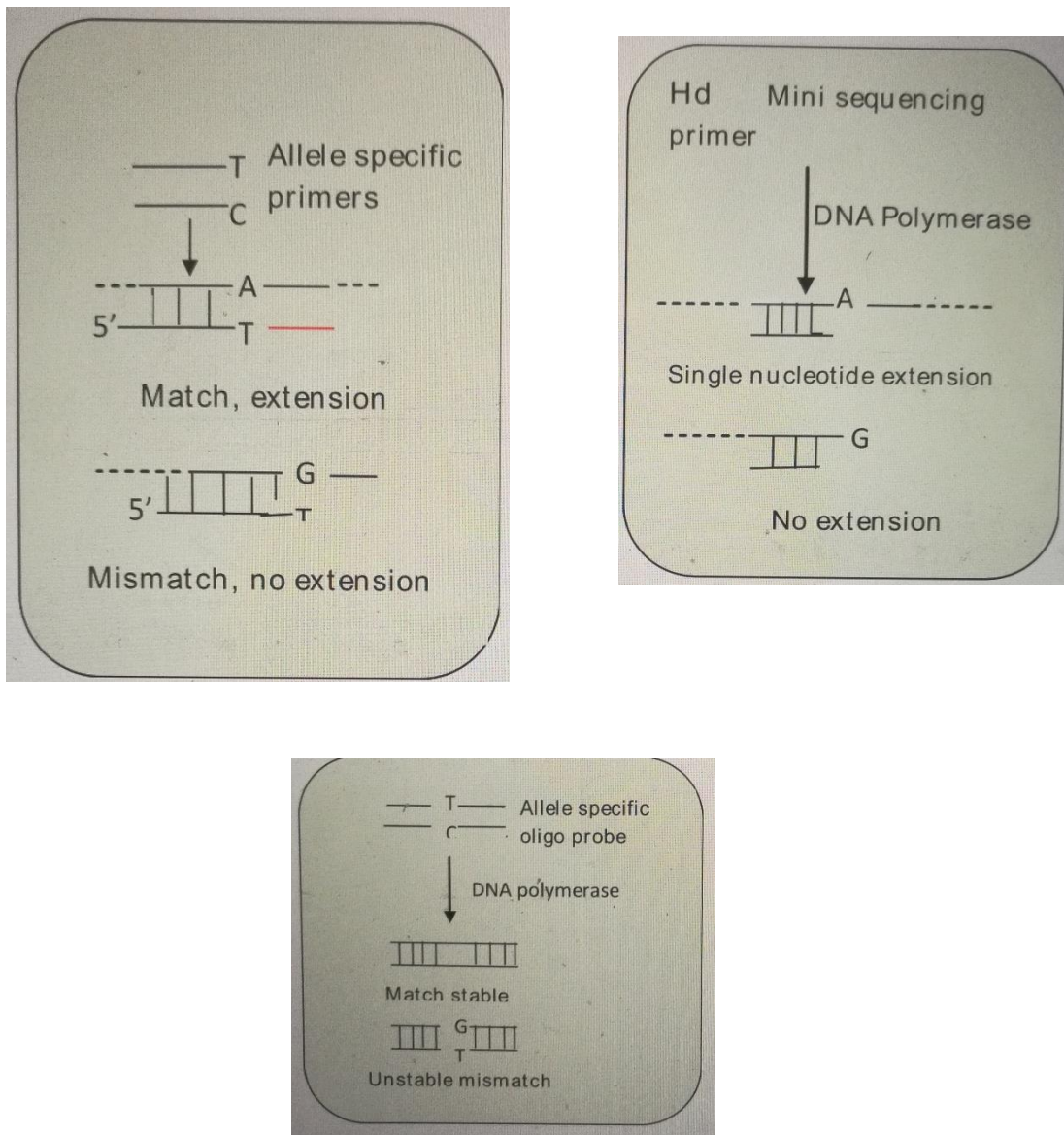


Figure 10 Biochemical reaction principles underlying genotyping

(Adapted from Syvanen, 2001).

1.15. Rationale and aims of the study

Although SPAQ combination was used as a transitory measure to curb drug resistance at the time that the study was implemented (Program 2002), it is of major importance still because of a number of reasons. Firstly, artemisinin based combinations were the major policy treatments for malaria, and each component in this combination that was available was associated with the study drugs for the first line therapeutic management of uncomplicated malaria in most endemic countries including Cameroon. In this light therefore, the parasite is considered subjected to some level of drug pressure, especially because of the short half-life of artemisinins. Secondly, SP is still in use for the IPT prophylactic management of pregnant women based on the current policy and practice and also the premise that in vivo drug efficacy evaluated in children may underestimate the true efficacy of the drug used in intermittent treatment. Assessing the level and distribution of drug resistant forms of the parasite as well as how humans respond to therapy against this infection will provide a better understanding of not only how effective the control measure works but also its potential in generating long lasting protective immune responses. In addition, seasonal malaria chemoprevention has been recommended as a strategy for malaria control towards elimination in areas of unstable malaria transmission such as in the far northern parts of Cameroon. The current drug of choice is Artesunate-SP or SPAQ with the potential to rapidly clear parasites and provide prophylactic benefits while minimising the development of high grade resistant forms of the parasite. Thirdly, of particular importance, signals in the immune genes associated with pathology of malaria and treatment response will provide valuable insights into mechanisms of immune boosting of therapy. This can find application in the design of adjunct therapies for malaria in the background of failing therapies attributable to drug resistant falciparum malaria.

In this thesis therefore, we shall be examining variations in human and parasite genetic architecture associated with treatment response and/or susceptibility/treatment resistance to malaria. We studied variations in immune response genes known to be involved in malaria pathophysiology and for which prior information exist as to the feasibility of genotyping assays on the Sequenom's iPLEX genotyping platform. In addition, the prevalence of parasites resistance alleles in distinct geo-ecological regions in Cameroon will be assessed as well as the molecular association with treatment response and production of fever. Lastly, we will assess the combined effect of human and parasite determinants on the outcome of treatment to identify which factors are critical for response to therapy in different regions. These approaches are expected to provide insights into how differently unrelated individuals with similar exposures respond to a combination of SP

and AQ used in the treatment of uncomplicated malaria in Cameroon in the background of differing parasite resistant allelic distribution

1.16.Objectives of the study:

1. Describe and determine the prevalence of major Plasmodium falciparum drug resistant mutations in pfcrt-codon 76, pfmdr-1-codon 89, 184 and 1246 ; pfpfdhfr-codons 51, 59 and 108 and pfdhps codons 437 and 540 among circulating parasites in five geo-ecological sites in Cameroon.
2. Assess the association between the molecular markers of Plasmodium falciparum drug resistance and treatment response in Cameroon.
3. Determine the molecular correlations between host candidate immune gene polymorphisms and treatment response parameters in children with AQ or SP resistance conferring Plasmodium falciparum mutants.
4. Identify candidate immune genes that may be implicated in differential clinical and parasite response to antimalarial treatment in different ecological sites in Cameroon.

CHAPTER 2: RESEARCH DESIGN AND METHODOLOGY

2.1.Study Area

The study area included represented the northern and southern regions of Cameroon; three ecologically different regions with different malaria endemicities: Garoua/Ngaoundere, Yaoundé and Mutengene. In addition, two other sites included in the molecular analyses were Ngaoundere and Bangolan, earmarked as sentinel sites for malaria drug resistance surveillance in Cameroon. These last sites were also selected based on their pattern of malaria transmission and contribution in understanding the molecular epidemiology of drug resistance in *Plasmodium falciparum*. Thus, Ngaoundere, although in the north represent a short guinea savannah type geography, while Bangolan being in the south is located within a closed community in the plains of Noun with rice farms and continuous high malaria transmission.

2.2.Study Design

2.2.1.Design types incorporated in the study.

This study broadly has a mixed epidemiological design, derived from firstly a randomised controlled drug trial and a case control study nested within another randomised clinical trial involving another class of antimalarial drug. The randomised trial consisted of a non-inferiority efficacy study comparing artemisinin based combination therapy in Cameroon and carried out in Ngaoundere, Bangolan, Mutengene and Garoua while the case control design was nested within another randomised double blind placebo controlled trial comparing sulphadoxine pyrimethamine alone or in combination with amodiaquine in Garoua, North Cameroon. This latter study was performed as an interim measure to curb chloroquine resistant malaria when artemisinin based combination were not yet available to the public. The randomised studies in Ngaoundere and Bangolan were the first studies assessing artemisinin based combinations at the time when they were being introduced in public health facilities in Cameroon.

2.2.2.Patient enrolment, drug administration and follow up procedures.

The patients who fulfil all of the following criteria were included in the study, irrespective of the drug administered:

- Children of either gender, between 6 months (> 5kg) and 10 years of age.
- 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/ μ l.

- Presenting with fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) or having a history of fever in the preceding 24 hours.
- Able to ingest tablets orally (either suspended in water or uncrushed with food).
- Willing to participate in the study with written informed consent from parent/guardian.
- Willing and able to attend the clinic on stipulated regular follow-up visits.

Patients who presented with one of the following criteria were excluded from the study:

Any of the following “danger signs of severe malaria”:

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

Patients were excluded on the basis of reported prior treatment with other antimalarials drugs within the past two weeks even if they had a fever and parasitemia.

A patient who was previously included in the study was subsequently withdrawn from treatment with the study drug if he/she had one of the following criteria:

- Adverse events (AEs) with severity grade > 3.
- Serious adverse events (SAEs).
- Marked deteriorated clinical conditions or unexplained clinical manifestations.
- Vomiting of replacement dose.
- Serious or repeated non-compliance of the patient with protocol specification.
- At the request of the parent/guardian (withdrawal of consent).

- At the discretion of the principal investigator.

Patients who were withdrawn from the study were not replaced and whenever possible were followed up for safety assessments (clinical and laboratory assessments) until day 42 or until the clinical problem was resolved; except otherwise, decided by the parent/guardian. Reasons for any withdrawal were fully documented in patient study file. The withdrawn patient was visited if they failed to show up for follow up.

A patient who was included in the study and had completed the treatment course was discontinued from further assessments according to the study protocol if any of the following criteria was met:

- Endpoint was met, i.e., treatment failure.
- Development of severe malaria during follow-up.
- Loss to follow-up.
- At the request of the parent/guardian (withdrawal of consent)

Patients who were discontinued from the study for any reason were not replaced. The reasons for study discontinuation were fully documented in CRF.

2.3. Drug administration

Table 4 Drug administration dose table

Treatment	Medication	D0		D1	D2
AQ-SP	Amodiaquine	10mg/kg body weight	10mg/kg body weight	10mg/kg body weight	
	Sulphadoxine- pyrimethamine	1.25mg/kg body weight			
Coarsucam (ASAQ)	Artesunate	4mg/kg	4mg/kg	4mg/kg	
	Amodiaquine	10mg/kg	10mg/kg	10mg/kg	
Quinine	Quinine base (6days)	10mg/kg x 3 times daily x 5-7			

2.4. Treatment follow up procedures

Patients enrolled in the study were required to return to the clinic for follow up and further treatment. They were asked to return on days 1, 2, 3, 7, 14, 21 and 28. The rationale for the initially close programmatic visits is to allow for close monitoring of patients for early events like adverse drug event occurrence, fever and parasite dynamics and subsequent early treatment failure. On each of the follow up days, the patients were reviewed both clinically and biologically. The clinical review consisted of a complete physical examination and a clinical examination. The biological review consisted of a laboratory analysis of vital parameters including liver and kidney function test, full blood count and parasitemia. Any other test deemed necessary by the physician was also included in the biological review. In the event of a failure, the patient was treated as a case of severe malaria and rescued with quinine as prescribed by the study physician and excluded from the study. In the case of a withdrawal, the reasons for withdrawal was documented and the participant was left to go after ensuring that he/she is in good health.

2.5.Parasite density determination

Children visiting the clinic with symptoms of malaria were screened for clinically for the disease. Thick and thin blood films were made and stained with 10 % Giemsa for 25 minutes. These smears were then read under a light microscope under objective 100. At least 100 high power fields of the smear were read and parasites counted in the thick smear against 200 leukocytes. The parasite density was calculated assuming that 8000 leukocytes were present in one microlitre of whole blood as follows

$$\begin{array}{l}
 x \text{ (parasites)} \longrightarrow 200\text{wbc} \\
 y \text{ (parasites)} \longrightarrow 8000\text{wbc}
 \end{array}
 \left. \vphantom{\begin{array}{l} x \\ y \end{array}} \right\} \longrightarrow \text{Parasite Density } y = X \times 8000/200 \text{ given as } y/\mu\text{L of blood.}$$

The thin smear was used to distinguish between be present in the parasite population. A second microscopist read slides to confirm parasitemia and speciation. Where there was more than 5% disparity between both readings, a third reading will be done by a chief microscopist. The mean value of the two closest readings was recorded as the right value. Approximately 10% of the slides were read as a measure of quality control.

2.6.Outcomes Classification

Patient’s treatment outcome was classified according to the WHO guidelines (W.H.O) 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 parasitemia; or
- Parasitemia on day 3 with axillary temperature $> 37.5^{\circ}\text{C}$; or
- Parasitemia 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 danger signs or severe malaria after day 3 in the
- presence of parasitemia without previously meeting any of the
- criteria of ETF; or
- Presence of parasitemia and axillary temperature $>37.5^{\circ}\text{C}$ (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 parasitemia on any day from days 7 onwards and axillary temperature $<37.5^{\circ}\text{C}$, without previously meeting any of the criteria of early treatment or late clinical failure.

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

- Absence of parasitemia at study endpoint (day 28) with axillary temperature $<37.5^{\circ}\text{C}$
- Absence of any significant clinical concern.

Specific efficacy parameters evaluated were as follows:

PCR-adjusted parasitological treatment response:

- Cure rate on day 28: defined as the proportion of patients who cleared asexual parasites parasitemia within 28 days follow up after treatment
- Cure rate on day 14: defined as the proportion of patients with complete clearance of asexual parasitemia within 14 days follow up after treatment. Of course, it should be noted that absence of asexual parasites mean no parasites observed within the microscopic detection limit of 10^8 parasites in circulation.

Prevalence of ‘early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF)’ and ‘adequate parasitological and clinical response (ACPR).

2.7. Molecular methods

To investigate the ecological distribution of single nucleotide polymorphisms in drug metabolising genes, samples designated for molecular analysis were used to isolate total parasite genomic DNA which served as the principal source of genes for further genotyping of mutations. Samples for genotyping studies included filter paper blood spots from three clinical trials between 2004 and 2008. Samples were used to characterize the genetic diversity of drug resistant mutations within different ecological zones in Cameroon, notably between Garoua, Ngaoundere, Yaoundé, Bangolan and Mutengene. For resistant conferring haplotype with samples obtained from clinical trials, analysis was restricted to three sites (Garoua, Yaoundé and Mutengene while for analyses involving northern and southern ecologies, either Yaoundé or Mutengene or both were involved in the analysis. In relation to analysis of host responses to therapy, combined samples from Garoua, Yaoundé and Mutengene were used as well as results from the efficacy trial.

2.7.1. Genotyping Parasite Single Nucleotide Polymorphisms.

Filter paper processing.

For all children who fulfilled the criteria and were duly enrolled in the study after consent, thorough clinical and biological examination was carried out to set the baseline clinical and biological characteristics. Samples to be used for parasite DNA extraction were spotted on filter paper (Whatman N3) and allowed to dry away from sunlight and flies in a clean environment. These samples were collected during blood collection procedures for microscopy and full blood cell count. Additional samples were collected from patients if they fulfilled the conditions of ETF, LCF, and LPF as described in the WHO document for surveillance of drug resistance in malaria. The filter papers were packaged in individual ziplock bags and stored inside a plastic sealable dish together with silica gel desiccator. This was transferred to the laboratory in Yaoundé for further analyses.

DNA Extraction

Parasite DNA was extracted using the chelex-100 based method as described by Djimde et al. (Djimde 2001). In this method, a detergent, Saponin is used to disrupt cell membranes and liberate the parasite on the filter paper which is then captured by the cationic resin Chelex-100 in phosphate buffered saline. The DNA is further liberated by agitation, several centrifugations and re-suspended in Tris EDTA or nuclease free water and stored at -20°C until use. The detailed procedure for DNA extraction using this method is presented in annexe 10.

2.7.2. PCR correction of treatment response.

In the event of a treatment failure during the field trial, the identified parasites cannot be distinguished microscopically as the same parasites observed before treatment or new infections due to high transmission. The efficacy results obtained in the field therefore may not represent

the true treatment response. The crude treatment response rate can be corrected by genotyping polymorphic markers such as merozoite surface protein 1 (msp1), merozoite surface protein 2 (msp2) and Glutamine rich protein (glurp) in the pre-treatment and failure parasite samples. If the genotype profile of the paired samples are the same for these markers, it is concluded that the treatment failure was due to recrudescing parasites and if not, on new infections. In the context of this study, this procedure was used to correct the treatment response.

2.7.3. PCR amplification of msp1, msp 2 and glurp genes.

For the outer PCR amplification reaction, the following were mixed together in a total volume of 18,25µl of nuclease free water in a pre-PCR clean area deprived of DNAase : 0,5µl of dNTPs, 0,25µl of each of the various primers (for example S2 and S3 for msp-2) at a concentration of 2.5 µM each, 0,25µl of One Taq™ hot start polymerase at 5units/µl and 2.5µl of Thermopol buffer and 2 µl DNA template. They were subjected to the following reaction conditions : Initial denaturation at 94°C for 3 minutes; denaturation at 94°C for 30s, primer annealing for 42°C for 1 minute, extension at 65°C for 2min for a total of 30 cycles and final elongation at 72°C for 3minutes.

The master mix for the nested reaction contained 0,25µl of each primer (for example S1 & S4 for msp-2) at a concentration of 2.5 µM; 0,5µl each of dNTPs ; 0,25µl of One Taq™ hot start polymerase (5units/µl) in 2,5µl of 10X Thermopol buffer and 20,25µl of nuclease free water. To this was added 1-2 µl of reaction product of outer PCR amplification and incubated under the following conditions: Denaturation at 94°C for 30s, primer annealing at 50°C for 60 seconds, extension at 72°C for 2 minutes for a total of 30 cycles and final elongation at 72°C for 3minutes. The reaction was kept on hold at 4°C until agarose gel electrophoresis was carried out.

2.7.4. Determination of pfmdr1-86 polymorphism in Ngaoundere.

While part data for dhfr, dhps and pfert from the towns of Bangolan and Ngaoundere were issued from Sanger sequencing studies done at the Genome Laboratory at the University of Washington, USA, the pfmdr1-86 mutation was amplified using a T3 thermocycler (biometra). The product of the first PCR (external PCR) was used as a substrate for the second in Cameroon. The primers used were those described by the "Malaria Group" of the Center for Vaccine Development at Maryland University School of Medicine. 2012 version (<http://medschool.umaryland.edu/CVD/plowe.html>).

For the outer PCR, each tube was made of 18.25µl of nuclease-free water, 2.5µl of 10X Thermopol buffer, 0.5µl of each of four dNTPs, 0.25µl of each 2.5 µM primer (MDR1 and MDR2, as shown below), 0, 25µl of hotstart Taq polymerase at 5units / µl and 3µl of DNA extract to constitute a final volume of 25µl. These tubes were subjected to the following amplification conditions:

pre-denaturation at 95 ° C for 5 min; denaturation at 95 ° C for 30 s, fixation of the primers 45 ° C for 30 s, elongation at 65 ° C for 45 s x 30 cycles and termination at 72 ° C for 5 min. For internal PCR, each tube consisted of 20.25µl of nuclease-free water, 2.5µl of 10X Thermopol buffer, 0.5µl of each of four dNTPs, 0.25µl of each 2.5µM primer (MDR3 and MDR4), 0, 25µl of hotstart Taq polymerase (5units / µl) and 1µl of amplicon from the first amplification, to constitutes a total volume of 25µl. These tubes were subjected to the following amplification conditions: denaturation at 95 ° C for 3 min, fixing of the primers at 95 ° C for 30s, elongation at 45 ° C for 30s x 25 cycles and termination at 65 ° C for 45s.

2.7.5.Determination of single nucleotide polymorphisms in drug metabolizing genes and construction of haplotypes.

To determine mutations in parasite gene fragments associated with drug metabolism, we employed the PCR and dotblot analysis. The Dotblot assays were carried out at the London School of Hygiene and Tropical Medicine.

Table 5: Genes, codons of interests and haplotypes of wild and mutant forms of the parasite.

Gene	Codon of interest	Haplotypes of interest (in 3'-5' direction of gene)	
		Wild type	Mutant
<i>Chloroquine resistance transporter</i>	K76T	CVMNT,	CVIET,
<i>multidrug resistance transporter gene 1</i>	T86Y, Y184F, 1034C, 1042D, 1246Y,	YFYD, YN,	TY
<i>dihydrofolate reductase</i>	L51I, 59R, S108N	IRN, IRD	NCS,
<i>dihydropteroate synthase</i>	A437G, L540E	,AAK SAK, , FAK	AGK, SGK, SGE

PCR and restriction fragment length polymorphism.

The primers and polymerase reaction conditions for the amplification of the above genes are provided in annexe 7. Briefly, each gene fragment was amplified using stated primers in a thermocycler and the sequences targetted the mutations represented above, for the different genes of interest.

2.7.7.Dot Blot Assay and Haplotype Construction.

Production of dot blot DNA template for resistance determination by PCR

To determine resistance mutations, PCR amplifications were performed in suitable PCR buffers containing a final concentration of 700 mM Tris pH 8.8, 200 mM (NH₄)₂ SO₄, 2.5 mM MgCl₂, 10 mM dithiothreitol (DTT), 1 % Triton X-100 and 1 mg/ml BSA. Deoxyribonucleotides (dNTPs) obtained from Sigma^R were reconstituted in nuclease free-water prior to use. The acidity of the 10 mM dNTP solution was adjusted to pH 7.0 and used at a final concentration of 2 mM for each of the four dNTPs. The DNA extract (3 µl) was used to perform amplification reactions using 6.25 IU/ µl and 5U/µl of Taq DNA polymerase from Promega, for the outer and nested reactions respectively. The amplifications were performed in a final volume of 25 µl, containing 30 - 40 picomoles of oligonucleotide primers. Primers for various genes used for the various gene loci for primary and nested PCR reactions are shown below (see table...) Primary PCR for the *pfdhfr* gene was performed through forty cycles involving DNA denaturation at 94 °C for 3 minutes for the first cycle and 1 minute in subsequent cycles, primer annealing at 52 °C for 2 minutes and 72 °C for 1 minute and a final elongation step at 72 °C for 10 minutes. In the nested reaction, 2 µl of the amplicon from the primary PCR reaction was used as the DNA template. The primary denaturation was done at 94 °C for 3 minutes for the first cycle and 1 minute for subsequent cycles; primer annealing at 44 °C for 2 minutes and primer elongation at 72 °C for 1 minute for the next 4 cycles. After the first 5 cycles, the annealing time was changed to 1 minute at the same temperature for the next 34 cycles. The reaction was stopped after a final elongation step at 72 °C for 10 minutes.

The same conditions for PCR were applicable to the amplification of the *pfdhps* gene with the exception of the nature of the primers used described earlier¹⁹. Conditions were a primary denaturation step at 94 °C for 3 minutes was done for the first cycle and for the 40 subsequent cycles. For the other cycles, it was done for 1 minute, while the primer annealing was done at 51 °C for 2 minutes and primer elongation at 72 °C for 1 minute. Final elongation was done for 10 minutes at 72 °C. The primer sequences and reaction conditions for *pfert* and *pfmdr-1* amplifications are represented in Table 1.

Amplifications generated a 711 bp and a 594 bp fragment of *pfdhps* and *pfdhfr* respectively, containing the target single nucleotide polymorphisms. In both the outer and nested PCR reactions, 23µl of PCR mix containing primers (0.25 µM final concentration), MgCl₂ (2 mM), 250 µM each deoxynucleoside triphosphate (dNTPS) and 1 x Bioline Taq polymerase was prepared. Template DNA (2 µl) was added to the outer reaction mixtures. Two microlitres of the outer PCR product of the different genes was introduced into a 23µl of secondary amplification mixture. The PCR products were subjected to electrophoresis in a 1.5 % agarose gel in the presence of ethidium bromide. A 100 bp ladder from digestion prepared by New England Biolabs was used as DNA size standard. Molecular weights of sample bands were obtained by interpolation from a standard curve.

Dotbot assay using single strand oligonucleotide probe and haplotype determination.

To identify point mutations in targetted genes associated with resistance in *P. falciparum*, Sequence-specific oligonucleotide polymorphism (SSOP) was used and point mutations were in *pfprt*, *pfmdr-1*, *pfdhfr* and *pfdhps* genes. Amplification using SSOP resulted in a nested amplicons for each of four genes. They were heat denatured at 95 °C for 2 minutes, and spotted onto nylon membranes in 1 µl volumes in a 12 by 8 membrane grid. Replica blots were made from each array and assayed simultaneously following DNA crosslinking (exposure to 1200 Joules of UV light), blocking with non-fat dry milk, and overlaid with the corresponding 18-mer sequence-specific oligonucleotide probes labelled at the 3' end with digoxigenin (DIG) for hybridisation. The wild and mutant samples were hybridised separately. Stringent washes were performed between assay steps with tetramethyl ammonium chloride in buffer. Subsequently, alkaline phosphatase-conjugated anti-DIG Fab fragments were used to detect hybridised DIG-labeled probes as described by Conway et al., 1999 (20). A fluorogenic substrate, CSPD, was added to the membrane preparation and incubated. If there was hybridisation of the alkaline phosphatase conjugate, the CSPD will be broken down and the product will be visualised in Chemidoc based on enhanced chemluminescence. The presence or absence of the variant sequence polymorphism was thus recorded by the presence or not of chemluminescence units after exposure of the membrane in a phospho-imager. The final chemluminescence was obtained for each assay by subtracting the background chemluminescence. The sample mutant status were categorised as pure or mixed and whether they contained minority or majority haplotypes. Samples were considered to be mixed but containing a majority SNP when the minority SNP was less than half of the majority value but higher than that represented by the first gridline on the chart. Samples with mixed but no major haplotype were excluded from the analysis.

Table 6: Probes and probe sequences and target gene haplotypes in *P. Falciparum* dhfr and dhps

Probe	Probe sequence	Wild type haplo-type	Mutant haplo-type
Probes targeting <i>pf dhfr</i> Codons 50 and 51			
50/51CN 50/51CN2 50/51RN 50/51RN2 50/51RI 50/51CI	TGG AAA TGT <u>A</u> AT TCC CTA TGG AAA TGT <u>A</u> AC TCC CTA TGG AAA <u>C</u> GT AAT TCC CTA TGG AAA <u>C</u> GT AAC TCC CTA TGG AAA TGT ATT TCC CTA TGG AAA TGT <u>A</u> TT TCC CTA	CNCS	CIRN
Probes used to target <i>pf dhfr</i> Codons 59 and 108			
59C 59R 108N 108S 108T	AAT ATT TTT GTG CAG TTA AAT ATT <u>T</u> C GTG CAG TTA A AGA ACA <u>A</u> AC TGG GAA AG A AGA ACA <u>A</u> GC TGG GAA AG A AGA ACA <u>A</u> CC TGG GAA AG		
Probes targeting <i>pf dhps</i> Codons 436 and 437 (9 probes)		SAK,	AAK, SGK, SGE, AGE, AGK, FAK, FGK, FGE
436/437SA 436/437SG 436/437FA 436/437FG 436/437AA 436/437AG 436/437CA 540K 540E	GAA TCC <u>T</u> CT GCT CCT TTT GAA TCC <u>T</u> CT GGT CCT TTT GAA TCC <u>T</u> TT GCT CCT TTT GAA TCC <u>T</u> TT GGT CCT TTT GAA TCC GCT GCT CCT TTT GAA TCC GCT <u>G</u> GT CCT TTT GAA TCC <u>T</u> GT GCT CCT TTT ACA ATG GAT AAA CTA ACA CAC ATG GAT <u>A</u> AA CTA CAC		

2.8.Molecular genotyping of candidate human immune genes

2.8.1.Selection of host candidate immune response genes

Below, Table 7 summarises the various genes used for the genome wide association study. The list included a selection from a growing list of cytokines and other immune mediators. Candidate genes were chosen in order to reflect a wide spectrum of cytokines together with their receptors and promoters that are thought to be involved in the development of malarial pathogenesis. Also included among the candidate genes selected were lymphokines that regulate the expression of these cytokines and immune mediators, the adhesion molecules and inflammatory mediators of pathological effects during infection.

Table 7 Selected human immune candidate genes

Gene	Chr*	Chr location	rs Number	SNP sequence
DARC	1	157441307	rs2814778	TGCTTCCAAG[A/G]TAAGAGCCAA
RGS2	1	191036449	rs2179652	TCCAGCCCTG[C/T]GGCCAGCCTC
IL-10	1	205007454	rs3024500	TCCTGGGGGT[A/G]GGGGGTAGCT
IL-10	1	205015988	rs1800890	ATTTTAAATG[A/T]ATTTTCCAG
IL-10	1	205013520	rs1800896	CTACTTCCCC[C/T]TCCCAAANAA
CR1	1	205849479	rs17047660	AAATGCAATT[A/G]GAGTACCAGG
GBP7	1	89355278	rs7537937	CCATTGACTG[C/G]AATGCCACCT
IL-1A	2	113259694	rs17411697	GCCTAGGTCA[G/T]CACCTTTTAG
IL-1B2	2	113306861	rs1143634	CTATCTTCTT[C/T]GACACATGGG
TLR9	2	52231737	rs352140	CGGAGCTACC[A/G]CGACTGGAGG
TLR9	3	52236071	rs187084	TCACTGCCCT[C/T]AAGAAGCTGA
IL-1IL-17RD	3	57113459	rs6780995	GCAGCTGGTC[A/G]TCTCTGTAGT
IL-1IL-17RE	3	9935070	rs708567	CTCCACCCCT[A/G]AGTCAGCTGC
TLR1	4	38476105	rs4833095	TAAGGTAAGA[C/T]TTGATAACTT
TLR1	4	38476609	rs5743611	TCTCATAATA[C/G]AATCCAGTAT
TLR6	5	38506745	rs5743810	CACCAGAGGT[C/T]CAACCTTACT
TLR6	5	38506909	rs574380	CTACAAATTC[C/T]GAATGCAAAA

IRF1	5	131854779	rs2706384	CCGGGCGATC[A/C]CCTCGCCTGC
IL-1IL-13	5	132023863	rs20541	TCAGTTGAAC[C/T]GTCCCTCGCG
IL-4	5	132037053	rs2243250	AGAACATTGT[C/T]CCCCAGTGCT
C6	5	41235716	rs1801033	CCATGCACTG[A/C]GCCTCTGGTA
IL20RA	6	137367540	rs1555498	GTAATAGATA[C/T]GGGCAAACA
LTA	6	31648120	rs2239704	AGGACACTGC[G/T]GGGCGGTAGT
LTA	6	31648292	rs909253	CAGAGAGGAA[C/T]CATGGCAGAA
TNF α -1031	6	31650287	rs1799964	GCTGAGAAGA[C/T]GAAGGAAAAG
TNF- α -376	6	31650943	rs1800750	CTGTCTGGAA[A/G]TTAGAAGGAA
TNF- α -308	6	31651010	rs1800629	GAGGGGCATG[A/G]GGACGGGGTT
T	6	31651080	rs361525	CCTCRGAATC[A/G]GAGCAGGGAG
T	6	31652168	rs3093662	GTGAATACAC[A/G]GATGAATGGA

C	6	31947288	rs2242665	ATGGACCAAC[A/G]T TACTCCACC
C	7	117017519	rs17140229	AATAGTGTTA[C/T]TTCAGTGAAT
N	7	30458762	rs2075820	GCGGGACCCC[A/G]AGGAGGTGTT
hCD36_T126G	7	80138385	rs3211938	GTACATCATA[G/T]GGTGTGCTAG
hCD36_G143C	7	80140046		TTTACAATTT[C/G]CAANNCGGCT
hCD36_I1444D	7	80140051		AATTTSCAAG[-/A]CGGCTGCAGG
T	9	119515123	rs4986790	TACCTCGATG[A/G]TATTATTGAC
T	9	119515423	rs4986791	TTTGGGACAA[C/T]CAGCCTAAAG
A	9	135121143	rs8176746	TTTCTACTAC[A/C]TGGGGGSGTT
H	11	5204749	rs33950507	AGTTGGTGGT[A/G/T]AGGCCCTG

h	11	5204808	rs334	CTGACTCCTR[A/T]GGAGAAGTCT
T	11	5675093	rs7935564	GATAAAGGAG[A/G]ATCAAGAGGC
R	11	63243962	rs542998	GTGAAAGTGG[C/T]TTTACCTGAT
IL	12	66928914	rs2227507	ATCTCTCCAC[C/T]CTCTCCAAGC
IL	12	66930885	rs1012356	CCATTAACT[A/T]TAATAAATCT
IL	12	66932788	rs2227491	CACACGGCCC[C/T]GTTCGTCACA
IL	12	66933980	rs2227485	TNCTATAGTG[A/G]CTGAGTAAGC

IL	12	66934889	rs2227478	GTGCCACTGC[A/G]AAGGGTCGGA
SP	14	64333053	rs229587	CTGGTTTTCA[C/T]TGAGCCAGGT
IL	16	27281681	rs1805015	CTTCAGCAAC[C/T]CCCTGAGCCA
A	16	3973437	rs2230739	CACAGGTCAT[A/G]AAGAACTCCC
A	16	4019824	rs10775349	G TTCAGCTTT[C/G]TTACACATTT
A	17	15802057	rs2535611	GGTACACTTC[C/T]TTTTTTTTTT
N	17	23120724	rs2297518	CATGAAGAGC[A/G]ATTTCTTCAG

N	17	23152636	rs1800482	CAGAGTTCGA[C/G]ACCAGCATGG
N	17	23152855	rs9282799	AGTGTTGGGA[C/T]GGTGAGATCA
N	17	23153339	rs8078340	AAGGCAGAAC[C/T]AGCTACATCA
IC	19	10255792	rs1799969	TTCCCTGGAC[A/G]GGCTGTTCCC
IC	19	10256683	rs5498	GGTCACCCGC[A/G]AGGTGACCGT
E	19	6870624	rs373533	TGGTGGTCTT[G/T]GAAGAAGCGC

E	19	6870753	rs461645	CAAACCTGAA[C/T]GTTCTCCAGA
G	20	56905438	rs2057291	AGCTCCTTAT[A/G]GAGAATTCTA
G	20	56919207	rs8386	AACTGAGAA[C/T]ATCCGCCGTG
D	22	22509132	rs3177244	TTCTGCAGGG[A/G]CATCCAGGAG
C	X	135557275	rs3092945	TACAWCAGCA[C/T]CAACAATTAT
C	X	135558221	rs1126535	TCATAGAAGG[C/T]TGGACAAGGT
G	X	153416686	rs1050829	TGGAGGGCAT[C/T]CATGTGGCTG

G	X	153417411	rs1050828	GCATAGCCCA[C/T]GATGAAGGTG
a		11223656		TTNCACNCNT[A/G]CCTCCTCTCT
a		11226027		GTGAGAGTAA[C/T]AATACTTGCC
a		11226571		GTAAACCTGC[A/C]TCTCTGTTTC

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For gender SNPs, homozygotes=females, heterozygotes= males, chromosome positions, rs numbers correspond to the August 2009 build of the SNP database, dbSNP hosted in the NCBI website (www.ncbi.nlm.nih.gov/projects/SNP/). Additional SNPs in some genes e.g. IL-1 and IL-13 could not fit into multiplex reaction. Sequences represent short stretches at points where SNPs occur in the various genes. Within the short sequences, they are shown in square brackets. Chr*= chromosome number, Chr= chromosome.

Single Nucleotide Polymorphisms (SNPs) were selected using information from the literature and the SNP web database of the National Centre for Biological Information hosted by the National Library of Medicine in the National Institutes of Health (USA) (www.ncbi.nlm.nih.gov/projects/SNP/). The SNPs were selected for each investigated gene based on their previously reported or anticipated functional relevance for cytokine expression and/or structure. This was done to reflect a compromise between SNP function, the marker spacing and minor allele frequency (MAF). The initial SNP selection consisted of validated markers with $MAF \geq 5\%$. These validated markers were further narrowed down to an economic 66 known SNPs for which genotyping assays could be designed on the mass spectrometry platform, as this was the preferred method of choice for high throughput genotyping. A list of the structural references of the various SNPs as well as their locations can be seen in annexe 8.

2.8.2. Multiplex genotyping of immune response genes

With the objective of genotyping single nucleotide polymorphisms in immune genes previously selected, human DNA was extracted, purified, quantified and used to build multiplex reactions in 96 well microplates to obtain SNP calls on an iPLEX MALDI-TOF platform. The overall aim of this process was to identify by sequencing, the different alleles of the immune genes from isolates of individual with differential outcome to treatment.

2.8.3.Human DNA extraction.

Human DNA extraction was purified from blood specimen by the same method as for parasite DNA namely; Chelex-100 extraction. The difference with human DNA was the treatment of the final extract following purification. It was necessary for the DNA extract to be purified and quantified because of the downstream applications that the extract was going to undergo.

2.8.4.PicoGreen ds DNA quantitation assay.

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A₂₆₀). The PicoGreen ds DNA quantitation method is based on the detection of double stranded DNA bound to a fluorescent stain that once excited emits wavelengths of light which is captured and represented as an emission spectrum. From this curve, the fluorescence measured is related to the DNA concentration as an extrapolation of a standard curve of fluorescence versus DNA concentration prepared from a DNA standard solution.

A 2 µg/mL stock solution of dsDNA was prepared in TE using standard preparation protocol. The DNA concentration was determined on the basis of absorbance at 260 nm (A₂₆₀) in a cuvette with a 1 cm path length; an A₂₆₀ of 0.04 corresponded to 2 µg/mL dsDNA solution. Then 1.0 mL of the aqueous working solution of PicoGreen reagent was added (prepared in Reagent Preparation) to each cuvette. Subsequently, 1.0 mL of the aqueous working solution of the Pico-Green reagent was added to each sample and incubated for 2 to 5 minutes at room temperature, protected from light. The fluorescence of the sample was measured using instrument parameters that correspond to those used when generating the standard curve. To minimise photobleaching effects, the time for fluorescence measurement was kept constant for all samples. The fluorescence value of the reagent blank was subtracted from that of each of the samples to get the true sample value. Then, using the standard curve generated, the DNA concentration of each sample was determined. The assay was repeated using a different dilution of the sample to confirm the quantitation results.

2.8.5.Amplification of parasite genome to increase DNA template

One of the challenges with multiplex genotyping resides in the low initial quantities of DNA to be subjected to genome sequencing. Whole-Genome Amplification by Improved Primer Extension Pre-amplification PCR (I-PEP-PCR) was used to increase the amount of DNA template to be used in the downstream sequencing reactions. This was necessary because human DNA was obtained from blood spots on filter paper collected primarily to genotype infecting malaria parasites. The method used was adapted from Hughes and Moody (2007).

Principle

The goal of the PCR-based whole-genome amplification (WGA) was to generate microgram quantities of genome-representative DNA fragments from very small quantities of starting material. Primer extension preamplification PCR (PEP-PCR) uses degenerate 15-mer PCR primers. The effectiveness of PEP-PCR was increased by the use of a DNA polymerase cocktail that includes Taq DNA polymerase and a proofreading DNA polymerase (to provide 3'-to-5'-exonuclease activity, thereby excising mis-incorporated nucleotides that slow the progression of Taq DNA polymerase). The result is a reaction that generates a smear of DNA fragments visible on an agarose gel. The goal was essentially to establish that the results generated from the amplified DNA are indistinguishable from the results obtained from the original genomic DNA.

Ten microlitres of genomic DNA was added to each tube containing 50 μ L master mixed solution. The solution consisted of 6 μ L of 10X Thermopol buffer, 6 μ L of Magnesium Chloride, 0.6 μ L of dNTP mix, 3.4 μ L of oligo-dT 15-mer (5'NNNNNNNNNNNNNNNN3') random primer, 3 μ L of enhancer gelatine and 1.4 μ L of high fidelity Taq polymerase and nuclease free water to make 60 μ L final volume in each tube. PCR-amplification of the sample(s) in a thermal cycler used the following conditions:

Table 8 PCR Amplification and cycling conditions for the Long PCR reaction

Number of cycles	Temperature	Time
1	94°C	3 min
50	94°C	60 sec
	37°C, ramping to 55°C	>2 min (0.1°C/sec)
	55°C	4 min
1	72°C	5 min

PCR products were run in 1% agarose gel electrophoresis to ascertain of the size and purity of the DNA. Before the DNA was subjected to sequencing, excess deoxyribonucleotide triphosphates following whole genome amplification were dephosphorylated with 2 μ L shrimp alkaline phosphatase by incubation at 37°C for 60 min, and stopped by inactivation at 85°C for 10 min. The selected SNPs were analysed in one primer extension reaction, with the following conditions: 94°C for 15 min an initial denaturation, 40 cycles of 94°C for 5 s each including 5 cycles of annealing at 52°C for 5 s, and extension 80°C for 5 s, followed by a final extension at 72°C for 3 min.

2.8.6.Strategy for mass spectrometry based genotyping.

The strategy for mass spectrometry genotyping includes four principal reactions:

Amplification of target gene loci by PCR.

- Materials

- DNA source plate: 384-well deep-well PCR plate containing 2.5 ng/ μ l DNA of interest (stored at 4°C)
- 100 mM dNTPs (Applied Biosystems; stored at -20°C)
- 25 mM MgCl₂ (QIAGEN; store at -20°C)
- Ultrapure PCR-grade H₂O (Invitrogen)
- 5 U/ μ l HotStar Taq Plus DNA polymerase with 10 \times PCR buffer (QIAGEN; stored at -20°C)
- Forward and reverse primers: 1 μ M each in multiplex pool stored at 4°C)
- 384-well PCR reaction plate (Eppendorf twin. Tec)
- 96-tip or 384-tip pre-PCR Tomtec or Hydra-type (Matrix Technologies) robotic workstation.
- 96-tip pre-PCR robotic pipette with stacker (Beckman-Coulter) and disposable aerosol-barrier.
- 96-well ABGene Thermo-Fast skirted plates (AB-0800)
- 1.5-ml microcentrifuge tubes or 15- or 50-ml conical polypropylene centrifuge tubes (e.g., Falcon)
- MicroAmp adhesive plate sealers (Applied Biosystems)

- Pre-PCR table top centrifuge with microtitre plate carriers
- Thermo Scientific Hybaid 384-well blocks, PC-controlled thermal cyclers

- **Methods**

A Master mix was prepared for the reaction as shown in the following table:

Table 9 Protocol table for master mix preparation for amplification of target loci by PCR

	Target DNA	H2O	PCR Buffer	MgCl	dNTP mix	Primer mix	HotStarTaq	Total	Per well in 96 well plate
Well	2	2.222	0.825	0.429	0.132	0.66	0.22	4.4	
plate		1194.55	443.52	230.63	70.96	354.82	70.96	2365.44	22

Using the computer aided robotic workstation, 2ul of the 2.5ng template DNA was added in each well followed by 4ul of mastermix. The plates are placed on the plate carrier and then centrifuged using the table top centrifuge at 425xg for one minute. The setup was then loaded on the 384 well ABI Thermocycler block and amplified according to the following reaction conditions.

Table 10 Amplification reaction condition for target loci

1 cycle:	5 min	94°C	(initial denaturation)
45 cycles:	20 sec	94°C	(denaturation)
	30 sec	56°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	3 min	72°C	(final extension)
Final step:	indefinite	4°C	(hold).

The plates were further centrifuged to bring the reaction products to the bottom of the wells and then subjected to post PCR clean up using shrimp alkaline phosphatase.

2.8.7. Post PCR product clean-up by Shrimp Alkaline Phosphatase assay.

The purpose of the SAP clean-up is to remove unincorporated dNTPs through cleaving of phosphate groups at the 5' terminus from amplification products. This is achieved enzymatically using shrimp alkaline phosphatase according to the following setup.

Table 11 Protocol for clean-up of PCR amplification products for downstream analysis

	H2O	10X SAP buffer	SAP (1.7U/ul)	Total amount in mix
Well	1.53	0.17	0.3	2
Plate	1107.72	123.08	217.2	1448

After combining the components as shown, the plates were loaded in 384 well reaction plates and placed on the plate carrier and vortexed. For each well, 6ul of PCR product was added to 2ul of SAP reaction mix and then centrifuged at 425 x g for 1 minute. The setup was then incubated in thermocycler under the following conditions.

Table 12 incubation conditions for optimal activity of Shrimp Alkaline Phosphatase

Step 1	1 cycle:	40 min	37°C
Step 2	1 cycle:	10 min	85°C
Step 3	Final step:	indefinite	4°C.

Following this reaction, the products are centrifuged at 425 x g for one minute and stored at 4°C for the time required to extend products in a subsequent step.

2.8.8. Primer Extension of PCR products

The iPLEX primer extension reaction is a standard method for detecting single nucleotide polymorphisms or small insertion/deletion polymorphisms in amplified DNA. Following SAP clean-up, a primer extension reaction mastermix (containing extend primer, buffer, enzyme, and mass-modified ddNTPs) is added to the PCR amplification products. During the iPLEX reaction, the primer is extended by one mass-modified nucleotide depending on the allele interrogated and the design of the assay.

- Materials

- Ultrapure PCR-grade H₂O (Invitrogen)
- iPLEX enzyme (Sequenom)
- 10× iPLEX buffer (Sequenom)
- iPLEX Extension Mix (Sequenom)
- Extend primers: from 5 to 10 μM each in multiplex pool
- PCR products in 384-well PCR plates, cleaned up by SAP reaction .
- 1.5-ml microcentrifuge tubes or 15- or 50-ml conical polypropylene centrifuge tubes
- 96-well ABGene plates
- 96-tip post-PCR SpectroPREP (Sequenom) with stacker
- MicroAmp adhesive plate sealers (Applied Biosystems)
- Post-PCR tabletop centrifuge with microtiter plate carriers
- ABI or Hyaid Thermal cycler with 384-well blocks.

- Methods

Table 13 Mastermix preparation protocol for primer extension reaction

	H2O	10x iPlex buffer	iPlex extension mix	Probe mix	iPlex enzyme	Total	Per well of 96 well plate
Well	0.4926	0.222	0.2	1.044	0.041	2	13
plate	346.79	156.29	140.8	735.26	28.86	1408	

The reaction components were combined in a 1.5ml microcentrifuge tube in the order shown above (Table 13). The cocktail was aliquoted into 96 well plates, placed in 384 well plates

(4 mixes) and then placed on the plate carriers. To each well, 2ul of extension mix was added to each well automatically, sealed, vortexed and centrifuged at 425 x g for 1 minute to bring solution at the bottom of the well.

The primer extension reaction was carried out under the following thermal cycling conditions.

Table 14:PCR condition for primer extension reaction

1 cycle:		30 sec	94°C	initial denaturation
40 cycles:		5 sec	94°C	Denaturation
	5 cycles:	5 sec	52°C	Annealing
		5 sec	80°C	Extension
1 cycle:		3 min	72°C	final extension
Final step:		indefinitely	4°C	hold.

Following extension reaction, extension products must be cleaned up to optimise mass spectrometry based detection. This was achieved by using a resin known as SpectroCLEAN (Sequenom). This consist of a cationic resin which is pre-treated with an acidic solution used with the goal to remove salts such as Na⁺, k⁺ and Mg²⁺ ions which in spectrometric measurements create high background noise.

The slurry of the resin was prepared by adding 56g of resin to 80ml of ultrapure water and bubbles removed firs by swirling and then by applying slurry slowly unto a nonmagnetic mixing device that operates via a vacuum diaphragm pulsing geysers at variable amplitude and capable of protruding to the surface of the reservoir liquid. 16ul of this slurry preparation was added to each of post extension product wells of the 96 well plates and the plates rotated using a rotator for 30 minutes. The plates are then centrifuged again for 1 minute at 425 x g at room temperature. The clean extension products are removed from the supernatant automatically using Spectro-Point. These clean extension products are now ready for spotting and detection using mass spectrometry.

2.8.9. Detection of primer extension products by matrix assisted light desorption time-of-flight mass spectrometry.

Spotting Primer extension products on SpectroCHIP.

For the extension products to be effectively incorporated with the appropriate matrix for time-of-flight mass spectrometry, the oligonucleotide products (25nL) must be arrayed onto existing matrix spots on silica chips based on capillary action of slot pins and contact dispensing. The process involved computer controlled automatic spotting. In this process, the nanopoint is pre-conditioned with 100% ethanol and then washed through ten cycles. The silica chips are labelled and selected loaded in the deck for spotting with both extension product and calibrant. The appropriate program selected from the software is used to spot analyte (extension products) and then calibrant onto appropriate chips. This is done such that the pins do not hit the resin. The chips are then covered with the sealer.

2.8.10. MALDI-TOF Mass Spectrometry.

The Sequenom MassARRAY system relies on MALDI-TOF (matrix assisted laser desorption/ionization-Time-of-Flight mass spectrometry). In principle, a focused laser beam, either in the UV or infra-red ranges is applied to the chip. The matrix absorbs the light energy heating up and causing the illuminated substrate to vaporise into the vacuum. The expanding matrix assists the ionisation of the substrate which is transferred electrostatically into a time-of-flight mass spectrometer. Here they are separated from matrix ions and detected based on their time of flight proportional to their mass-to-charge ratio (m/z).

- Methodology

The chips were loaded into the compact Spectrometer. After loading the chips, the software was opened and the chips to be analysed selected in the computer interface. The appropriate assay name was then inserted and analysis autorun. The resulting spectra were analysed by SpectroTyper which combines base calling and cluster algorithms.

2.8.11. SNP and genotype calling.

The process of converting base calls and quality scores into a set of genotypes for each individual in a sample is often divided into two steps: genotype calling and SNP calling. SNP calling aims to determine in which positions there are polymorphisms or in which positions at least one of the bases differs from a reference sequence. Genotype calling, on the other hand, is the process of determining the genotype for each individual and is typically only done for positions in which a SNP or a 'variant' has already been called. Genotype calls were obtained in real time during chip detection. Spectrometry traces can be visualised immediately after detection using

SpectroTYPER-RT (Sequenom). The software allows simultaneous visualization of all assays in a multiplex reaction and which can be decomposed into specific desired assay(s). The software uses assay design information to calculate the expected position of analyte (extension product) peak in the spectra. Therefore, it was important to carefully design assays before the runs.

2.8.12. Genotype and data quality control

. Association studies require rigorous assessment of genotyping quality to minimize sources of error. Difficulties include sample misidentification, failure of specific assays, low genotyping call rates and poor reproducibility. Low call rates for a SNP may indicate the assay did not work well and a low call rate for a set of genotypes from an individual might be indicative of a problem with DNA quality.

Routine quality control of data generated in the sequencing laboratory was performed on a daily basis. Quality control was done to identify if SNP call or genotype call met the minimal criteria for acceptance.. Results were initially evaluated according to the fraction of assay successes per plate. For genotyping plates, an assay failed if it did not meet minimal criteria of mass spectral quality as determined in real-time by the MassARRAY software. If greater than 10% of assays on a plate failed, then the plate failed quality control, and the spectra was inspected before a repeat run was done. In addition to assay success rates, genotyping plates were reviewed for results from positive- and negative-DNA control wells that were organized in specific patterns to assist in the quality control process and to ensure correct plate orientations during processing and data review. The choice of threshold for identifying significant deviations from HWE depends on the sample size and other indicators of data quality. The actual distribution of the HWE statistics in the sample was used to set an appropriate threshold. Genotyping accuracy in this present study was assessed by testing the conformation of the observed genotype distributions in the controls to the expected distributions under Hardy-Weinberg equilibrium (HWE). Assays which deviated from HWE at the 0.1% significance threshold were excluded from further analysis.

2.9. Data Analysis.

2.9.1. Definition of explanatory variables.

Two major discrete analyses were conducted. The first analysis involved the association between parasite mutations in specific defined genes and treatment response classified as failure or success. In this analysis, the independent variable was the presence of parasites with drug resistant mutations in defined parasite genetic loci.

The second major analysis concerned the association between candidate immune genes and clearance of parasite with drug resistant mutations at defined parasite genetic loci. The independent variable in this case was the candidate immune gene polymorphisms.

2.9.2. Definition of the dependent or response variable

Clearance of parasites with drug resistant mutations was considered as the primary dependent variable during the analysis. Clearance of parasites denoted a slightly different notion from parasite clearance estimations. Clearance of parasites was considered to be the proportion of those who successfully resolved their fever and parasite loads during the 28 days follow up period, as opposed to the parasite clearance time estimated during the treatment period. Clearance was categorised into “Failure” or “Success”. In this study, failure was defined as the proportion of patients with the combined treatment failure obtained by adding proportions of children in failure categories. i.e. ETF, LCF, LPF while “Success” was defined as the proportion of patients experiencing an ACPR irrespective of their parasite drug resistance status.

In addition, anaemia, measured using a portable Smartsan haemoglobin analyser on days zero, seven, and twenty-eight and any day of failure or adverse outcome was evaluated. Routine haematology included white blood cell count, platelet count and white blood cell differential. Since data obtained from the study sites with different response rates to antimalarials will be aggregated for analysis, study factors which apparently could modify treatment response were accounted for in the sample size estimation and efficacy analysis using corrected statistics for confounder effects.

2.9.3. Statistical analysis

A number of different statistical tools were used in this study to achieve our results. First, to evaluate the levels of different molecular markers in different study ecologies from genotyping parasite isolates, the proportions were calculated, expressed as the fraction of isolates expressing the genotype per hundred. This was expressed as the prevalence of the mutation or haplotype when the isolates genotyped for the mutations were collected on day 0, i.e. before treatment. This proportion was expressed as the frequency of occurrence when the isolates genotyped for the various mutations were sourced from participants failing treatment during the study period. A comparison of the prevalence of alleles between three sites in the SP/AQ study used to investigate biological factors related to treatment response was done by using the following statistical test of hypothesis comparing the frequency.

where :

In addition, since we were interested in studying the relationship between the prevalence of molecular markers and treatment outcome, two categorical variables, we used the Chi square test to evaluate this relationship and to test for significance using the P value including the confidence interval. The difference in distribution of different drug resistant alleles between ecologies was also tested for by the use of the Chi square statistic. For this reason therefore, our variables were expressed as frequency counts as described above.

For drug resistant markers and associated treatment response, the Chi Square test was used to test the hypothesis that selection of resistant variants was associated with decrease response to antimalarial therapy.

For human SNP genotyping, we categorised participants as people who cleared and people who did not clear parasites with resistant mutations and people with high and persistent versus. non-persistent day 0 fevers, as the outcome. We used the presence or absence of SNPs in different immune genes as exposures in multiple pair wise analyses.

The effect of the above analysis was examined for confounders by stratification for variables such as ethnicity (Fulani Vs. non-Fulani) and site using the Mantel Haenzel common odds ratio. Because these exposure and outcome measures are categorical variables, the Chi square test or Fisher's exact test was used to determine the relationship between them. In addition, the strength of the relationship was determined by a calculation of the odds ratio. The odds ratio was chosen because of its beneficial quality of being insensitive to controls (people without the outcome), which for a case control design can influence the risk of developing the outcome of interest. Confounder effects were adjusted for by randomization, during participant selection into different arms of the clinical trials, double blinding and placebo control before the start of the study. A further statistical confounder problem in candidate gene association studies arises from multiple testing. This affects the interpretation of the data in the sense that we cannot, statistically speaking attribute a significant relationship between one exposure (e.g. a SNP in a particular gene) and an outcome measure (clearance of drug resistant parasites) when making several comparisons between SNPs combinations and treatment outcome .One way of going around this was to perform a pairwise analysis, and this was chosen for this study given that the sample size was not sufficiently powered to allow for significant differences to be expected from multiple testing analyses. However, for some SNPs, this was attempted. It is estimated that sample sizes for genome wide studies for association analysis should be in the order of millions to provide the necessary power to interpret significant marker-disease associations (Marquet S 2001). Since our

sample size was small, we perform correction for multiple comparisons using the Bonferroni statistics. In this case, the probability of a type I error is generally set at 5%. In a set of n tests, the overall risk of a type I error becomes higher than 5%. This overall type one error level for a set of n tests is defined by an upper bound determined by Bonferroni's inequality stated below:

Overall- $\alpha < n \times \alpha'$ where α' is the level of type 1 error for each of the n tests and was set at 5%.

Therefore the corrected α level for each SNP analysis after Bonferroni correction is given by α'/n , in order to keep the overall α -level at 5% (Shi 2012). This was applied in the analysis of the correlation between SNPs and treatment outcome. The Chi square P value as well P value of the Odds ratio and confidence intervals were calculated simultaneously for each SNP analysis. The Hardy Weinberg Equilibrium analysis was evaluated to test for the probability that the independence of two alleles at a particular locus is arising from a large random mating population with no selection. This was done to show that the study population before analysis was not suffering from effects that may influence the outcome of analysis and therefore blur the observations inferred from the population. This assessment led us not to consider doing a population sub-structuring to evaluate the effect of P values in different population backgrounds. A P-value of the odds ratio less than 0.05 for both pair wise analysis and Bonferroni corrected analysis was considered significant. The confidence intervals of all P values calculated were considered in the interpretation of significance levels. The study was carried out according to standard operating procedures following the guidelines of Good Clinical and Laboratory practices promoted by the World Health Organisation.

2.9.4. Sample size calculations:

Sample size for this research was primarily determined by the efficacy studies from which samples were collected. The sample size was calculated a priori with the assumption that the comparator drug would have a cure rate above 94%. To demonstrate with 95% confidence ($\alpha = 0.05$) that tested drug was acceptable if they are at worst 10% (d) inferior in the occurrence of failures, we would accept a 10% risk (β) to rule out the null hypothesis of the lack of inferiority.

$$\begin{aligned} \text{Using the formula with } f(\alpha, \beta) \text{ statistics: } N(\text{sample size}) &= [2p \times (100-p) \times f(\alpha, \beta)]/102 \\ &= (2 \times 94 \times 6 \times 10.5)/102 \\ &= 118 \text{ individuals in the smallest arm} \end{aligned}$$

Considering that other trials have reported loss to follow-up and withdrawal rates of 10% in 28 days follow-up periods, we assumed that a 20% loss to follow up and withdrawal for 42 days follow-up was reasonable. This allowed a size of 142. The least number of patients in the comparator arm was 170. In total, at least 284 patients were recruited for efficacy studies, 250 used

for characterising drug resistance genes per site, and 750 used in evaluating associations with treatment response and candidate gene association studies in the nested case control analysis.

2.10.Ethics Review

Ethical approval was obtained from the Institution Review Board of the Cameroon Baptist Convention Health Board, the National Ethics Committee of the Ministry of Public health, Cameroon and from the Ethics Review Committee of the London School of Hygiene & Tropical Medicine. The trial objectives and procedures were explained in French, English or a relevant local language (Pidgin, Fulfulde) to the parents or guardians of each potentially eligible study subjects and answers to any questions were given. Informed consent was obtained from the parents/guardians by signature or thumb mark. The interests of the patients were safeguarded by a local safety monitor and by an international Data and Safety Monitoring Board. The trial was also registered in the NIH clinical trials database (NCT00146718).

CHAPTER 3: RESULTS AND DISCUSSION

Results

3.1.Characteristics of study participants at baseline.

Table 15: Baseline characteristics of study participants in Garoua, Yaounde and Mutengene

Site	Mut			Yao			Gar		
Drug Groups	SPAQ	SP	AQ	SPAQ	SP	AQ	SPAQ	SP	AQ
Age (mean in months \pm SD)	29 \pm 15.5	27 \pm 14.8	27 \pm 15.2	29.4 \pm 14.5	30 \pm 15.1	29 \pm 13.8	27 \pm 15.4	26.6 \pm 16.5	27.4 \pm 17.2
Female/Male ratio	1	0.9	1	0.7	1.2	0.82	0.74	0.82	1.2
Weight (kg) (mean \pm SD)	12.5 \pm 3.5	12.2 \pm 3.2	12.2 \pm 4.1	13.02 \pm 3.0	12.6 \pm 3.4	12.9 \pm 3.2	10.84 \pm 3.3	10.86 \pm 2.7	10.59 \pm 3.3
GMP (\pm SD)	39601 (\pm 48160)	27014 (\pm 49560)	22072 (\pm 30260)	7776 (\pm 27120)	6721 (\pm 25200)	5364 (\pm 25720)	7264 (\pm 27730)	5668 (\pm 19040)	6294 (\pm 26890)
Temp $^{\circ}$ C (mean \pm SD)	38.9 \pm 1.1	38.7 \pm 1.1	38.7 \pm 1.0	38.2 \pm 1.2	38.3 \pm 1.0	38.4 \pm 1.2	37.9 \pm 0.6	38.0 \pm 0.6	37.7 \pm 0.7

Yao:Yaounde, GAR:Garoua, Mut: Mutengene.

In the study involving sites in Garoua, Mutengene and Yaounde, a total of 3,183 children were screened of whom 760 were included in the study. About three quarters of the participants were excluded on the basis of severe malaria (17.4%), parasite negativity (37.6%), residence outside the study area (17.1%), fresh scarification marks (3.6%), less than six months of age (19.5%) and refusal of consent (5.8%). Two hundred and fifty five children were allocated to receive SP, 253 to received AQ and 252 to receive SP+AQ as shown in Table 15 above.

3.2. Baseline characteristics of children included in the study in Ngaoundere:

The following table represents the baseline characteristics of 150 children enrolled in the drug trial in Ngaoundere.

Table 16 Baseline characteristics of 150 children enrolled in the Ngaoundere study

Characteristic		AS/AQ (n = 73)	AL (n= 77)	P - value
Sex	<i>Male</i>	39 (53.4)	39 (50.6)	0.73
	<i>Female</i>	34 (46.6)	38 (49.4)	
Age (years)	<i>Mean ± SD</i>	04.93 ± 03.64	04.94 ± 03.32	0.98
	<i>Range</i>	01.0 – 13.0	01.0 – 13.0	
Weight (kg)	<i>Mean ± SD</i>	16.87 ± 08.36	16.06 ± 06.95	0.52
	<i>Range</i>	07.0 – 41.0	06.0 – 35.0	
Temperature (°C)	<i>Mean ± SD</i>	37.95 ± 01.19	37.91 ± 0.79	0.80
	<i>Range</i>	31.1 – 40.5	35.5 – 39.8	
Haemoglobin (g/dl)	<i>Mean ± SD</i>	10.63 ±2.65	10.38± 2.42	0.55
	<i>Range</i>	4.8 – 28.0	4.9 – 17.8	
Anaemia	<i>(Hb < 9.5 g/dl)</i>	21/73 (28.8)	26/77 (33.8)	0.51
Parasitemia(/µl)	<i>GMPD</i>	4628.30	3886.36	0.18
	<i>Range</i>	1340 – 184 000	1240 – 156 000	

A total of 150 children were recruited during the study period. As observed in Table 16, no significant differences in sex, age, weight, temperature haemoglobin levels, parasite density and anaemia status between AS/AQ group and the AL group existed. There were 78 (52%) male and 72 (48%) female patients enrolled in this study. Their overall mean (\pm SD) age, weight and temperature values were 04.95 (\pm 3.47) years, 16.6 (\pm 7.66) kg and 37.9 (\pm 1.002) °C. The geometric mean baseline parasitemia was 4231 parasites / μ L of blood. With respect to age, 56% of patients were \leq 5 years of age, and 44% were 6–14 years of age. Using these categories there was no

difference between response to the two drugs used and number of subjects in each category ($p > 0.05$).

3.3. Baseline characteristics of children in the Bangolan study

The following table provides the baseline characteristics of 300 children enrolled in the Bangolan study.

Table 17 Baseline characteristics of children enrolled in the Bangolan study

Drug Arm	ASAQ	AL	p-value
Male/Female	110/116	38/36	0.506
Age(months)	41.45(\pm 23.79)	43.15(\pm 25.99)	0.275
Weight	13.83(\pm 4.52)	14.6(\pm 5.72)	0.748
Temperature($^{\circ}$ C)	38.5(\pm 0.8)	38.3(\pm 0.7)	0.904
Parasitemia(/ μ l)	15539,18	14425.58	0.528
Haemoglobin (g/dl)	8.58(\pm 1.99)	8.66(\pm 2.00)	0.973
Glycaemia (mg/dl)	105.2(\pm 27.1)	109.3(\pm 25.3)	0.236
WBC(cells/ml)	7165(\pm 1851)	7568(\pm 1294)	0.328
Platelets ($\times 10^3$)	2243(\pm 271.1)	237.5(\pm 291)	0.463
Neutrophils (%)	47.8(\pm 11.9)	44.35(\pm 12.4)	0.616
Eosinophils (%)	1.78(\pm 0.87)	1.00(\pm 1.0)	0.860
Basophils (%)	0.25(\pm 0.24)	0.4(\pm 0.32)	0.580
Lymphocytes (%)	43.03(\pm 11.31)	43.5(\pm 14.74)	0.665
Monocytes (%)	3.84(\pm 2.81)	3.38(\pm 1.94)	0.498

Out of the 1416 children screened, a total of 1116 children were not eligible for the study. The commonest cause for exclusion from the study were (i) low parasite count ($n=306$, 27.4%), (ii)

distance from study facility (n= 196, 17.6%), (iii) out of age range (n=216, 19.4%), (iv) Concomitant infections (n=52, 4.7%), (v) danger signs (n=69, 6.2%), (vi) refuse consent (n=15, 1.3%), (vi) previous drug treatment (n=76, 6.8%) and (vii) others (n=186, 16.6%). Three hundred (300) children were enrolled for the study. There was no significant difference between the two treatment groups with respect to the baseline characteristics (Table 17). Of the total number enrolled, 79.14 % were less than 5 years.

3.4. Distinction between recrudescence and re-infections based on the highly polymorphic *msp-2* gene.

Pre- and post-treatment parasite genetic profiles of the *msp-2* gene were compared to distinguish recrudescence from reinfections. Previously, and in other reports, we showed that distinction based on patterns of *msp-2* were not significantly different compared to using *msp-1*, *msp-2* and *glurp* genes. Thus, using only *msp-2* represented for us a cost-effective way of distinguishing recrudescence from reinfections. Figure

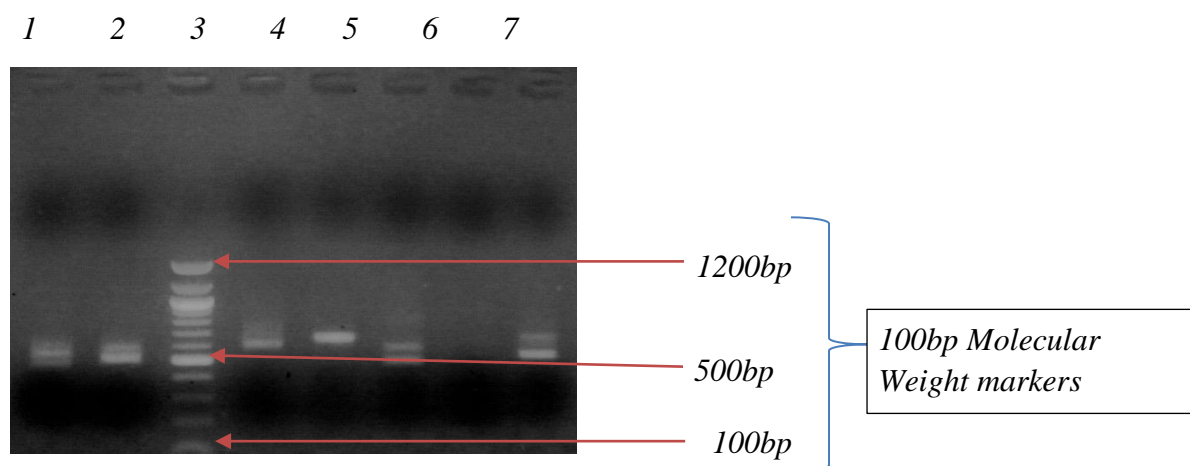


Figure 11: Distinction between recrudescence and reinfection based on genotyping *msp-2* genes in pre-and failure samples.

Legend. The bands represent patterns of *msp-2* for patients 1, 4 and 5. The first two lanes (1 and 2) represent *msp-2* at pre-treatment (1) and at time of failure (2) and therefore indicates a recrudescence based on the similarity. On the other hand, bands on lane 4 and 5 are dissimilar, representing a reinfection in patient 4. Bands in lane 5 and 7 are also similar, and represent an indeterminate result. Lane three represents the molecular weight marker.

3.4.1. Allelic variation in pre-treatment parasites based on *msp-2* gene

Allelic variation of infecting parasites based on the *msp-2* gene varied between Garoua, Yaounde and Mutengene. Alleles in infections in Garoua varied from 219bp to 818bp and about more than 50% of alleles were between 480-620bp with a peak around 570bp. In Mutengene or Yaounde,

although the majority of alleles observed ranged between 415bp – 690bp, there were no alleles less than 400bp in the infections (Data not shown). The mean number of alleles per infection as a proportion of the total number of patients obtained, also known as the multiplicity of infections was statistically different between the sites. In Mutengene, double infections were majority, although difference in the multiplicity of infections was not significant. The histogram in Figure indicates these differences.

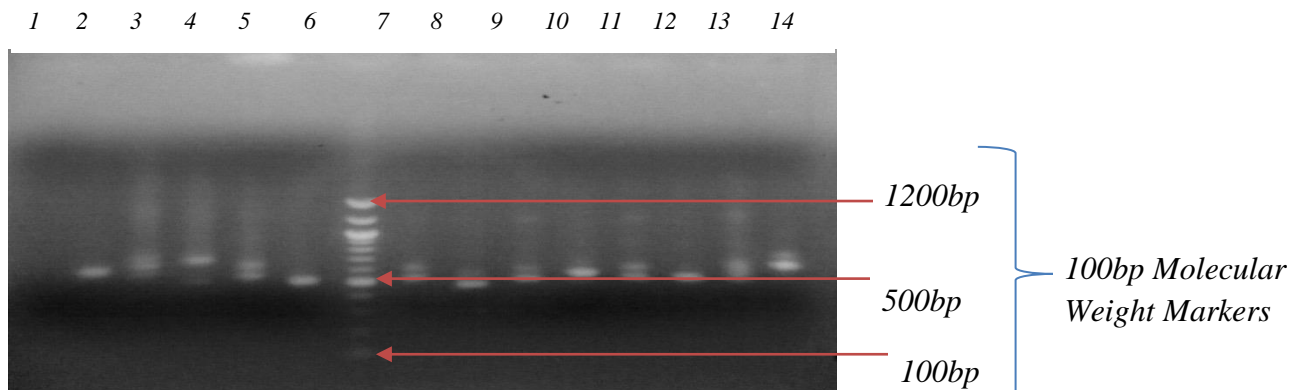


Figure 12 :Cross-section of bands patterns of msp-2 in samples used to determine parasite diversity in the study population

Legend. Lanes 1-15 (except band at lane 7) represent migrations of pre-treatment samples based on the msp-2 gene. The number of bands in each lane indicates the number of different parasite clones in the infection. Lane 7 represents the migration of 100bp molecular weight marker.

We also sought to characterise the parasite genetic diversity as a function of msp-2 genetic profile for three sites in Cameroon. The figure below provides a snapshot of the genetic diversity.

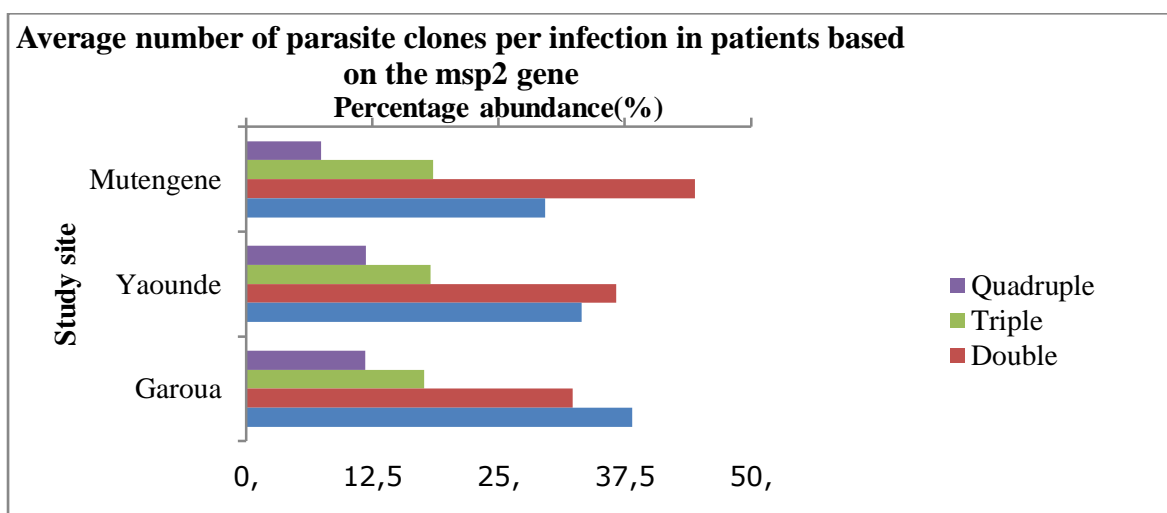


Figure 13: Parasite genetic diversity as a function of the msp-2 genetic profile

3.5. Baseline prevalence of drug resistance mutations in five genes in five ecological sites of Cameroon.

Mutations conferring resistance to fansidar (SP) and amodiaquine (AQ) were genotyped and constructed into haplotypes for some of the combinations of mutations in the three study regions. The prevalence of and distribution of these mutations was studied and compared for all the regions. In addition, the distribution of mutations at efficacy endpoints of both day 14 and day 28

were compared to attempt an effect of the drugs on the distribution of these mutations. Even though a detailed effect of drug selection on genes was not presented, it was necessary to observe the changes in parasite allelic frequency between day zero, day 14 and day 28.

3.6. Comparison of baseline prevalence of resistance conferring mutations to AQ between the northern and southern ecologies.

The prevalence of resistance conferring mutations to AQ involved the genotyping and percentage expression of the Pfcrt and pfmdr-1 haplotypes among isolates collected on day 0 in the three study sites. As seen in the table below, the baseline prevalence show some differences between the sites.

Table 18 frequency of resistance conferring mutation to AQ the northern and southern ecologies

Table 18

Mutation/Site	Garoua (G)	Mutengene(M)	Yaounde (Y)	M-G	P-value	Y-G	P-value
pfcr-76T	53.8	96.8	92.1	40	<0.001	38.3	<0.001
pfmdr1-86Y	46.4	83.6	94.3	36.6	<0.001	47.9	<0.001
pfmdr1-184F	78.6	97.3	97.6	18.7	<0.002	19	<0.001
pfmdr1-1246D	1.5	3.1	8.9	1.6	>0.05	8	0.017

We observe from the above table that for both *pfmdr1-86Y* and *pfcr-76T* mutations, implicated in in vivo resistance to AQ, there is a significant difference in frequency of alleles from Garoua and Mutengene. Indeed, we observed a two-fold frequency of *pfcr-76T* in Mutengene compared to Garoua and this was significant. The same significant observation between the two sites was made for *pfmdr1-86Y* (Table 18). With regards to *Pfmdr-1-184F* and *pfmdr-1-1246Y* in Mutengene and Garoua, the *pfmdr 1-184F* known to be key in artemether-lumefantrine resistance was found to be significantly different between the north and the south. The prevalence of *pfmdr-1-1246Y* was not different between the sites ($P=0.55$). It should be noted however, that the latter two mutations do not contribute as much as the first two in the resistance phenotype.

The same observation is made when isolates from Yaoundé were compared with isolates from Garoua for the studied AQ resistance mutations. The *pfmdr 1-86Y* and *pfcr-76T* mutations were significantly different ($p<0.0001$) between the two sites, further highlighting the differences observed when comparing isolates from Garoua and Mutengene. Furthermore, there was no significant difference between isolates from Yaoundé and Mutengene with respect to these AQ resistance defining mutations among isolates. Meanwhile the prevalence of the 1246Y mutation in all three sites was low; the difference was significant comparing the prevalence in Mutengene and Yaoundé ($P=0.017$, 95%CI: 1.10-10.81).

On the basis of these observations, parasites circulating in the north region of Cameroon significantly differ from parasites circulating in the southern region when comparing the prevalence of AQ resistance defining mutations.

3.7. Baseline prevalence of resistance conferring mutation and clinical responses

The baseline prevalence of the various mutations conferring resistance to AQ was put in the context of *in vivo* resistance to AQ observed during the clinical trial efficacy analysis. The *in vivo* resistance to AQ was defined as the proportion of patients enrolled in the AQ treatment arm that experienced a treatment failure. This was expressed as the combination of early treatment-, late clinical and parasitological failures as a percentage of the total patients enrolled in the AQ arm following WHO criteria. Parasite failures were corrected by genotyping polymorphic markers to make a difference between true failures and re-infections in this analysis.

Table 19: Baseline occurrence of resistance conferring mutations and clinical resistance levels to amodiaquine in the five sites.

	<i>Pfcr</i> t-76T	<i>pfmdr</i> -1-86Y	<i>pfmdr</i> -1-184F	<i>pfmdr</i> -1-1246Y	N86Y
Mutengene	97,0	83,6	97,3	3,1	84
Yaoundé	92,1	94,3	97,6	8,9	94
Garoua	53,9	46,5	78,6	1,5	47

The above table (Table 19) represents the prevalence of all the mutations related to AQ resistance considered in the analysis as well as the level of *in vivo* resistance observed in the study. For Mutengene and Yaoundé where mutation rates are comparable for the major resistance defining mutations to AQ, the level of AQ resistance is also comparably similar, though the prevalence of mutations is inversely related to the level of AQ resistance. This observation suggests that other factors may be involved in the expression of *in vivo* resistance. When only one mutation, the N86Y of the *pfmdr*-1 gene was considered, the same observation was obtained, suggesting that this mutation may be the principal mutation modulating resistance levels to AQ in the regions in the background of the other mutations notably the *pfcr*t-76T mutation. Figure 14 summarises the baseline levels of various mutations contributing to AQ and CQ resistance in Cameroon.

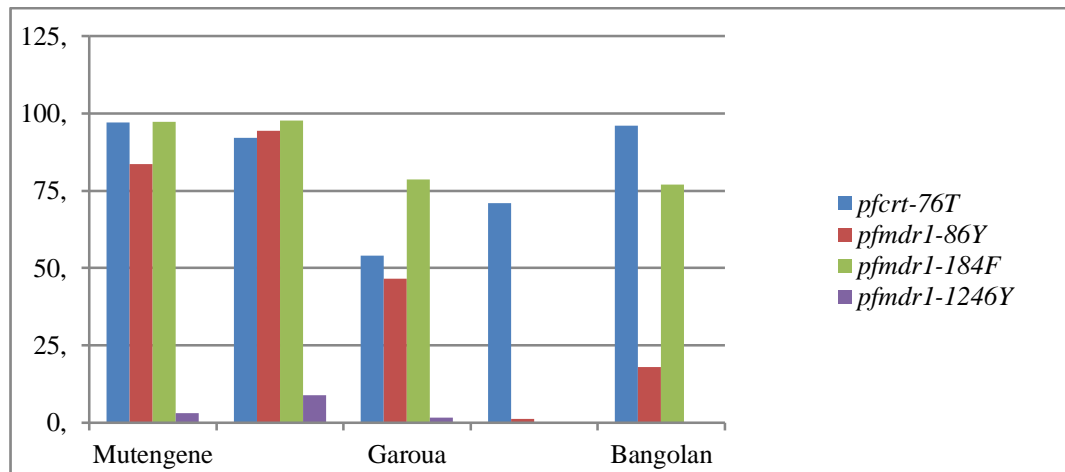


Figure 14: Baseline chloroquine and amodiaquine resistance mutations in five geo-ecological sites in Cameroon

In Bangolan, the *pfmdr1-184F* mutants predominantly circulate, with low levels of the 86Y mutation. Similar observation was made for Ngaoundere, where the resistant *pfmdr1* was found in only 1% of isolates. Generally speaking, parasites circulating here carry the wild type alleles associated with increased sensitivity to AQ. Of note, we observe close to 78.2% of the wild type alleles at the position 86 in Bangolan. Alleles carrying the 1246Y mutation were not found among isolates from this region, as opposed to other regions albeit in low levels. Therefore, more AQ sensitive parasites circulate in Bangolan compared to the other areas under study. On the other hand, CQ mutant parasites circulate widely within the five regions, with the observation that Garoua had the lowest prevalence. In Bangolan, the mutant allele appears to be fixed in the population of parasites circulating there.

Considerable variations in frequencies of mutations in genes associated with reduced sensitivity to fansidar were observed. The haplotypes, a group of genetic determinants inherited together and that influence a particular outcome, better reflects resistance or sensitivity. In describing fansidar resistance, point mutations in *dhfr* and *dhps* genes constructed by combination presents the various haplotypes. The figure below presents constructed haplotype variations between various sites.

From Figure 15 below, there are considerable differences in the occurrence of haplotypes among isolates from different regions. Firstly, the SGK haplotype includes mutations at codon 437 and principally implicated in resistance to fansidar in confirmed studies in Africa and Asia. Its lowest prevalence occurs in Garoua, and highest in Mutengene, greater than four times its occurrence in Garoua and almost twice its occurrence in Yaoundé. This haplotype occurs at a frequency of 33.5% in the marshy rice farming environments of Bangolan, close to twice the fre-

quency in Garoua. Generally speaking, the prevalence of the SGK haplotype decreases from south to north and from littoral forest through the savannah to sahelian regions. On the other hand, this finding is corroborated by the variation in other haplotypes. For example, the AAK haplotype, considered as the wild type haplotype occurs more frequently in Garoua. Indeed, this haplotype is five times more frequent in Garoua compared to Mutengene and almost twice more frequent than in Yaoundé. On the other hand, the AGK haplotype, representing a mutation at codon 436 and known to contribute only marginally to fansidar resistance appears to follow a different trend. It is lowest in Mutengene and highest in Bangolan, with no discernable trend in the various sites.

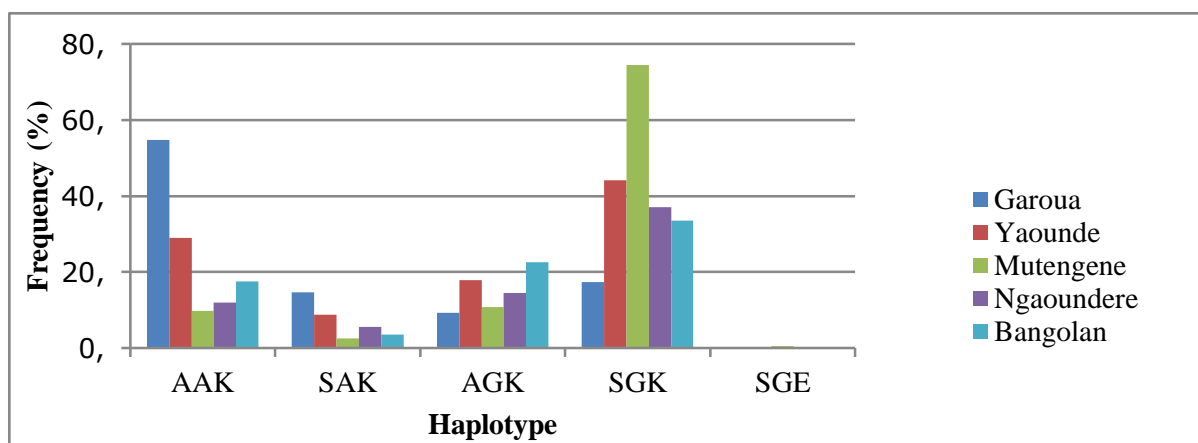


Figure 15: Frequency of *pfdhps* haplotypes in five ecological sites in Cameroon.

It is widely considered that mutations in the *pfdhps* gene occur after mutational events in the *pfdhfr* gene of parasites with reduced sensitivity to fansidar. In Africa, mutations occur principally at codons 51, 59 and 108 in the *pfdhfr* gene and contribute to resistance to fansidar. Haplotypes were constructed for various combinations of mutations among circulating parasites.

Below, we find haplotypes constructed for parasites in the five regions.

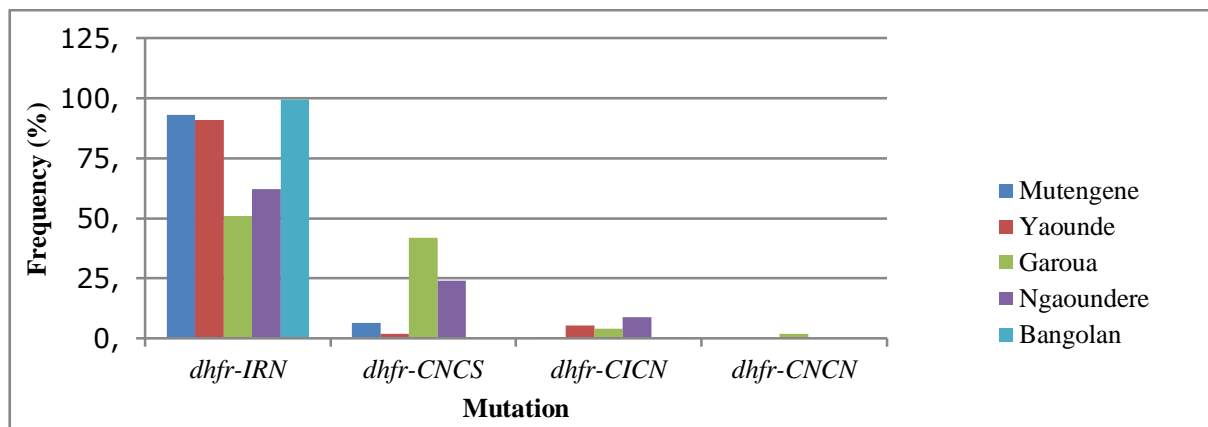


Figure 16: Key *dhfr* haplotypes observed in five study regions.

Key haplotypes of the *pfdhfr* gene were observed in all the study sites, although in various proportions. Haplotype IRN which corresponds to point mutation changes in the *pfdhfr* codon 51, 59 and 108 was the dominant haplotype observed. The haplotype frequency decreased from south to north. In one site (Bangolan), all parasite isolates tested possessed the IRN haplotype. A few mixed parasite isolates were obtained in Bangolan, but were left out because haplotypes of mixed infection could not be constructed.

Based on the above histogram, we observe a greater than 90% frequency of the IRN haplotype in isolates coming from all study sites except in Garoua where the haplotype frequency was 50%. In Bangolan, the frequency was found among 100% of isolates while 95% of isolates circulating in Mutengene carried the haplotype. It appears therefore that in three of the four regions considered for this analysis, the majority of circulating parasites already carry mutant alleles to sulfadoxine resistance. On the other hand, the sensitive NCS haplotype occurred at low frequency, highest in Garoua (33%), followed by Yaoundé (3.9%) and was not seen in Bangolan (0%).

3.8. Distribution of mutations according to drug response classification

Parasite mutations and haplotypes in genes implicated in drug resistance of isolates among those from whom a treatment outcome could be determined were also studied. The efficacy endpoints for this study were the Day 14 and Day 28 cure rates. Treatment success was defined as the proportion of patients who responded with an adequate clinical and parasitological response while failure was defined as the proportion of all patients who experienced either an early or late clinical or parasitological failure based on the same WHO criteria.

3.9.Haplotype frequencies for the *pfcr* gene among patients with Day 14 and Day 28 outcome.

The following histograms(Figures 17 and 18) show the variations in the frequencies of the various haplotypes of the *pfcr* gene studied. The haplotype frequency distribution for the *pfcr* in Garoua shows the same trend when we consider those patients who failed or cleared their parasites adequately by day 14 and 28. Hence, we observed that there is no change in the frequency of the CVIET (10%) or the mixed haplotype (4%) at day 14 or 28 parasite endpoints. However, there appear to be a non-significant decrease in the CVMNK haplotype both among those who cleared their parasites and those who failed by day 28. On the other hand, although the same trend of no change in haplotype frequencies is observed by day 14 and day 28 parasite endpoints for the Mutengene site, there are some differences.

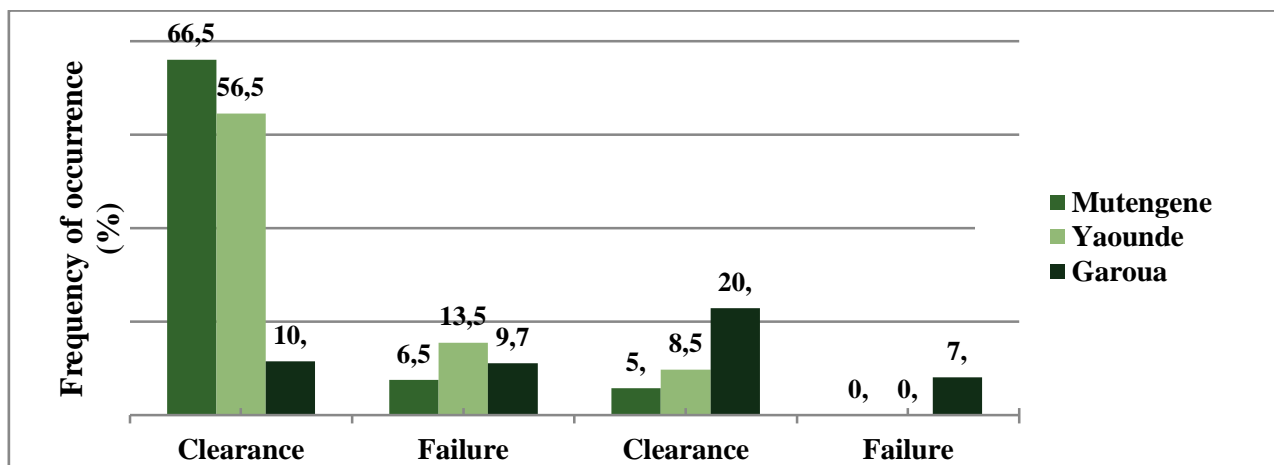


Figure 17 Haplotypes frequencies for the *pfcr* in isolates from patients with Day 14 clearance or failures in Mutengene, Yaounde and Garoua

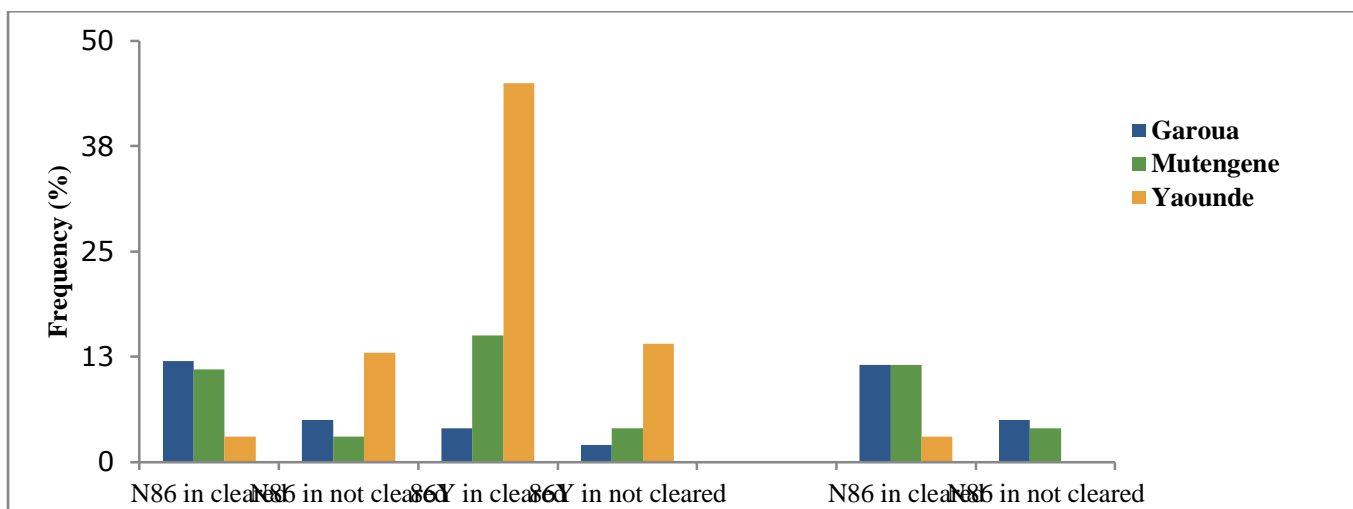


Figure 18 Haplotypes frequencies for the *pfmdr-1* gene among in patient who clered or not comparing Day (left panel) and endpoints in Mutengene Yoaounde and Garoua

Analysis of the *pfmdr-1* haplotypes among isolates from different study sites and during different endpoint classifications is presented in Figure 16. As shown in the above histograms, the prevalence *pfmdr-1* mutations differed between sites and days of follow up after treatment for both cleared and failure parasites. However, the distribution of mutation frequencies at each site shows a general recognizable pattern. In this light, we observe increasing frequencies for both the failures and clearance in all studied loci in the *pfmdr-1* gene on day 28 compared to Day 14 for Garoua. At the 1246 loci, the day 14 frequency for clearance is lower than the Day 28 frequency. On the other hand, in Mutengene, there tended to be a general reduction in the frequency of the mutations at all studied loci in the *pfmdr-1* gene in the clearance and apparently no change in the frequency among failures. On day 28, we observe the appearance of the 1246Y allele in the Garoua failure parasites although the frequency was less than 3%. This indeed is an indication that with increasing amodiaquine treatment pressure, *pfmdr-1* 1246Y alleles may be selected for. Of particular note, the *pfmdr-1* 1042 mutation associated with AQ resistance in different areas is absent from both sites considering isolates from those who cleared or did not clear their parasites adequately at both day 14 and day 28.

3.10. Haplotype frequencies of the *pfdhfr* and *pfdhps* genes in isolates classified for day 14 and day 28 endpoints

Figure 19 below shows the variation in frequencies of haplotypes in the *pfdhps* and *pfdhfr* genes in isolates after day 14 and day 28 classification of treatment response

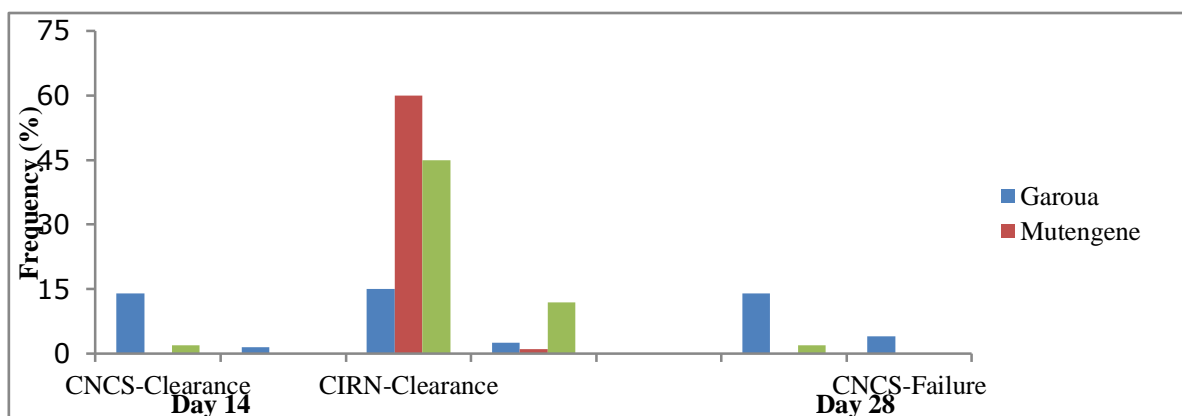


Figure 19: Haplotype frequencies of different *pfdhfr* mutations between Mutengene, Yaounde and Garoua in both failure parasites and ACPRs at day 14 and day 28 classification endpoints.

Key haplotypes of *dhfr* observed for Garoua, Mutengene and Yaounde were

Figure 19 Haplotypes frequencies of the *pfdhfr* and *pfdhfr* genes in isolates classified for day 14 and day 28 endpoints

Legend CIRN(mutant) and CNCS(wild). The histogram shows that the haplotypes were prevalent prior to treatment, but in low frequencies for those who failed treatment.

The haplotype frequency distribution in Garoua between day 14 and day 28 for both failures and ACPRs followed a general trend. These frequencies tended to be fairly stably distributed with a generally greater than 20% AAK through very low frequencies (less than 1%) for the FAE, FAK and SGE haplotypes to a stable 10% for the SGK haplotype. On the other hand, we observe a decrease in the frequency of all haplotypes between day 14 and day 28 for both failure and ACPR parasites in the sites.

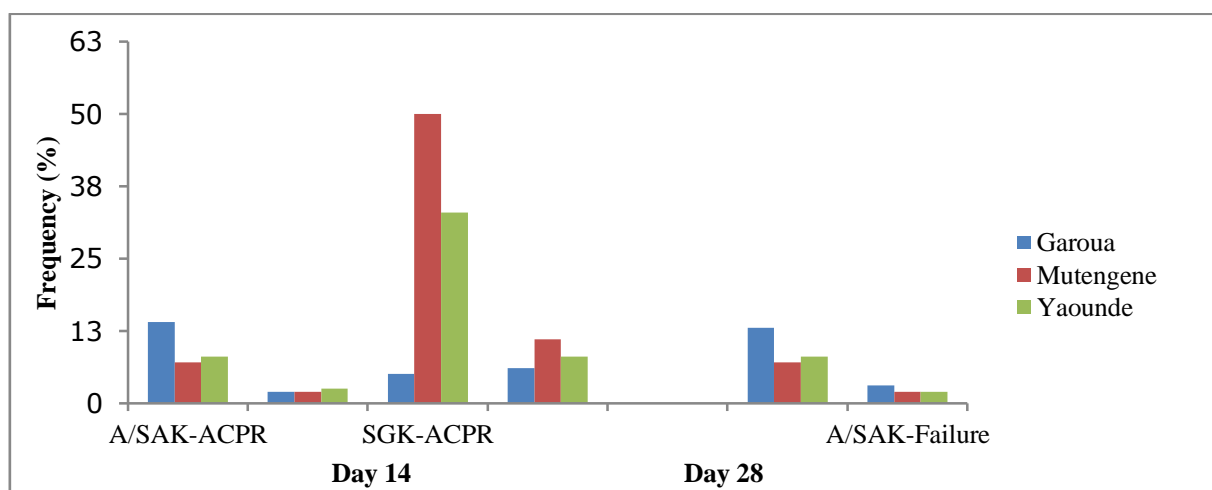


Figure 20: Haplotype of *dhps* in different sites were not by isolated in clinical failures.

We remark in Mutengene the very high frequency of the SGK haplotype among parasites on day 14 compared with the frequency on day 28 for parasites with ACPRs. Indeed, this haplotype shows the greatest frequency among isolates at both efficacy endpoints and across the sites irrespective of whether the patients infected with these isolates adequately cleared their parasites or failed to do so. Furthermore, the number of haplotypes was found to be lower for the Mutengene and Yaoundé sites compared to the Garoua site considering the day 28 endpoint; with some haplotypes completely disappearing in among the Mutengene isolates (FAE, FAK, and SGE, not represented). We also remark that the magnitude of decrease in the frequency of haplotypes for all haplotypes differs between isolates from Mutengene and Yaoundé compared to those from Garoua.

As can be observed in the figures above, there are also marked differences in the prevalence of certain haplotypes between sites and different endpoints. Of note, all the haplotype have

a greater frequency in Mutengene and Yaoundé compared to Garoua for both failures and ACPRs on day 14 and day 28. The prevalence of the SGK haplotype linked with SP failure for example was more than four times that for Garoua on Day 14 and only slightly different from Garoua at Day 28 classification. Following the same pattern of reasoning, we observe a disappearance of parasites with the SGE haplotypes by day 28. Parasites with mixed haplotypes (both sensitive and resistant mutations) were of comparable frequency at day 14 for both Garoua and Mutengene while at day 28, this frequency decreased for parasites from Mutengene.

Of particular interest, we notice the apparent inexistence of the SGE haplotype, which has been implicated in high resistance to fansidar in East Africa. In Garoua, there was no trace of the haplotype at different endpoints or failure and ACPRs meanwhile this allele was barely observed (less than 0.5%) in Mutengene at baseline at day 14, but completely disappeared when isolates were classified by Day 28.

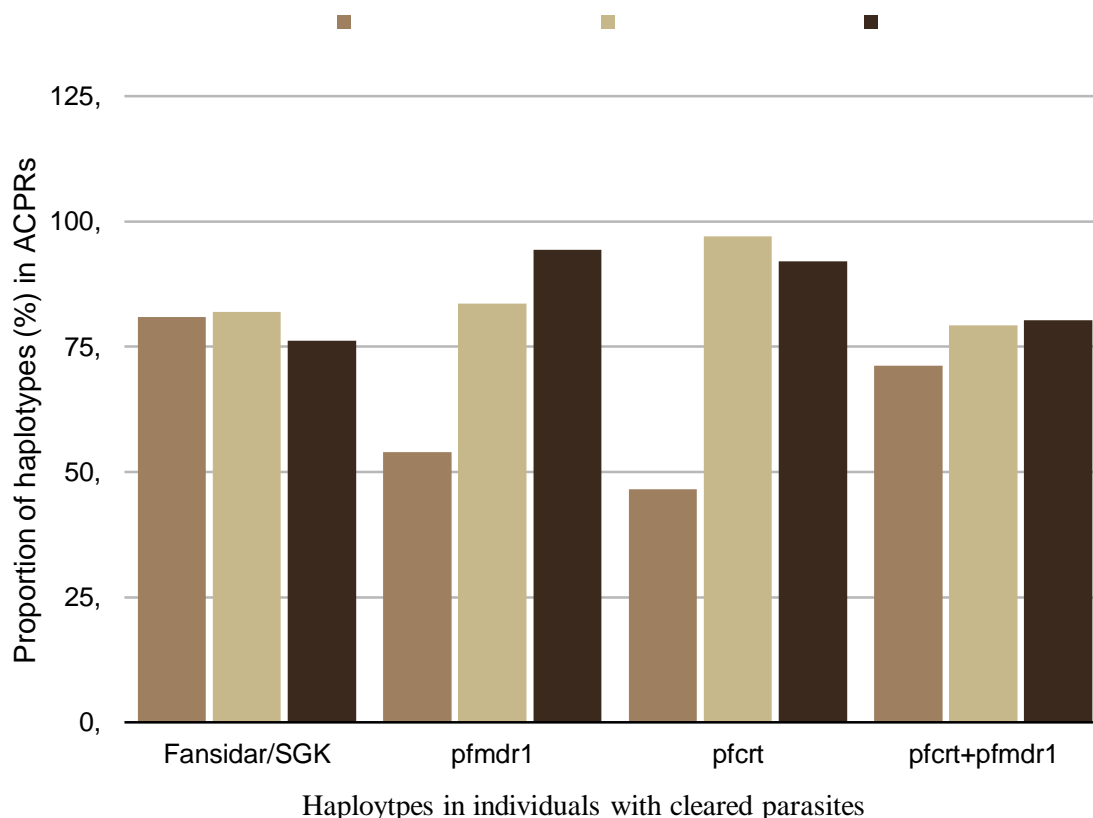
3.11. Combined resistance conferring haplotypes in adequate clinical and parasitological responses.

The following figure (Figure 19) shows levels of circulating mutant haplotypes conferring AQ and SP resistance and the cure rates expressed as a proportion of all ACPRs in various study sites.

We observe very high levels of *pfprt-76T* haplotype in Mutengene and Yaounde respectively compared to the levels in Garoua (less than 55%). The same observation can be made when we consider the *pfmdr-1-86Y* haplotype in the different ecologies. However, the frequency in Yaounde is highest, followed by Mutengene. The level of circulating parasites with *pfmdr-1-86Y* was lower in Garoua than the *pfprt-76T* haplotype. When compared to the level of adequate clinical response among the patients, the same trend as above was followed. However, the differences between the ecologies appeared to be non-significant ($P>0.05$). When analysed for levels

Figure 21: Resistance conferring haplotypes in adequate clinical and parasitological response.

of resistant haplotypes and ACPRs, we observe an inverse significant relation ($P<0.05$) between the frequency of the two mutant haplotypes and the observed ACPR for AQ in Garoua. In Yaounde and Mutengene sites, this relation was direct and non-significant. However, the level of circulating resistant haplotypes in each case was slightly higher than the observed ACPR re



sponse for AQ. In the same vein, we sought to understand how the *pfdhps* haplotype would distribute among those who adequately cleared parasites as registered in the efficacy outcome

classification. Figure 19 also indicates the SGK haplotype as a function of the ACPR is highest in Garoua, and lowest in Yaounde. When compared to Mutengene, The proportion is similar to that in Garoua. Under normal circumstances, regions with high SGK haplotype frequency should have a lower ACPR and vice versa. We observe that this trend is not respected for Garoua. Despite differences in frequencies of SP resistant mutations between Garoua and Mutengene, the proportion of SGK/ACPR is similar, and is markedly different for Yaounde; which compares better with Garoua compared to Mutengene. This observation lends to the hypothesis that differences in certain biological factors might help explain better the contradictory findings observed. Factors that may interfere in this relationship between resistant mutations and cure rates include immunity, ethnicity and geography.

3.12.Cure rates for amodiaquine, fansidar and the combination of both drugs combined for the three study sites.

The combined efficacy outcome following treatment of children in Garoua , Mutengene and Yaounde. Data were pooled together for each of the arms in each of the three sites and the cure rates calculated as the proportion of patients experiencing an adequate clinical and parasitological response by day 28 after drug intake. Classification of response followed criteria by WHO(W.H.O 2003) protocol for evaluating therapeutic response to antimalarial drugs. The following histograms indicate how children respond for each of the drug arms in the study.

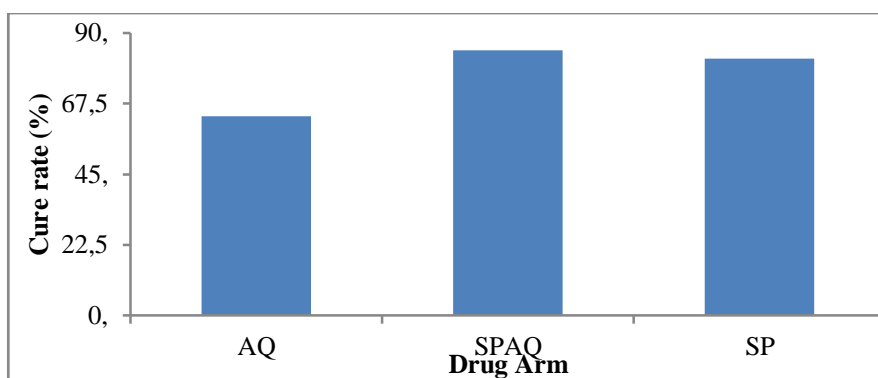


Figure 22: Combined Cure Calculated for the Three Study Site

The cure rates at endpoint with amodiaquine used alone was lowest (63,6%), followed by SP and then the combination of the two (Figure 20). The differences in the cure rates were not significant between SP used as monotherapy and SP used as a combination with AQ. The cure rate for SP monotherapy was found to be higher compared to the cure rate for AQ monotherapy. Overall, the cure rates with AQ-SP combination was superior compared to cure rate obtained

with AQ or SP. However, the cure rate for this combination was significantly superior only when compared to treatment with AQ alone (Figure 23).

3.13. Association of drug resistant mutations with response to treatment.

The prevalence of different drug resistant alleles was assessed for their contribution in the occurrence of treatment failure within the populations irrespective of the population geography. Haplotypes conferring drug resistance status were combined for both the *pfert*, *pfmdr-1*, *pfdhps* and *pfdhfr* genes. The tables in the following page summarise the analysis.

Table 20: Association between the prevalence of resistant haplotypes in the *pfdhfr* and *pfdhps* genes and treatment failure to SP combination.

Genes	Genotype Status	Haplotypes	SP Treatment response				OR(95% CI)
			Failure	ACPR	X ² value	P value	
<i>pfdhps</i>	Wild	AAK +SAK	18	47	0.4	0.52	0.81(0.44-1.51)
	Mutant	SGK+SGE + AGK	59	188			
<i>pfdhfr</i>	Wild	CNCS	7	18	0.18	0.67	0.82(0.32-2.04)
	Mutant	CIRN+ CNRN + CICN	74	232			
<i>pfdhfr+pfdhps</i>	Mutant	CIRN/SGK	75	201	0.11	0.74	0.83(0.28-2.45)
	Mixed	CIRN/SAK	18	45			
	Wild	CNCS/SAK	5	11			

We used the WHO drug efficacy assessment protocol (W.H.O 2003) as the basis for classifying therapeutic responses including treatment failures. Haplotypes of mixed infections (wild type and mutant at each locus) were considered to contribute equally between the two outcomes, success and failure for all the genes in the analysis.

Table 21: Association between the prevalence of resistant haplotypes in the *Pfcrt* and *pfmdr-1* gene and treatment failure to AQ combination.

Genes	Genotype status	Allele/Haplotype	AQ treatment response				
			Failure	Success	X ² Value	P value	OR (CI)
<i>Pfcrt</i>	Mutant	<i>Pfcrt</i> 76T	66	241	0.035	0.85	0.94(0.46-1.88)
	Wild	<i>Pfcrt</i> K76	12	41			
<i>Pfmdr-1</i>	Wild	<i>Pfmdr-1</i> N86	11	67	3.74	0.03	0.50(0.25-1.02)
	Mutant	<i>Pfmdr-1</i> 86Y	66	209			
	Mutant	<i>Pfmdr-1</i> 184F	47	162	0.004	0.94	0.96(0.36-2.54)
	Wild	<i>Pfmdr-1</i> Y184	6	20			
	Wild	<i>Pfmdr-1</i> 1246D	69	268	6.90	0.75	
	Mutant	<i>Pfmdr-1</i> 1246Y	7	7			
<i>Pfmdr-1</i> + <i>Pfcrt</i>	Mutant	<i>Pfcrt</i> 76T + <i>Pfmdr-1</i> 86Y	60	214	0.24	0.0.62	1.23(0.52-2.93)
	Mixed	<i>Pfcrt</i> 76T N86+ <i>Pfmdr-1</i>	13	47			
	Wild	<i>Pfcrt</i> 76K + <i>Pfmdr-1</i> N86	7	31			
	Mutant	<i>Pfmdr-1</i> 76T+86Y+1246Y	10	23	0.13	0.72	1.16(0.51-2.59)
	Mixed	<i>Pfmdr-1</i> 76T+86Y+1246D	63	268			
	Wild	<i>Pfmdr-1</i> 76K+86N+1246D	8	37			

The same observation could be made for the association between resistant haplotypes of the *Pfcrt* and *pfmdr-1* genes and treatment failure to either AQ. The association tests between different AQ resistance conferring haplotypes did not indicate any significant relationship with treatment response to amodiaquine. Table 23 above shows that both wild type and resistance confer-

ring mutants of *pfmdr-1* and *Pfcrtr* genes were proportionately distributed among those who succeeded or failed to clear parasites.

The distribution of various alleles among isolates at enrolment and isolates at treatment outcome also indicated the disappearance of some resistant forms and the persistence of other resistant forms in all the sites. This phenomenon is not different in terms of which resistant alleles disappeared or persisted with treatment with either of the drugs under study. Overall, these observations indicate that irrespective of ecology, other factors are necessarily influencing the occurrence of treatment failure or success in the study population.

In summary, the baseline prevalence of SP and AQ resistance mutations in Ngaoundere and Bangolan show distinct patterns. The frequency of *pfmdr-86Y* mutation was less than 20% in Bangolan and was not found among parasites in Ngaoundere. On the other hand, the *pdmdr1-1246D* sensitive allele was present in 100% of circulating parasites. The mutant *Pfcrtr-76T* and *pfdhfr* triple mutant (IRN) haplotype tend to be reaching a fixation point in the parasite population in these two localities. The *pfdhps* resistant haplotype SGK occurred among less than 40% of the parasite population in Bangolan while 10% of the parasite population had mixed alleles (wild and mutant). In the parasite samples obtained from the SP and AQ efficacy study, the differences in the frequencies of the mutations studied by day 14 and day 28 post-treatment were minimal for *pfdhfr* ($p > 0.05$) but significant ($p = 0.017$) for *pfdhps* SGK in Mutengene (Figure 1b) indicating that parasites with *pfdhfr* mutations were cleared at a similar rate in response to SP drug treatment, but parasites with the *pfdhps* SGK mutation in Mutengene were not. The prevalence of the quintuple resistant allele (SGE) was found in one isolate in Mutengene and did not influence the treatment outcome. The combined frequency of drug resistant haplotypes in the *pfdhfr*, *pfdhps*, *Pfcrtr* and *pfmdr-1* genes for all sites shows that AQ and SP resistance conferring haplotypes were high with the *pfdhfr/pfdhps* combined resistant haplotype IRNG haplotypes being more prevalent than the amodiaquine (combined *Pfcrtr/pfmdr-1*) TY haplotypes in the parasite population. The distribution of *pfmdr-1* resistance conferring mutant (*pfmdr-1-1246Y*) was not different among the sites and occurred only among very few parasite isolates at each site. It did not constitute a risk factor for AQ treatment failure alone or when combined with other relevant mutations. When looking at the prevalence of drug resistant mutations among those who had an ACPR, we noticed a generally inverse relation between these two phenomena. Amodiaquine resistant mutants as a proportion of the ACPR was comparable between Yaounde and Mutengene, but differed compared to Garoua. The proportion of fansidar resistant mutations as a function of the ACPR on the other hand was comparable between Garoua and Mutengene, but differed in comparison to Yaounde. This pointed to the contribution of host immunity in clearing drug resistant parasite popu-

lations in children who are being treated. This observation drives the hypothesis that drug resistant mutants among children in Cameroon are cleared in different ways depending on host factors including genetics and ethnicity. Genetic characterisation of these host factors may point a light into how children in Cameroon succeed to clear parasites with resistant mutations in different ways at a period in their lives when sufficient protective immunity to malaria has not yet been assured.

3.14. Single Nucleotide Polymorphisms in Host Immune Genes and treatment response.

Given the results obtained that pointed to the involvement of human/environmental factors in explaining further the efficacies of the therapies under study, we undertook to genotype a set of 67 single nucleotide polymorphisms in candidate immune genes in human samples and analyse for correlations with treatment outcome, notable parasite clearance and fever outcome.

3.14.1. Verification of the Hardy Weinberg Equilibrium Law

To verify that the sample population under study was in Hardy Weinberg equilibrium, the SNP reference rs7537937 that identifies a G to C mutation alongside their frequencies within the sample population was used.

Table 22 : Different alleles of the rs7537937 used to verify Hardy Weinberg Equilibrium in our sample population.

Allele (<i>rs7537937</i>)	Frequency
C	0.51
G	0.49

Based on this information, the allelic frequencies expected for the SNP were calculated as follows:

From the HW equilibrium law is stated mathematically by the relation given by $P^2 + 2pq + q^2$, Where p= frequency of dominant allele, q= frequency of recessive allele and pq being the heterozygote,

Computing the above numbers, we obtained observed values as follows:

$$P^2 = 0.492(396) = 94.94$$

$$Q^2 = 0.512(396) = 103.13.$$

$$2pq = 2(0.49)(0.51)(396) = 197.91.$$

The Chi squared value was calculated using the relation

$$\chi^2 = \sum_{i=0}^n \frac{(O - E)^2}{N}$$

Where n= number of observations, N is population sample size for the HW test, O=observed frequency, E=expected frequency.

Computation of the above values and reading from a Chi square table gives a χ^2 value of 1.96. As the value obtained is less than 3.84, the null hypothesis, stating that the population under study is in Hardy Weinberg Equilibrium is not rejected.

3.14.2. Distribution of SNPs across the study population

The distribution of the 67 SNPs in 17 chromosomes identified in the candidate gene analysis across the population was studied. Table 25 represents the SNPs found to be generally showing any correlations with treatment outcome in the efficacy study.

Table 23: SNPs represented in our study population showing correlations with treatment outcome.

Gene	SNP dbID	Success		Failures		X ² P value	OR Allele A	95%CI	P for OR
		A	B	A	B				
		Allele designation							
		A	B	A	B				
IL-10	rs3024500	26	6	28	30	0.002	0.59	0.43-0.81	P<0.05
GBP2	rs7537937	25	7	19	39	0.0	0.41	0.47-1.04	P<0.05
TLR9	rs352140	18	14	6	52	0.0	0.18	0.08-0.41	P<0.05
IL-1IL-17RD	rs6780995	26	6	29	31	0.002	0.60	0.43-0.81	P<0.05
TLR6	rs5743809	3	29	0	56	0.020	NA	NA	NA
C6	rs1801033	26	6	32	26	0.013	0.68	0.51-90	P<0.05

IL-4	rs2243250	21	11	9	51	0	0.23	0.12-0.43	
LTA	rs909253								
		25	7	14	42	0	0.32	0.19-0.52	
TNF α , - SM2	rs1799964	12	20	6	50	0.003	0.28	0.12-0.68	
TNF α , - SM2	rs1800629	9	23	2	58	0	0.12	0.02-0.51	
TNF α , - SM2	rs361525	10	22	2	56	0	0.11	0.02-0.47	
TNF α , - SM2	rs3093662	32	0	41	15	0.001	NA	NA	
NOD 1	rs2075820	25	7	14	46	0	.029	0.18-0.48	
IL-22	rs1012356	22	8	26	32	0.01	0.61	0.43-0.87	
IL-22	rs2227491	11	19	39	19	0.006	1.84	1.11-3.03	
IL-4IL-4R		19	13	10	46	0	0.30	0.16-0.56	
	rs12720463	7	25	1	57	0.001	0.08	0.01-0.61	

- OR=Odds Ratio.

- Allele A: allele carrying single nucleotide polymorphism.

- Allele B: Reference allele which refers to the wild type allele.

- reference: allele of reference with OR=1. $P < 0.05$ is considered significant.

- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

When we considered only those who failed with a late parasite (occurrence of parasites any day between day 7 and day 28 without overt signs of pyrexia and without prior meeting the criteria for early treatment failure), we observed two SNPs that showed variable distributions among those who experienced LPF and those who did not. The mutant heterozygote and homozygote genotypes of the rs17411697G/T of IL-1- α gene were associated with increased risk to failure (G/T OR 0.68, 95%CI 0.36-1.30; T/T OR 5.30, 95%CI 1.38-20.37, $P=0.028$) as well as the rs17047661G/A of CR1 (G/A OR 0.78, 95%CI 0.45-1.34; A/A OR 0.38, 95%CI 1.80-13.5, $P=0.035$).

Table 24: Association between SNPs and late parasitological failure.

Gene/SNP	Non-LPF	LPF	Odds Ratio (95% CI)	P value(OR)
rs 17411697 IL-1a				
G/G	358(73.2)	54(76.1)		reference
G/T	126(25.3)	13(18.3)	0.68 (0.36-01.30)	0.028
T/T	05(01.0)	04(05.6)	5.30 (1.38-20.37)	
rs17047661 CR1				
G/G	130(26.4)	26(35.6)		reference
G/A	243(49.3)	38(52.1)	0.78 (0.45-1.34)	0.035
A/A	120(24.3)	09(12.3)	(1.80 – 13.5)	

3.14.3. Association of SNPs and pre-treatment patient temperature

Pyrexia as an immune modulator is the consequence of several immune players. We determined if there were associations between SNPs genotyped and pretreatment temperature among children in the study. We assessed the correlation between SNPs and high and persistent pre-treatment fever defined as day 0 temperatures greater than 39°C for two days. Among the alleles with differentially distributed in those with high and persistent pre-treatment fevers (Table 27), mutant alleles of G6PD, IL-13 and IL-22 were found to be associated with persistent hyperpyrexia(OR 1.34, 95%CI: 0.94-2.05; 1.85, 95%CI: 0.85-4.0; OR 1.85, 95%CI: 0.94-2.31) while

alleles of the ABO blood group determinant rs8176746-A/C, IL-4R-rs1805015-C/T and GNAS-rs2057291-C/T were more positively correlated with the ability to resolve fevers during the two days. (P=0.006, OR: 0.6, 95%CI: 0.4-0.89). However, on applying the Bonferroni correction, the adjusted P values were not significant.

Table 25 : SNPS showing correlations with high pre-treatment temperature defined as temperature below or above 39°C for 48 hours.

Gene	SNP dbID	T>39 °C		T<39°C		X ² P value	OR(A allele)	95%CI	p(OR)
		Allele designation							
		A	B	A	B				
ABO	rs8176746	29	93	92	484	0.022	0.61	0.38-0.98	P<0.05
IL-22	rs1012356	76	48	298	268	0.040	0.70	0.47-1.04	P<0.05
IL-22	rs2227491	66	60	347	227	0.048	1.39	0.94-2.05	P<0.05
IL-4R	rs1805015	59	61	206	354	0.006	0.60	0.4-0.89	P<0.05
IL-13	rs2057291	8	74	65	325	0.055	1.85	0.85-4.02	P<0.05
G6PD	rs1050829	30	90	180	366	0.043	1.47	0.94-2.31	P<0.05
IL-4	rs2243250	30	62	64	222	0.048	1.46	1.0-2.1	P<0.05

- OR=Odds Ratio.
- Allele A: allele carrying single nucleotide polymorphism.
- Allele B: Reference allele which refers to the wild type allele.
- reference: allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

3.14.4. Frequency distribution of alleles in Fulanis and non-Fulanis in the North of Cameroon.

We sought to understand how SNPs were distributed in the Fulani population compared to the non-Fulani population in our sample. Table 28 provides the results of the analysis.

Table 26: SNPS most frequently encountered among Fulanis clearing resistant parasites.

Gene/SNP	Fulani	Non-Fulani	OR allele A (95% CI)	P value(OR)
rs17411697 Allele	IL-1 α			
G	22(25.0)	254(85.8)		reference
T	66(75)	42(14.1)	0.87 (0.76-0.99)	0.017
rs187084 Allele	TLR9			
C	56(62.2)	220(72.9)		reference
T	34(37.7)	82(27.1)	1.39 (1.00-1.92)	0.050
rs20541 Allele	IL-13			
C	5(5.8)	43(15.3)		reference
T	83(94.3)	237(84.6)	1.11 (1.03-1.19)	0.018
rs2230739	ADCY9			
Allele				
A	18(19.6)	34(11.3)		reference
G	74(80.4)	266(88.6)	0.90 (0.81-1.01)	0.042
rs7935564	TRIM5			
Allele				
A	30(34.9)	133(45.6)		reference
G	56(65.1)	159(54.4)	1.19 (0.99-1.44)	0.079
rs10775349	ADCY9			
Allele				
C	29(32.3)	52(75)		reference
G	61(67.7)	248(25)	0.82 (0.70-0.95)	0.002
rs1050828	G6PD+376			
Allele				
C	1(1.1)	26(10.7)		reference
T	87(98.9)	268(89.3)	1.08 (1.04-1.13)	0.013

rs2243250	IL-4*			
Allele				
C	62(67.3)	22(25.0)		reference
T	30(32.7)	64(75.0)	1.45 (1.01-2.09)	0.048

- OR=Odds Ratio.
- Allele A: allele carrying single nucleotide polymorphism.
- Allele B: Reference allele which refers to the wild type allele.
- reference: allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

Table 25 : Above indicates a total of eight SNPs which were found to be differentially distributed between the Fulani study population clearing resistant parasites compared to the non-Fulani study population clearing resistant parasites. Some of the alleles such as IL-4C>T were almost twice represented among the Fulanis. The frequency of gene SNPs was computed among the Fulanis and the most frequent reported. A total of 8/64 (9.3%) SNPs in seven immune related genes were most frequently encountered among the Fulanis, namely Adenyl cyclase 9, interleukin 4, Glucose-6-phosphate dehydrogenase, twinfilin actin binding protein homologue 2, interleukin 13 and interferon regulatory factor 1. The SNP in interleukin 4 was found to be almost twice as likely to be encountered among the Fulanis (OR=1.46, 95%CI: 1.012-2.098) compared to non-Fulanis. The SNPs in IL-1 α ,IL-13 and IL-4, located on the same chromosome segment were also observed to be more frequent among the Fulanis compared to non-Fulani study population. Other SNPs in the IL-1IL-13 gene could not be built on the multiplex assay.

3.14.5. Association of SNPs and clearance of drug resistant parasites among the Fulani population in North ecology

The association between allele frequencies and clearance of drug resistant parasites was performed for individuals of the Fula ethnicity in the Northern ecology. Alleles showing significant correlation with clearance belonged to genes associated with pro- and anti-inflammatory responses, pathogen receptor of the CpG motif, complement system components and transcription factor. Table 29 below provides a summary of the analysis.

Table 27: Association analysis of SNPs and Clearance of drug resistant parasites among the Fulanis in North Ecology

rs1012356	IL-22*			
Allele	Clear	Not clear		
C	32(55.1)	8(26.6)		reference
T	26(44.2)	22(73.4)	0.61 (0.42-0.87)	0.011
rs2227491	IL-22*			
Allele				
C	19(31.6)	19(63.3)		reference
T	39(68.4)	11(37.7)	1.83 (1.10-3.03)	0.006
rs1799964	TNF α -1031*			
Allele				
C	50(89.2)	20(62.5)		reference
T	6(10.8)	12(37.5)	0.28 (0.12-0.68)	0.003
rs1800629	TNF α -308			
Allele				
	58(96.6)	23(69.6)		reference
	2(3.3)	9(30.4)	0.11 (0.02-0.51)	
				0.02
rs361525	TNF α -238			
Allele				
A	56(96.5)	22(68.7)		reference
G	2(3.5)	10(31.3)	0.11 (0.02-0.47)	0.01
rs3093662	TNF α +851			
Allele				
A	15(22.7)	0(00.0)		reference
G	41(87.3)	32(99.9)		0.001

- OR=Odds Ratio.

- Allele A: allele carrying single nucleotide polymorphism.

- Allele B: Reference allele which refers to the wild type allele.
- reference: allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

Prominent among the inflammatory genes, the rs1012356 T-allele of the IL-22 gene was positively associated with clearance of drug resistant parasites (OR 0.61, 95%CI: 0.42-0.87, P=0.011) while the rs2227491 T-allele of the same gene correlated with risk of failure to clear drug resistant parasites (OR 1.83, 95%CI:1.01-3.03, P=0.006). In addition, alleles of the tumour necrosis factor alpha gene cluster showed a positive correlation with clearance of drug resistant parasites (Table 32). In particular, Fulani participants carrying the -1031 mutant T-allele of the TNF α gene were 72% more likely to clear AQ resistant parasites compared to those who failed (OR 0.28, 95%CI: 0.12-0.68, P=0.003).

Table 28 : Association between SNPs and treatment response classified as success or failure.

rs number	Failures	Success	OR (95%CI)	P value
rs2243250	IL-4			
Allele				
C	51(85.0)	11(34.3)		reference
T	9(15.0)	21(65.6)	0.23 (0.11-0.43)	0.002
rs1805015	IL-4R			
Allele				
C	46(82.1)	13(40.6)		reference
T	10(17.8)	19(59.4)	0.30 (0.16-0.56)	
rs1801033	CC6			
Allele				
A	26(44.8)	6(19.3)		reference
C	32(55.2)	26(80.7)	0.67 (0.51-0.90)	0.013
rs909253	LTA			
Allele				

C	42(75)	7(21.8)		reference
T	14(25)	25(79.1)	0.32 (0.19-0.52)	0.013
rs3024500	IL-10			
Allele				
A	30(51.7)	6(18.4)		reference
G	28(48.3)	26(80.9)	0.59 (0.43-0.81)	0.002

- OR=Odds Ratio.
- Allele A: allele carrying single nucleotide polymorphism.
- Allele B: Reference allele which refers to the wild type allele.
- reference: allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

Regarding the SNPs among the Fulani who either succeeded or failed treatment, the frequency of the rs909253 mutant T-allele of the lymphotoxin alpha gene was significantly higher among the Fulani participants clearing drug resistant parasites with the wild type allele as reference allele. On the other hand, producers of alleles in IL-4 as well as its receptor showed association with clearance. In particular, the rs2243250 T-allele of the IL-4 gene was 75% more frequent among Fulani clearing resistant parasites, compared to Fulanis no clearing resistant parasites; indicating a positive correlation with the parasite clearance phenotype (OR 0.23, 95%CI:0.11-0.43, P=0.003). A similar positive correlation could be observed with the 1805015 mutant T-allele of the IL-4R gene as shown in Table 33 above.

3.14.6. Differences in the distribution of candidate gene polymorphisms and treatment outcomes by geo-ecological regions

The sites wherein the study was conducted were differentiated following their ecology and ethnicity. In this regard, the Mutengene and the Yaounde sites with equatorial and forest ecology and a greater heterogeneous mix of ethnic populations were considered southern ecology while the Garoua site with sudano-sahelian ecology and predominantly mbororo and other ethnicities was considered the northern ecology. The basis of this difference was the degree of population movements.

Table 29: Geo-ecological differences in clearance of resistance conferring mutants and SNPs

Gene/SNP	South	North	Odds Ratio (95% CI)	P value(OR)
	N(%)	N(%)		
CR1 rs17047660				
A/A	207 (47.9)	54(41.9)		reference
A/G	191 (44.2)	52(40.3)	1.04 (0.68-1.60)	0.007
G/G	34(07.9)	23(17.8)	2.63 (1.43-4.84)	
IL-1B2 rs1143634				
C/C	324 (77.1)	85(67.5)		reference
C/T	91(21.7)	36(28.6)	1.51 (0.96-2.38)	0.034
T/T	05(01.2)	05(4.0)	3.83 (1.80 – 13.5)	
IL-17RC rs708567				
A/A	117 (26.9)	39(29.8)		reference
A/G	207 (47.6)	74(56.5)	1.07 (0.68-1.68)	0.012
G/G	111 (25.5)	18(13.7)	0.49 (0.26 – 0.90)	
TNF α -1031rs1799964				
T/T	357 (81.9)	91(68.9)		reference
T/C	75(17.2)	37(28.0)	1.94 (1.23-3.06)	0.005
C/C	04(0.9)	04(03.0)	3.99 (0.98 – 16.3)	

- OR=Odds Ratio.
- reference : Allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as current as the August 2009 version of the dbSNP build.

In the southern ecology, there are greater chances of population movement between sites as compared to the northern ecology geographically more isolated compared to the south with significantly less mixed marriages. Besides differences in parasite ecology and treatment response due to climatic type, both southern and northern ecologies have equal potential to receive influx from neighbouring Nigeria, frequently implicated in the traffic of substandard medication. Single nucleotide polymorphisms in the genes tested showed variable genotype frequency distribution between the ecologies with significant associations with clearance of parasites with amodiaquine resistance conferring mutations. As shown in Table 28, we observe that the rs17047660 G/G mutant homozygote genotype of the complement receptor gene 1 (CR1) was more frequent among the individuals in the northern ecology compared to those in the southern ecology with the A/A wild type genotype as reference.

Table 30: Geo-ecological differences in clearance of AQ resistant mutants

Gene/SNP	South	North	Odds Ratio	P val-
	N(%)	N(%)		
rs3093662 TNFα+851				
A/A	373(85.9)	98(73.1)		
G/G	3(0.7)	3(02.2)	3.81(0.76-19.17)	
hCD36_T1264G rs3211938				
T/T	334(77.3)	120(93.0)		
G/T	94(21.8)	09(07.0)	0.27 (0.13-0.54)	5.729e-05*
G/G	04 (00.9)	0(0.0)		
TRIM5 rs7935564				
A/A	110(25.6)	51(40.5)		
RTN3 rs542998				

G/G	172 (40)	43(33.6)		
G/G	56(13)	32(25)	2.29 (1.3 – 3.96)	

- OR=Odds Ratio.
- Allele A: allele carrying single nucleotide polymorphism.
- Allele B: Reference allele which refers to the wild type allele.
- reference: allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

Table 28 above indicates that among those who cleared parasites with AQ resistant mutations, all SNPs shown were consistently more frequent among the southern study population compared to the northern population. Of all these SNPs, only the homozygous recessive of the IL-17RC gene seems to be associated with a favourable outcome (clearance of resistant parasites). However, other SNPs were also observed to be positively associated with increased clearance of drug resistant parasites with higher frequency among the southern study population. These include the hCD36-T126G where the heterozygote mutant genotype was found to be significantly higher among those in the southern ecology who cleared resistant parasites compared to those in the northern ecology (21% Vs 7%, OR 0.27, 95%CI 0.13-0.54, P= 5.729e-05) despite adjustments for multiple testing. In addition, the recessive condition of this gene, also known as the null allele and the presence of which abrogates the production of CD36 occurred in 9% in the southern population but none in the northern population.

In addition, the rs7935564G/A heterozygote of the TRIM5 α gene was found to be more frequent among the participants in the southern ecology and positively correlated (G/A OR 0.53, 95%CI 0.34-0.84; AA OR 0.460.26-0.80, P=0.005) with clearance of AQ resistance parasites.

3.14.7.SNP Association with AQ resistant TYN haplotype by ecology.

We examined the distribution of SNPs among those who failed to clear parasites with TYN resistance conferring mutation and observed a higher frequency of the heterozygote genotype among those who failed treatment with a tendency of the rs1805015 IL-4IL-4R-63011T/C heterozygote genotype to be associated with risk to failure (Pearson $X^2 = 5.02$, df=2, LR=5.03, P=0.08). In addition, the rs17047661A/G heterozygote genotype of the CR1 gene also was higher among those who failed even though the opposite was observed among the homozygotes. This SNP was associated with risk of treatment failure (Pearson $X^2=6.18$, df=2, LR=5.90, P=0.042).

Table 31: Association of SNPs with clearance of AQ resistant mutants by ecological site

Gene/SNP	South	North	Odds Ratio	P val-
IL-4R-63011 rs1805015				
A/A	20(20.6)	65(14.2)		
A/G	50(51.5)	216(47.2)	5.02 (5.03)	0.08(2)
G/G	27(27.8)	177(38.6)		
CR1 rs17047661				
A/A	17(17.1)	37(06.7)		
A/G	45(45.5)	185(38.9)	6.18 (5.90)	0.045(2)
G/G	42(42.4)	258(54.3)		

- OR=Odds Ratio.
- Allele A: allele carrying single nucleotide polymorphism.
- Allele B: Reference allele which refers to the wild type allele.
- reference: allele of reference with OR=1. P<0.05 is considered significant.
- rs ID references for single nucleotide polymorphisms are as updated as the August 2009 version of the dbSNP build.

3.14.8. Differential correlation of SNPs and parasite or fever clearance among the Fulani

In summary, a number of SNPs in genes that are associated with early immune or early pro-inflammatory and anti-inflammatory response against infections were prominent, some significantly prominent while others were borderline and only tended towards a significant association. In the same vein, SNPs found among those who presented with high and persistent fever were associated with risk of failure while others were more associated with the ability to clear parasites. Associations between SNPs and clearance of resistance conferring mutants were different and specific for individuals who were in different study drug arms. This was marked among the Fulani population (Table 30) in the North of Cameroon. These SNPs initially found to be associated with parasite with SP or AQ conferring resistance mutants irrespective of the drug or among high and persistent fever producers. Lastly, geo-ecological differences in the ability to clear AQ resistant parasites were noticed, but marked for one SNP in the pleiotropic CD36 gene, which was consistently higher in the northern population clearing AQ resistant parasites compared to the other southern region. Related to this, the null allele of the same SNP was completely absent among the northern population although the general prevalence of the null allele was low.

Table 32: Summary of recurrent SNP associated with high and persistent fever and parasites with AQ drug resistant mutations among the Fulani.

SNP	Parameter	Gene	X ² P-value	Odds Ratio	95% CI for OR	Bonferroni adjusted p value
rs3024500	Fulani	IL-10	0.002	0,594	0,43-0,81	NS
rs6780995		IL-17	0.002	0,595	0,43-0,81	NS
rs1801033		C 6	0.013	0,679	0,51-0,90	NS
rs1799964		TNF -SM2	0.003	0,286	0,11-0,69	NS
rs2227491		IL-22	0.006	1,830	1,11-3,03	NS
rs1012356		IL-22	0.011	0.61	0.42-0.87	NS
rs1805015		SP	IL-4R	0.014	1.311	1,07-1,63
rs2227491	T°C (>39°C)	IL-22	0.013	1.502	1,80-2,01	NS
rs2227491	AQ	IL-22	0.017	1,440	1,07-1,94	NS
rs3211938	Ecology	hCD36	5.729e-05	0.27	0.13-0.54	S*

NS: $p > 0.0008$ indicating a non-significant association after adjustment for multiple testing.

3.15. Discussion

The outcome of treatment in malaria depends on a number of factors that are inherent in the parasite, the drug and in humans. It is well recognised that most of the pathophysiological effects of parasitic infections such as malaria is mediated by the immune system (19, 241). These mediators of immune origin or cytokines are meant to protect but can also cause significant disease. The outcome of infection with the malaria parasite as well as other infections hinges in the delicate balance between appropriate and inappropriate induction of these mediators. In our study, we have assessed a number of genetic polymorphisms in parasite genome involved in the metabolism of the drugs used for treatment and secondly human mediators that are thought to have a role in the pathogenesis of malaria or response to treatment. Our aim was to evaluate if there were such mediators that have an influence in the determination of the outcome of treatment in

children with the combination of amodiaquine and fansidar besides the effects of the drugs. These human immune mediators, sixty-four in number and selected from a growing list of cytokines, chemokines and their receptors span seventeen human chromosomes. The principal factors assessed in these genes were single nucleotide polymorphisms(SNPs) within promoter regions, exons, introns, untranslated regions and intergenic regions of these genes, whose presence is thought to influence, either alone or in a concert with other SNPs, the expression of this gene or the integrity of the resultant protein structure and hence its function. These SNPs were examined in patients with uncomplicated malaria under treatment in Garoua, Yaoundé and Mutengene, three distinct epidemiological zones of Cameroon.

3.15.1.Parasite identity, genetic diversity and resistance in different towns of Cameroon.

The samples used in this study came from clinical studies that determined recrudescence from re-infection to better characterise clinically resistant isolates for downstream molecular analysis. Polymorphic markers, particularly the the MSP-2 antigen marker was used to compare samples before and after treatment(Ranford-Cartwright 1997). Mixed infections, which were not infrequent in the samples were defined as the occurrence of multiple MSP-2 based strains in the same infection. Otherwise known as the multiplicity of infection, the results indicate that in different parts of Cameroon, malaria remains highly complex, with several parasite genotypes in infected individuals, a reflection of transmission patterns. Indeed, the results imply that parasite transmission in Cameroon is still heterogenous and there is no evidence of decrease in transmission found in any of the towns studied, confirming earlier reports (Ali 2005). The occurrence of this type of complexity has implications in the event of vaccine based interventions against malaria. Firstly, the efficacy of chimeric or multivalent vaccines targeting some of these protective antigens is jeopardized by antigen variations. In a study by Ouattara et al., (Ouattara 2010) exploring the reason for low vaccine efficacy to AMA-1based bivalent vaccine, the authors reasoned that this could be due to strain specific induced responses to protective haplotypes. Secondly, circulating variants not targeted by the vaccine will increase in the population posing a significant problem with effectiveness of the vaccine. Takala and Plowe, in 2009(Takala 2009) reviewed a growing number of important antigens including, MSP-1, MSP-3, AMA-1, CSP, as well as evidence of extensive diversity in these antigens and the importance in vaccines carrying these antigens. Of note, the authors recommend that for extreme polymorphic proteins like it may be better to engineer vaccine constructs that boost immune response to protective epitopes in conserved regions(Takala 2009). Therefore, it is important to continue to study the extent of genetic variations in circulating parasite antigens shown to be protective and which are likely to be included in subunit or multivalent vaccines for malaria control in the near future.

3.15.2. Single nucleotide polymorphisms in drug resistant genes and association with treatment response.

Key mutations associated with drug resistance in malaria were determined from different towns in this study. Single nucleotide polymorphisms in parasite drug metabolizing genes assessed were the *pfcr1* K76T, the *pfmdr-1* T89Y, N1046D and D1242Y, all proposed to be involved in resistance to amodiaquine. Besides these, we also examined the *pfdhfr* mutations (N51I, C59R, S108N) and the *pfdhps* mutations (positions A437G and K540E) linked with resistance to fansidar.

3.15.3. Differences in drug resistant SNP distribution by study site

On assessing the prevalence of the various mutations in the different regions, we observed differing haplotype frequencies of mutations within the regions with higher frequencies in the south western part of Cameroon (Mutengene) and lower frequencies in the northern regions of Cameroon (Garoua). These differences in haplotype frequencies have been observed in other studies (166, 246). This has been attributed to widespread drug use leading to selective drug pressure on the parasite targets and explained by differences in endemicity (Alifrangis 2003). Considering the *pfdhfr* and *pfdhps* mutant alleles for example, the increased prevalence of the alleles in the southern western region of Cameroon compared to the Northern region can be explained by the pattern of drug use in the region, notably drug prescription habits and self-medication among the population. Notwithstanding, this is thought to be driven also by the increased intensity of transmission of malaria within the region, leading to multiple infectious bites and the necessity for drug intake both for prophylaxis and for cure. Therefore, it is likely that in Mutengene where transmission is all year round, parasites are exposed to drugs much more frequently than in Garoua where transmission is intense and highly seasonal. A second reason for these differences relates to the dynamics of asymptomatic infections with resistance mutations. In effect, multiple clones of infection some of which carry resistant mutations will compete, and in the absence of drug pressure, resistant clones will be selected against because of fitness cost (Babiker 2013). This often happens during long seasons without malaria as found in Northern Cameroon. However, it is important to confirm this assertion by evaluating rates of asymptomatic infections and resistant mutations in both regions. Fansidar has been used extensively in Cameroon for prophylaxis before, and for curative purposes. Following the same reasoning, the low prevalence of mutant *pfcr1* alleles in the Northern part of Cameroon may be consistent with reduced drug use over time. Compared to the situation in Limbe (a neighbouring town to Mutengene) where chloroquine resistance was first described (Oduola 1989) the increased sensitivity of the malaria parasite to CQ in the Northern region was later shown confirming the hypothesis that complex dy-

namics of drug resistance occurs in circulating infections and selective drug pressure is driving the extent of mutation rates to CQ in these regions.

The results show a very high occurrence of the *pfdhfr* resistant haplotypes within the pre-treatment isolates from various sites. The CIRN for example was 91.6% in Yaoundé, 95.1% in Mutengene and 100% in Bangolan. On the other hand, this prevalence was significantly lower for Garoua (50%). The IRN haplotype confers reduced sensitivity to pyrimethamine. The occurrence of very high levels of resistant haplotypes in pre-treatment parasites could highlight the possibility that pyrimethamine use among the population could be high, inducing the mutational events within this haplotype. This may not be the only reason for this observation. The rate of genetic recombination events in the haploid blood stage *Plasmodium falciparum* is high in high and stable transmission areas like the Bangolan and Mutengene contributing to the occurrence of haplotypes with a selection advantage. Secondly, the differences in occurrence of the haplotypes between the sites could translate in the differences in drug sensitivity in combination therapy. Indeed, the failure rates of SP was higher in the south compared to the north in this present study. Because mutations in this gene have been used as predictors of SP sensitivity before, we can infer from the prevalence of the IRN haplotype in the pre-treatment parasites that *in vivo* resistance to SP should be appreciable, though no direct correlation between resistant haplotype and *in vivo* resistance to SP was attempted. These observations appear to tie with the fact that in the north, the prevalence of the sensitive haplotype CNCS was significantly higher (33%) compared to each of the other sites studied. However, another possible interpretation to the occurrence of the haplotype differences could be an increased immune clearance of resistant parasites in Garoua (Djimde 2003). This is an intriguing possibility because although transmission in the North is seasonal, infection rates are usually very high translating into significantly increased pattern of drug use in the population in the background of low healthcare seeking. This explanation could mean that increased drug use contribute to the induction of resistant mutations and secondly, short and high transmission scenario serves to “boost” vaccination inducing even greater and more diverse protective immune responses in subsequent transmission seasons. However, there has been no correlation between the clearance of infections and risk of clinical malaria in subsequent seasons. Lastly, human immune response could play a part in the generation of different haplotype scenarios.

The distribution of wild and resistance-conferring haplotypes to SP across the epidemiological blocks shows some interesting trends. The high prevalence of the SGK mutant haplotype for example appeared to be linked with resistance to fansidar in Mutengene. This haplotype occur as

a single allele. In east Africa however, the haplotype exist as a double allele (SGK and SGE) and has been implicated in high grade resistance to fansidar in the region (Kublin 2002). This indicates that the quintuple mutation is a necessary requirement for resistance to fansidar in that region. While this is a necessity for this region, the SGE allele was not found in our study except one mutant parasite from the Mutengene site. Many West African countries do not have this mutant as well(Naidoo 2010). Our study equally shows a very low prevalence of the SGE haplotype in only one of three regions(Mutengene) under study. In east Africa for example, the prevalence of this mutation is high in regions with low SP efficacy (Kublin 2002, Greenhouse 2009). Among parasites with decreased sensitivity to SP in Cameroon, this haplotype is low (Mutengene) or inexistent (Yaoundé, Garoua, Ngaoundere and Bangolan). Because resistance to SP occur in a step wise manner, the K540E mutation is thought to be the fifth mutation conferring very high resistance to SP. If we consider the distribution of this mutation in our samples in the various study sites, we observe that this is confirmed. In Mutengene where fansidar resistance is highest, we observe the occurrence of the K540E mutation, though at low frequencies. This may well be an indication that development of SP resistance in this region was earlier compared to the other regions with no K540E mutation. This observation gives rise to the hypothesis that the K540E mutation may not be a necessary molecular marker to predict resistance to fansidar in Cameroon or West Africa at the present time. However, the authors of the study described above highlighted the proposition that the absence of the SGE allele may just be a transition, and that it is possible for this allele to be present in increasing proportions in the future should heavy use of fansidar continue. One of the artemisinin based combinations includes fansidar and artesunate (co-artemate). If we consider the theory behind the effectiveness of combination therapy (White 1999), we may argue that artesunate will greatly down-modulate the effect of fansidar on SP resistant genes through rapid parasite clearance. In this scenario, we expect to observe a decrease in the occurrence of these SP resistance conferring mutations with time. However, this may be too speculative as concerns about the development of resistance to artesunate is increasing in regions considered hotspots emergence of resistance to antimalarial drugs notably Thai-Cambodia border and the Thai Burmese border(159). Similarly in Africa, Menard (Menard 2005) showed the potential for development of resistance to artesunate. In this study aimed at assessing the efficacy of a seven days course of artesunate against uncomplicated malaria, the authors found a five-fold increase in the IC₅₀ value and corresponding eight-fold lower IC₅₀/IC₉₀ ratio for recrudescant parasites compared to non-recrudescant parasites; although this did not correlate with mutations in the *pfmdr1* codon 86. This *in vitro* study only highlights the possibility of resistance development even with a short half-life drug like artesunate.

While studying the lineages of resistant parasite populations in Africa, Pearce et al, (166) showed by analysing microsatellites around resistant alleles that these resistant parasite populations have distinct origins and subsequently dispersed to other geographical regions. Hence, for the AGK/SGK alleles, there has been an independent origin and subsequent regional dispersal of parasites carrying this resistant haplotype throughout west and central Africa with unique allelic type in Cameroon at the confluence of the allelic gene pool, while the same line of reasoning exist for the east African AAK and SGE alleles. The geographical divide coupled with difficulties in population movement between the Eastern and Western Africa could explain also the rare presence of the SGE allele in our study as well as in West and Central Africa (Pearce 2009).

When comparing the distribution of haplotypes and treatment efficacy we observe that this study appear to show an inverse relationship between the prevalence of resistance conferring mutations and efficacy. In high transmission areas such as Mutengene and Yaoundé, the efficacy of treatment with AQ was found to be high compared to highly seasonal transmission region of Garoua. This is probably explained by the development of acquired host immunity to the parasites and also to the post-treatment prophylactic effect of AQ. In vivo, AQ is a pro-drug metabolized to its active desethylamodiaquine with a long half-life of several weeks. This means that the drug has a long terminal elimination phase after treatment that may also favour the selection of less sensitive parasites when the drug concentrations wanes in the system.

3.15.4. Frequency distribution of wild and mutant alleles by day of efficacy assessment

Among patients with Day 14 and day 28 clinical and parasitological outcomes, the frequency of haplotypes in genes associated with resistance to amodiaquine, namely *Pfcr1* and *pfmdr1* generally decreased in all sites compared to day 0. While a clear relation between the prevalence of this parasites and treatment efficacy at day 14 and day 28 could not be ascertained, the results point to a significant contribution of these haplotypes on the outcome with AQ related treatments. However, this result also showed no conclusive evidence of selection of parasites carrying resistant haplotypes in the *pfmdr-1*, *Pfcr1*, *pfdhps* and *pfdhfr* genes in all the sites.

The frequency of the *pfmdr-1* 86N mutation remains low for both endpoints and for all the sites irrespective. This mutation is associated with sensitivity to AQ selected for by treatment with artemether-lumefantrine (Happi 2009, sisowath 2009). As shown with the *pfmdr-1* 1246D mutation, it is possible for selection of various *pfmdr-1* variants to occur, given that both lumefantrine and amodiaquine containing artemisinin therapies are recommended and extensively used against uncomplicated malaria in the community. In the light of our findings therefore, it is important to monitor the occurrence of this mutations in the era of artemisinin based combination

therapy, especially with increasing concerns about the efficacy of artemether lumefantrine (Duraisingh M T 1998) and (Mwai L 2009)

The decrease in haplotype frequencies for the *pfdhps* gene by day 14 and day 28 analysis time points was more marked when assessing the SGK haplotype in Mutengene among those who failed treatment than among those who cleared parasites adequately. We observe a close to five-fold decrease in the frequency of this haplotype by day 28 among those who cleared parasites adequately. Compared to the prevalence on day 0, this haplotype appeared to have been selected significantly when analysing at the day 14 time point. The frequency of the haplotype increased from (76% to 100 %) by day 14 and decreased to 20% (3 times less frequent than at day 0) by day 28. This observation may well confirm the implication of acquired immune response in the clearance of these haplotype after day 14. However, the effect may not also be as straight forward as stated due to sample size limitations among those participants that failed treatment.

3.15.5. Association of selected resistance conferring haplotypes with treatment outcome.

Our data shows that no independent association could be made between the leading resistance conferring haplotypes and treatment outcome. Although the SGK haplotype tended to be associated with SP treatment outcome in Mutengene, this association was not significant at the 5% significance threshold. It is known in the literature that high level of resistance to SP is expressed when the triple/quadruple *pfdhfr* and *pfdhps* mutations get established. However, as observed in Cameroon, only triple mutant *pfdhfr* and single *pfdhps* mutant (437) alleles have so far evolved and this may not be sufficient to cause high level of resistance. One of the reasons why there is no good correlation between mutant genotypes and SP failure could be due to partial resistant genotypes in circulating parasites and we did not investigate to know for a given infection what proportion of the parasites carried resistant alleles. On the basis of previous considerations on our methodology for determining and assessing resistance and treatment outcome, we concluded that other factors (notably human biological factors) could be strongly influencing the outcome of treatment in the parasite population as was speculated in previous reports.

3.15.6. Single nucleotide polymorphisms in immune genes and correlation with treatment response.

This comprehensive study also describes the association between the extent of treatment failure or treatment outcome with amodiaquine and fansidar among children ill with uncomplicated malaria and polymorphic variants across a wide spectrum of genes encoding cytokines, chemokines and their receptors with inflammatory or immunomodulatory properties. The children were of diverse ethnic epidemiological background in the three major regions represented by Mutengene, Yaoundé and Garoua.

Our study reports varying prevalence of drug resistance mutations translating varying treatment failure rates within three distinct ecological zones of Cameroon for AQ, SP and AQSP. These findings advocate for regional policy as opposed to a single sweeping policy in the management of malaria in endemic zones, where transmission and disease complexity are high. Molecular markers appear to provide interesting information for the monitoring of drug resistance. The continuous mapping of the patterns of resistance to crucial antimalarials can be performed using data gained by fast and efficient molecular methods. This can be used as an early warning system for changes occurring in endemic areas, thus providing additional information that may be crucial for regional and international drug policy changes (Plowe 1997). The role of such information in the implementation of disease management policies however, is important only if there is evidence of an association between the prevalence of these molecular markers and therapeutic failure from clinical studies.

An additional factor that may interfere in the relationship between the prevalence of drug resistant markers and observed treatment failure is the contribution provided by the host immune system (Enevold 2007, Diarra 2012). In individuals exposed to malaria for their first time, infection with the malaria parasite does not always lead to severe disease. Rather, a wide range of outcomes are possible ranging from death to resistance to infection. This range of responses evokes different innate mechanisms of protection possible (257). One of these mechanisms includes genetic polymorphisms in molecules involved in the pathogenesis of malaria that alter the outcome of infection. Hence in some patients on antimalaria treatment, different treatment responses can be the outcome of variation in immune genes involved in malaria pathogenesis against a background of drug resistance. The balance between pro- and anti-inflammatory cytokines is critical for the immune system to function adequately and can greatly influence the outcome of the immune response to protect against disease development (Torre 2002). Thus, specific immunomodulatory gene polymorphisms associated with gene regulation and protein expression may influence clinical outcome of a number of disease states including response to malaria therapy (Torre 2002, Fortin 2002, Hill 2006, Kwiatkowski 2005). To further assess the role of these variations in the outcome of treatment, we also investigated the association in SNP variation among patients that either cleared or failed to clear their parasites and the prevalence of parasite markers thought to be involved in resistance to AQ/SP.

3.15.7. SNPs in IL-22, related to IL-10 and mediating Th17 cellular response is possibly associated with treatment response.

The results show a number of cytokines to be involved in parasite clearance. Among the tested SNPs, a number of gene polymorphisms including a SNP in IL-22 (rs2227491) showed a

significant association with parasite clearance overall, and specifically among the Fulanis. Some of the same gene SNPs including were associated with pre-treatment hyperpyrexia and were risk factors for reduced ability to clear AQ resistant parasites. These results suggest that this cytokine might be involved in host response network mounted in response to fevers and intracellular pathogens. IL-22 SNP rs2227491 is a missense mutation leading to an A to G change upstream exon 5 of the IL-22 gene. IL-22 is a proinflammatory immune regulatory cytokine that is related to IL-10 and mediating Th17 function (Spolski R 2009). This cytokine is abundantly produced by cells of the innate immune system including $\gamma\delta$ -Tcells, NK cells, Th17 cells, and LTI-like cells. IL-22 is capable of inducing inflammatory mediators such as serum amyloid A protein, alpha-1 antitrypsin and haptoglobin. IL-22 shares the IL-10RB, receptor chain with IL-10 but requires its own specific receptor chain (IL-22R) for signal transduction (Sonnenberg 2011). The involvement of this cytokine in the modulation of the acute phase reactants suggests that in malaria, this cytokine may mediate early pro-inflammatory responses (Ryan-Payseur 2011). In a recent study investigating the differential expression of gene and protein products in peripheral blood mononuclear cells among HIV discordant couples and long lasting resistance to HIV-1, Missé et al., (265) observed an over production of several proteins involved in innate response including IL-22 and a group of peroxiredoxins . They suggested a role for the involvement of IL-22 in resistance to HIV-1 including its effect in the production of Serum amyloid A, a formyl peptide receptor agonist that modulates CCR5 expression and hence HIV entry. In our study, IL-22 may well be involved in the clearance of parasite through its role in generating the acute phase response. In a study evaluating single nucleotide variations in IL-10RA and IL-22 and viral persistence in hepatitis C infection (Hennig 2008), showed that other SNPs with the exception of rs2227491 were in strong linkage disequilibrium, some of which contributed together with SNPs in IL-10RA in modulating viral persistence in Hepatitis C infection and treatment, though their study was limited by sample size. It is also possible that this cytokine may be required in immune network acting in synergy with antibodies in the clearance of parasites among the Fulanis.

3.15.8. Complement receptor polymorphisms, less frequent in the North represented risk alleles of adequate response to treatment.

The complement receptor 1, also known as CD35 is a large 200 kDa glycoprotein that belongs to the family referred to as regulators of complement activation (RCA) and binds complement cleavage products C3b and C4b, thus acting as a cofactor to inactivate them to iC3b and iC4b, respectively. This receptor is widely distributed in different cell types including lymphocytes, red blood cells, myeloid cells, and has been implicated in clearance of immune complexes from the human system. It is the principal mediator of binding of uninfected red blood cells to

infected red blood cells via pfEMP1 protein. Of the two CR1 polymorphisms analysed in our study, the heterozygote AG-genotype of the exon 22 polymorphism shows a lower frequency among participants in the northern region who cleared drug resistant parasites. By contrast, the homozygote mutant GG genotype was significantly higher among the northern participants compared to the southern participants. Increase in the frequency of the G-allele has been correlated with low RBC expression of CR1 protein. The amino acid change (H1208R) caused by the SNP introduces a potential tryptic protease cleavage site in the CR1 protein leading to low expression of the protein. Because this SNP lies in a solvent exposed region, the substitution may cause increase in local hydrophobicity. The influence of low or high levels of CR1 on the outcome of malaria is variable. Low levels of CR1 may lead to reduced resetting and protection from severe malaria, but at the same time, low levels of CR1 may compromise clearance of immune complexes by macrophages in the spleen and liver and thereby contributing to anaemia. The relative importance of the two mechanisms is not clear. Our results show correlation between high frequency G-allele of CR1 gene and clearance of drug resistant parasites when comparing people with drug resistant parasites irrespective of the region and among all those who cleared drug resistant parasites in the northern participants when compared to southern participants. But the CR1 polymorphism was disproportionately represented among those who were likely to experience late parasite failure as well pointing to this SNP as a potential risk factor for re-emergence of drug resistant parasites after treatment. This observation is in line with results obtained by Sinha et al.,(267) who found a correlation between the G-allele and severity of malaria and also with evidence that low expression of/or deficiency (GG genotype of CR1 polymorphism) CR1 correlates with protection from severe malaria(Cockburn 2004).

3.15.9. Interleukin 4 and IL-4R receptor polymorphisms investigated may have had conflicting roles in influencing response to treatment.

IL-4 is an anti-inflammatory cytokine with studied malaria associated effects. Single nucleotide variations in IL-4 have been linked to protection from severe malaria. An SNP upstream the open reading frame of the IL-4 gene (IL-4 -590C) has been shown to affect promoter activity and affect IgE production in malaria patients in Burkina Faso, West Africa(Modiano 2001, Verra 2004, Verra 2009). IL-4 receptor interacts closely with its ligand, IL-4 to achieve this. Upregulation of the expression of IL-4R on CD8+ cells has been shown to be required for the development of protective memory against liver stage parasites(Carvalho 2002). rs1805015 is a missense mutation(T to C) resulting to a Serine to Proline change at position 503 of the interferon regulatory factor 1 protein. This mutation may be functionally implicated in the increased expression of interferon gamma. The interleukin 4 receptor also binds to IL-13, which may contribute to the

many overlapping functions of this cytokine and IL-13. IL-4, together with IL-13, IL-3, IL-5, IRF-1 form a cytokine gene cluster in the 5q31-q33 chromosomal region shown to be strongly associated with the interferon G mediated control of blood parasite density in one study (Flori 2003). Although SNPs in IRF-1 and IL-13 signals were obtained from our studies, their association with treatment outcome was weak. It is therefore possible that variations observed in these cytokines might be a signal of their combined functional association in influencing treatment outcome and highlights the importance of testing haplotype and gene cluster polymorphisms rather than pairwise single gene mutations and disease outcomes in genomic studies. Indeed, because these SNPs may be in linkage disequilibrium within this chromosomal region, the combined contribution of this variation and that of the IL-4R could point to a functional association in modulating treatment outcome and warrant further study, especially as SNP variability is considerable across human populations. In the present study, a SNP in IL-4R was inversely associated with clearance of drug resistant parasites. Among those who carried parasites with AQ resistant mutations, the homozygote mutant genotype was significantly represented compared to those who did not carry the AQ drug resistant haplotype. Even though this correlation barely had a tendency towards significance, the effect of this mutation may be acting against a background of other anti-inflammatory regulatory mechanisms. The reduced expression of IL-4R on CD8+ T cells may lead to defect in the development of parasite specific memory after repeated exposure in line with the study mentioned above. The consequence may be that parasite specific CD8+ protective response is inhibited leading to greater susceptibility to infection. Other SNPs observed in IIL-4R notably the gain of function promoter SNP could lead to an increase in CD8+ cell receptor signalling favouring a T_H2 response limiting inflammation against a more parasite clearing pro-inflammatory T_H1 response.

3.15.10. TNF α enhancer haplotype (CACGG= -1031T>C, -851G>A, -308G>A, -238G>A) polymorphisms were disproportionately represented among those who cleared parasites in the North.

A range of TNF α polymorphisms were genotyped in the present study and analysed for their association with clearance of drug resistant parasites. TNF α is a pro-inflammatory cytokine with diverse effects on immune responses to parasites. It is produced by many cell types including dendritic cells, monocytes, macrophages, activated T cells etc. It is located within the class III sub-region of the highly polymorphic major histocompatibility region of the chromosome 6q21 between Lymphotoxin alpha and lymphotoxin beta genes. Single nucleotide polymorphisms in this gene have been associated with susceptibility or resistance to various diseases. Of note, the -238, -308, -851, -1031 mutations have been observed and associated with susceptibility to malar-

ia infection. The -308G/A polymorphism was linked to elevated TNF α transcriptional activity, while the -863C/A was associated with reduced promoter activity and lower serum levels of TNF α . However, these associations are not as straightforward because the effect of SNPs on the levels of TNF α can be tightly regulated beyond the effects of the SNPs on the activity of the gene promoters. When analysing the -1031 mutation in the TNF-alpha gene in our study, results reveal that the mutant homozygote of this SNP was associated with the risk of failure in individuals carrying parasites with AQ resistant haplotype. The correlation was significantly stronger when the association was adjusted for by site. The population in the northern ecology clearing drug resistant parasites more frequently harboured the minor allele compared those in the southern ecology. SNPs contributing to the TNF α enhancer haplotype (CACGG= -1031T>C, -851G>A, -308G>A, -238G>A) was disproportionately represented among those who cleared parasites in the North, (Sinha 2008) suggesting TNF- α may be implicated in parasite clearance in the North of Cameroon. This result is in agreement with those from India that studied the association of this SNP and risk of severe malaria disease manifestation. They found a significant correlation between the minor mutant allele and increased levels of TNF α at population level. Given multiple SNPs occurring with several different outcomes associated with disease, it appears therefore, that this mutation is an important correlate of malaria parasite clearance in the populations of Cameroon and warrants further investigations.

3.15.11. Single nucleotide polymorphisms in the CD36 gene may significantly influence immune response to malaria in children treated in Cameroon.

The pathophysiology of malaria is characterized by persistent effects of parasite derived factors in human systems with diverse consequences. One of the processes involves the binding of parasite derived molecules on erythrocyte surfaces to platelets, dendritic cells, uninfected erythrocytes, and leucocytes. The natural pathway for clearance of infected erythrocyte follow splenic network. Infected erythrocytes that adhere to other cells such as those above bypass this clearance pathway and contribute to the pathogenesis of malaria (Milner 2014, Vásquez 2012). The intracellular P falciparum exports to the infected erythrocyte surface a large multiclinal and multigene family of parasite specific erythrocyte membrane protein known as pfEMP1, which it uses to switch in order to evade immune attack and promote pathology(Angeletti 2013). This protein is responsible for binding CD36, a class B scavenger receptor, expressed on a range of cell types including leucocytes, platelets, dendritic cells and uninfected red blood cells. The adherence of infected erythrocytes via pfEMP1 to these cell types avoid splenic clearance of iRBCs and promote sequestration, microvascular clogging and triggering inflammatory responses typical of severe forms of malaria(Cserti-Gazdewich 2012, Patel 2007). Besides this, binding of iRBCs

to dendritic cell CD36 inhibits (Britta 2001) maturation and subsequent function in eliciting an effective parasite specific immune response. Binding of iRBC to CD36 to dendritic cells inhibit LPS induced upregulation of markers of dendritic cell maturation such as MHC Class 2, CD83, CD83 and CD86. On the other hand, binding of the iRBC to CD36 promote macrophage mediated clearance of iRBCs. Furthermore, this molecule has been shown to act as a co-receptor with TLR 2/6 complex and participate in the uptake of microbial components such as lipoteichoic acid thereby promoting innate sensing and clearance of bacteria.

Polymorphisms in the gene coding for CD36 have been found to have different consequences on the expression and function of this receptor. Several studies in Kenya, the Gambia and Nigeria have produced inconsistent findings on the influence of single nucleotide polymorphisms and susceptibility to malaria. In the Kenyan studies, reports show that the 1246G heterozygotes were protected from malaria symptomatology while evidence from the Gambia showed that deficiency of CD36 (homozygous recessive allele) was associated with susceptibility to disease(277). In the Nigeria study, no association could be concluded about susceptibility to severe disease and the frequency of T1246G allele (Amodu 2005). These findings suggest that CD36 may be implicated in the susceptibility to infectious diseases. In addition, Kajeguka et al(Kajeguka 2012) followed up children with or without CD36-T1264G genotypes for one year to investigate the acquisition of protective responses to MSP1₁₉ and found that CD36 deficiency (CD36-1264GG genotype) was associated with decreased antibody seropositivity to MSP1₁₉, and increased malaria attacks compared to normal or heterozygous(CD36-1264TG genotype) children. While the reports are contradictory, this clearly shows that CD36 could be involved, fully or in part in a mechanism of susceptibility to malaria or other infectious diseases Individuals heterozygous for 1264G were more likely to harbour parasites with AQ sensitive alleles (TYN-) compared to those without the SNP. This difference may suggest that this SNP may be a more important determinant in the rate of clearance of AQ resistant parasites in the Southern population compared to the Northern population. Furthermore the absence of CD36 deficiency in the Northern ecology lends support to the proposition that the heterozygote mutant SNP may be associated with a faster rate of clearance of AQ resistant parasites in the southern population in Cameroon. In the Southern parts of Cameroon, the 1246G allele is 21%, significantly higher than in other parts of Africa and comparable to its frequency in neighbouring southern Nigeria (Amodu 2005). This observation warrants further investigations in a larger sample size. If proven, the evolution of the alleles from its west/central African focus will be established although the reasons for this mode of expansion are yet unclear. While there is yet no clear evidence of associa-

tion between the prevalence of CD36-1246G mutation and clearance of drug resistant mutant parasites, the higher frequency lends support to the proposition that recent evolutionary determinants could be acting on this gene and influencing its spread from the West African focus. It is commonly known among the Nigerians that the Yoruba people are heavy consumers of oily foods, and several studies have not shown evidence of high risk factors for cardio-metabolic disease among these people compared to others. In Southern Cameroon as well, staple meals of individuals from localities contain significant amounts of oil raising the suspicion that nutrition related environmental factors may be exerting an additional selection pressure for this mutation in addition to malaria. Thus, CD36 may be linked to metabolism of carbohydrates and fats among these people living the same eco-regions in West Africa and associated with cardiometabolic disorders. This remains to be tested. In addition, another reason for the prevalence of the mutation and its absence to association with malaria susceptibility may be its evolutionary selection advantage against malaria has been abrogated, while only the selection signal remains. Although plausible, this does not explain why the allele appears to originate from West Africa and dispersing to other regions in Africa (280).

Our study also, should be interpreted with some caution due to challenges associated with genome wide association studies. One of these challenges relates to the sample size at which sufficient power would enable detection of common polymorphisms with significant effect on outcome parameter. In addition, the contribution of minor alleles to defined outcomes would be possible only with very large samples size and population sub-structuring as was observed in one analysis (Eid 2010) evaluating candidate malaria susceptibility/resistant genes in a hospital based and population based study. The effect of sub-structuring can alter observed associations. The small sample size also limits generalisations. The second relates to the association of haplotypes and treatment responses rather than single gene polymorphisms. The third relates to correction for multiple testing which when carried out in our analysis indicates only one SNP, the CD36-T1264G as significant considering the adjusted P value. However, the occurrence of the same SNPs in multiple outcomes related to treatment points to an important likely implication of these genes in treatment response. Overall, these findings provide further insights in to what factors contribute to treatment response in Cameroonian children and point to the necessity of further studies to elucidate the role of early host immunity in regulating antimalarial treatment outcomes.

CHAPTER 4: GENERAL CONCLUSIONS AND PERSPECTIVES.

4.1. General Conclusions

The goal of this research study was to investigate parasite and human factors that affect malaria parasite response to treatment of uncomplicated malaria with fansidar and amodiaquine among children in Cameroon. Among the known biological factors, gene polymorphisms in drug metabolizing systems in the parasite influence the resistance phenotype, often leading to treatment failure in drug therapy. Another biological factor relates to the response of the host to foreign pathogens, and variations in immune genes associated with response to infection can also influence treatment outcome. The principal findings of this research are presented below.

-Description and determination of the prevalence of major *Plasmodium falciparum* drug resistant mutations in *pfert*-codon 76, *pfmdr-1*-codon 89, *pfdhfr*-codons 51, 59 and 108 and *pfdhps* codons 437 and 540 among circulating parasites in five geographically distinct malaria endemic sites in Cameroon.

-Baseline drug resistant mutant alleles to several antimalarials circulate widely in Cameroon where the pattern and magnitude differ by region. The *pfert* and *pfpdhfr* mutations seem to be genetically fixed in Bangolan, Ngaoundere and Yaoundé.

-Assessment the association between the molecular markers of *Plasmodium falciparum* drug resistance and treatment response in a trial evaluating the clinical efficacy of AQ and SP in Cameroon.

No independent significant associations were found between parasite factors of drug response and levels of treatment response, although the SGK mutant haplotype tended to be associated with fansidar response in Mutengene. Widespread resistance to this drug was not observed because the *pfdhps*-540E mutant, implicated in high grade fansidar resistance, was also absent.

-Quantify the molecular correlations between host candidate gene polymorphisms associated with malaria pathophysiology and treatment response in children with AQ or SP resistance conferring *Plasmodium falciparum* mutants.

We observed found that based on the analysis of candidate immune genes, different sets of immune genes were operating to assist clearance of parasites with resistance conferring mutations. We observed that IL-22 alleles were associated with clearance of Amodiaquine resistance IL-4 alleles were more associated with clearance of fansidar resistance conferring mutants. The mechanisms observemaylargely include proinflammatory and cellular immunity driven for IL-22 and anti-Inflammatory and antibody mediated mechanisms for IL-4. The proinflammatory mediated mechanism involves IL-22 as corroborated by observations in immune gene polymorphisms associated with clearance among those with high and persistent pre-treatment fevers.

Decipher potential candidate immune genes that may be implicated in ecologic or treatment related responses to clearance of circulating *Plasmodium falciparum* drug resistant mutants

Human immunogenomic analysis revealed several molecular signatures including IL-22, IL-4, IL-4IL-4R, and CD36 as potential candidate factors associated with response to amodiaquine and fansidar resistant mutants in Cameroon. Different mechanisms of parasite clearance might operate based on drug resistant mutations, and also in the north or the south of Cameroon; and between the Fulani and the non-Fulani in Cameroon depending on the following.

1. The nature of the parasite mutant population including the proportion of mutant forms in the infection.
2. The ethnicity of the infected individual. Differences in CD1264G mutants between northern and southern ecology may reflect ethnic differences which may be related to diverse environmental and human factors such as in lipid intake and metabolism. This factor is associated with cardiometabolic diseases.

4.2.Perspectives.

Functional studies such as cytoadherence assays using peripheral blood from individuals with or without mutations of interest as well as among cohorts of northern Fulani and southerners are in perspective. This may throw more light in understanding fully the pathophysiology of malaria in the tropics and elucidate at a functional factors level those human factors that affect treatment with antimalarial drugs. A look at the association between CD36 and polymicrobial pyrogens and/or paradigms in communicable and non-communicable diseases might point to role of CD36 in modulating host parasite relationships from an evolutionary perspective. This may find application in immunotherapy or in stratified treatment of exposed populations.

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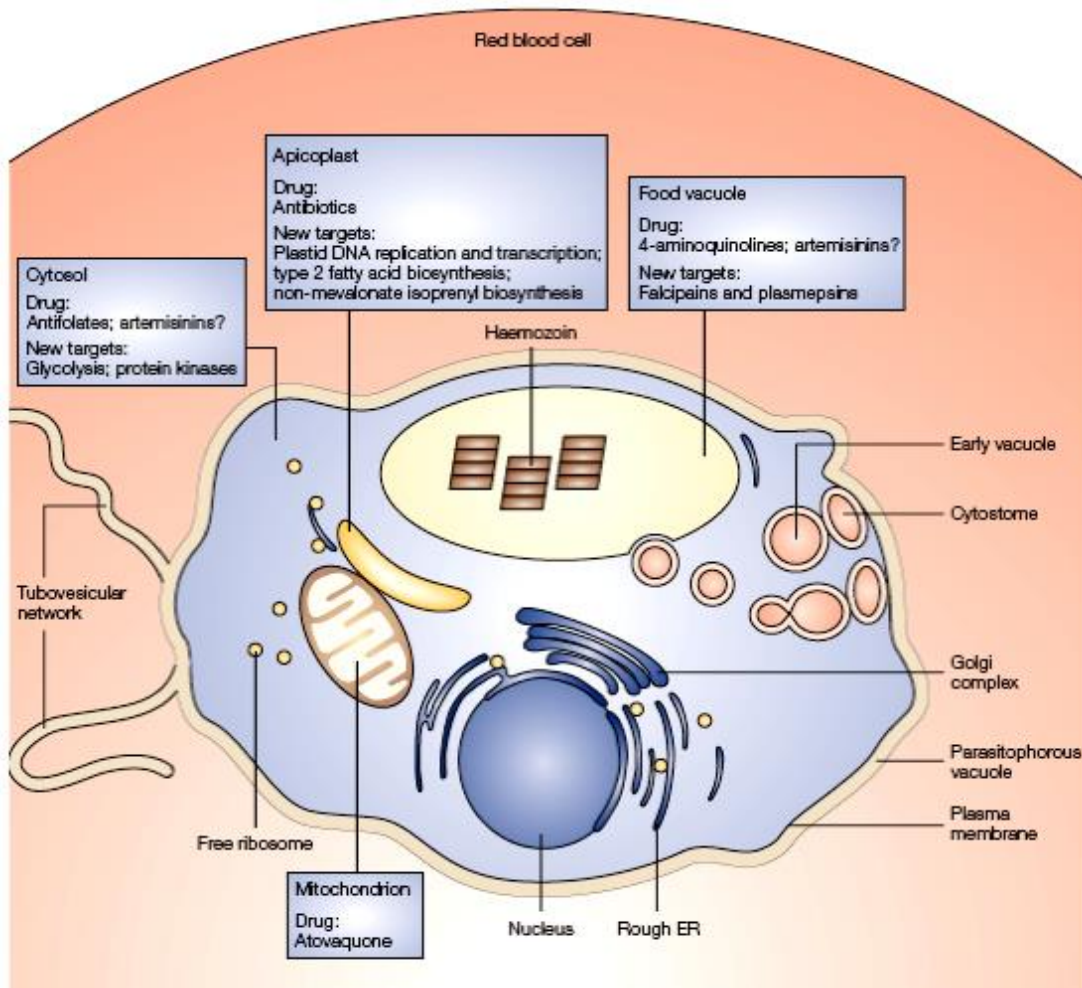
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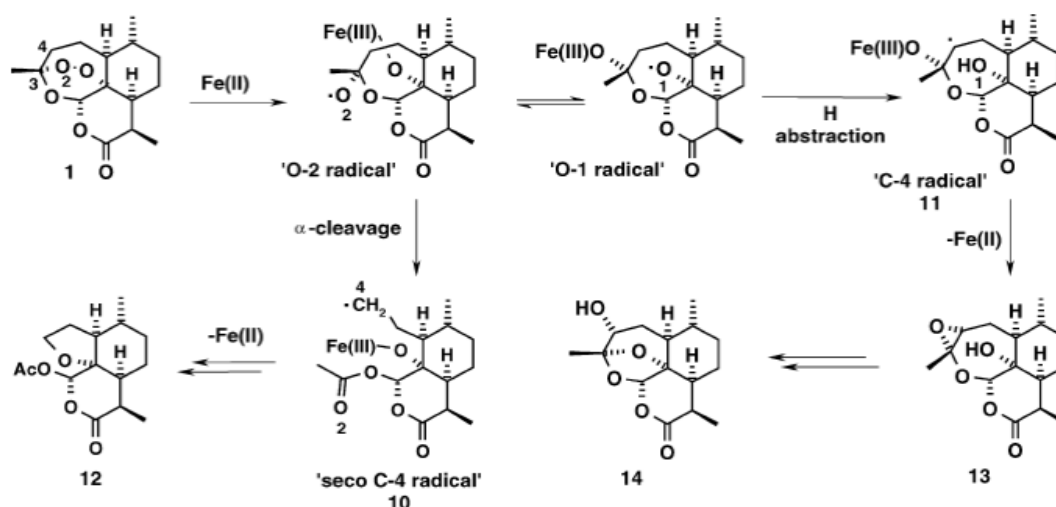
Annexes

annexe 1: Antimalarial Drug Targets



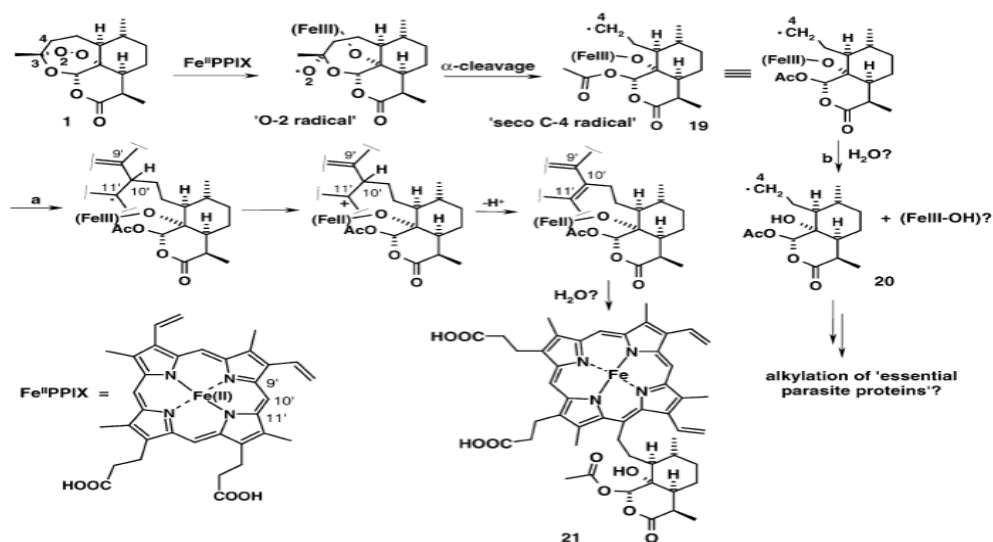
A representation of an intra-erythrocytic parasite showing the various compartments and drug targets as well as drug acting on them. Adapted from Fidock et al., 2004

annexe 2: Activation of Artemisinin



Proposed pathway for the activation of artemisinin by Fe^{2+} to produce carbon centred seco C-4 radicals and the end products 10 and 12. The antimalarial activity of artemisinin is claimed to reside in the radicals formed rather than the end products of this activation given that they have no antimalarial property. (Adapted from Krishna et al., 2004)

Annexe 3: Activation of artemisinin by haem (protoporphyrin IX).



Activation of artemisinin by haem (protoporphyrin IX) leading to formation of seco C-4 radical and alkylated adduct(21), or detachment of the seco C-4 radical(20) to attack essential parasite proteins.(Adapted from Krishna et al., 2004).

annexe 4: Resistance Markers, Genotypes and Resistance Status.

<i>Locus Name</i>	<i>Locus position</i>	<i>Marker type</i>	<i>Genotype</i>	<i>Label</i>	<i>Status</i>
<i>Pfcrt</i>	76	<i>SNP</i>	<i>T</i>	<i>Pfcrt 76T</i>	<i>Pure</i>
<i>Pfcrt</i>	76	<i>SNP</i>	<i>K/T</i>	<i>Pfcrt 76T</i>	<i>Mixed</i>
<i>Pfcrt</i>	220	<i>SNP</i>	<i>S</i>	<i>Pfcrt 220S</i>	<i>Pure</i>
<i>Pfcrt</i>	220	<i>SNP</i>	<i>A/S</i>	<i>Pfcrt 220S</i>	<i>Mixed</i>
<i>pfprdhfr</i>	16	<i>SNP</i>	<i>V</i>	<i>pfprdhfr 16V</i>	<i>Pure</i>
<i>pfprdhfr</i>	16	<i>SNP</i>	<i>A/V</i>	<i>pfprdhfr 16V</i>	<i>Mixed</i>
<i>pfprdhfr</i>	50	<i>SNP</i>	<i>R</i>	<i>pfprdhfr 50R</i>	<i>Pure</i>
<i>pfprdhfr</i>	50	<i>SNP</i>	<i>C/R</i>	<i>pfprdhfr 50R</i>	<i>Mixed</i>
<i>pfprdhfr</i>	51	<i>SNP</i>	<i>I</i>	<i>pfprdhfr 51I</i>	<i>Pure</i>
<i>pfprdhfr</i>	51	<i>SNP</i>	<i>N/I</i>	<i>pfprdhfr 51I</i>	<i>Mixed</i>
<i>pfprdhfr</i>	59	<i>SNP</i>	<i>R</i>	<i>pfprdhfr 59R</i>	<i>Pure</i>
<i>pfprdhfr</i>	59	<i>SNP</i>	<i>C/R</i>	<i>pfprdhfr 59R</i>	<i>Mixed</i>
<i>pfprdhfr</i>	108	<i>SNP</i>	<i>N</i>	<i>pfprdhfr 108N</i>	<i>Pure</i>
<i>pfprdhfr</i>	108	<i>SNP</i>	<i>S/N</i>	<i>pfprdhfr 108N</i>	<i>Mixed</i>
<i>pfprdhfr</i>	164	<i>SNP</i>	<i>L</i>	<i>pfprdhfr 164L</i>	<i>Pure</i>
<i>pfprdhfr</i>	164	<i>SNP</i>	<i>I/L</i>	<i>pfprdhfr 164L</i>	<i>Mixed</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>C</i>	<i>pfprdhps 436C</i>	<i>Pure</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>S/C</i>	<i>pfprdhps 436C</i>	<i>Mixed</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>A/C</i>	<i>pfprdhps 436C</i>	<i>Mixed</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>F</i>	<i>pfprdhps 436F</i>	<i>Pure</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>S/F</i>	<i>pfprdhps 436F</i>	<i>Mixed</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>A/F</i>	<i>pfprdhps 436F</i>	<i>Mixed</i>
<i>pfprdhps</i>	436	<i>SNP</i>	<i>C/F</i>	<i>pfprdhps 436C/F</i>	<i>Pure</i>
<i>pfprdhps</i>	437	<i>SNP</i>	<i>G</i>	<i>pfprdhps 437G</i>	<i>Pure</i>
<i>pfprdhps</i>	437	<i>SNP</i>	<i>A/G</i>	<i>pfprdhps 437G</i>	<i>Mixed</i>

<i>pfpdfhps</i>	540	SNP	<i>E</i>	<i>pfpdfhps 540E</i>	<i>Pure</i>
<i>pfpdfhps</i>	540	SNP	<i>K/E</i>	<i>pfpdfhps 540E</i>	<i>Mixed</i>
<i>pfpdfhps</i>	581	SNP	<i>G</i>	<i>pfpdfhps 581G</i>	<i>Pure</i>
<i>pfpdfhps</i>	581	SNP	<i>A/G</i>	<i>pfpdfhps 581G</i>	<i>Mixed</i>
<i>pfmdr-1</i>	86	SNP	<i>Y</i>	<i>pfmdr-1 86Y</i>	<i>Pure</i>
<i>pfmdr-1</i>	86	SNP	<i>N/Y</i>	<i>pfmdr-1 86Y</i>	<i>Mixed</i>
<i>pfmdr-1</i>	184	SNP	<i>F</i>	<i>pfmdr-1 184F</i>	<i>Pure</i>
<i>pfmdr-1</i>	184	SNP	<i>Y/F</i>	<i>pfmdr-1 184F</i>	<i>Mixed</i>
<i>pfmdr-1</i>	1034	SNP	<i>C</i>	<i>pfmdr-11034C</i>	<i>Pure</i>
<i>pfmdr-1</i>	1034	SNP	<i>S/C</i>	<i>pfmdr-11034C</i>	<i>Mixed</i>
<i>pfmdr-1</i>	1042	SNP	<i>D</i>	<i>pfmdr-11042D</i>	<i>Pure</i>
<i>pfmdr-1</i>	1042	SNP	<i>N/D</i>	<i>pfmdr-1 1042D</i>	<i>Mixed</i>
<i>pfmdr-1</i>	1246	SNP	<i>Y</i>	<i>pfmdr-1 1246Y</i>	<i>Pure</i>
<i>pfmdr-1</i>	1246	SNP	<i>D/Y</i>	<i>pfmdr-1 1246Y</i>	<i>Mixed</i>
<i>pfpdfhps</i>	613	SNP	<i>S</i>	<i>pfpdfhps 613S</i>	<i>Pure</i>
<i>pfpdfhps</i>	613	SNP	<i>A/S</i>	<i>pfpdfhps 613S</i>	<i>Mixed</i>
<i>pfpdfhps</i>	613	SNP	<i>T</i>	<i>pfpdfhps 613T</i>	<i>Pure</i>
<i>pfpdfhps</i>	613	SNP	<i>A/T</i>	<i>pfpdfhps 613T</i>	<i>Mixed</i>
<i>pfpdfhps</i>	613	SNP	<i>S/T</i>	<i>pfpdfhps 613S/T</i>	<i>Pure</i>
<i>pfmdr-1</i>	<i>CN 1</i>	<i>pfmdr-1</i>	<i>CN</i>	<i>pfmdr-1</i>	<i>CN=1</i>
<i>pfmdr-1</i>	<i>CN 2</i>	<i>pfmdr-1</i>	<i>CN</i>	<i>pfmdr-1</i>	<i>CN=2</i>
<i>pfmdr-1</i>	<i>CN > 2</i>	<i>pfmdr-1</i>	<i>CN</i>	<i>pfmdr-1</i>	<i>CN>2</i>
<i>pfpdfhps,</i>	<i>437, 540</i>	<i>SNP pfdhps</i>	<i>G,E</i>	<i>pfpdfhps G,E</i>	<i>Pure</i>
<i>pfpdfhps</i>	<i>437, 540</i>	<i>SNP pfdhps double</i>	<i>G,K/E</i>	<i>pfpdfhps G, K/E</i>	<i>Mixed</i>

annexe 5: Resistance Marker Genotypes

<i>Genotype Locus</i>	<i>Wild type</i>	<i>Mutant</i>	<i>Mixed</i>			
<i>Pfcr72</i>	<i>C</i>	<i>S</i>	<i>C/S</i>			
<i>Pfcr73</i>	<i>V</i>					
<i>Pfcr74</i>	<i>I</i>	<i>M</i>	<i>I/M</i>			
<i>Pfcr75</i>	<i>N</i>	<i>E</i>	<i>N/E</i>			
<i>Pfcr76</i>	<i>K</i>	<i>T</i>	<i>K/T</i>			
<i>Pfcr220</i>	<i>A</i>	<i>S</i>	<i>A/S</i>			
<i>pfprdhfr16</i>	<i>A</i>	<i>V</i>	<i>A/V</i>			
<i>pfprdhfr50</i>	<i>C</i>	<i>R</i>	<i>C/R</i>			
<i>pfprdhfr51</i>	<i>N</i>	<i>I</i>	<i>N/I</i>			
<i>pfprdhfr59</i>	<i>C</i>	<i>R</i>	<i>C/R</i>			
<i>pfprdhfr108</i>	<i>S</i>	<i>N</i>	<i>S/N</i>			
<i>pfprdhfr164</i>	<i>I</i>	<i>L</i>	<i>I/L</i>			
<i>pfprdhps436</i>	<i>S</i>	<i>A</i>	<i>F</i>	<i>S/A</i>	<i>S/F</i>	<i>A/F</i>
<i>pfprdhps437</i>	<i>A</i>	<i>G</i>	<i>A/G</i>			
<i>pfprdhps540</i>	<i>K</i>	<i>E</i>	<i>K/E</i>			
<i>pfprdhps581</i>	<i>A</i>	<i>G</i>	<i>A/G</i>			
<i>pfprdhps613</i>	<i>A</i>	<i>S</i>	<i>T</i>			
<i>pfmdr-186</i>	<i>N</i>	<i>Y</i>	<i>N/Y</i>			
<i>pfmdr-1184</i>	<i>Y</i>	<i>F</i>	<i>Y/F</i>			
<i>pfmdr-11034</i>	<i>S</i>	<i>C</i>	<i>S/C</i>			
<i>pfmdr-11042</i>	<i>N</i>	<i>D N/D</i>				
<i>pfmdr-11246</i>	<i>D</i>	<i>Y</i>	<i>D/Y</i>			
<i>pfmdr-1 CN</i>	<i>1</i>	<i>2</i>	<i>> 2</i>			

Annexe 6: Molecular Probes used for Genotyping pfmdr, Pfcrt, pfdhfr and pfdhps Genes

<i>Genotype</i>	<i>Sequence</i>
<i>IEK</i>	5'-TA ATT GAA AAA ATT TTT G
<i>IET</i>	5'-TA ATT GAA <u>ACA</u> ATT TTT G
<i>MNK</i>	5'-TA ATG AAT AAA ATT TTT G
<i>MNT</i>	5'-TA ATG AAT <u>ACA</u> ATT TTT G
<i>MEK</i>	5'-TA ATG GAA AAA ATT TTT G
<i>MET</i>	5'-TA ATG GAA <u>ACA</u> ATT TTT G
<i>INK</i>	5'-TA ATT AAT AAA ATT TTT G
<i>INT</i>	5'-TA ATT AAT <u>ACA</u> ATT TTT G

<i>Genotype</i>	<i>Sequence</i> 5'-3'
86 ASN	5'-AG AAC ATG AAT TTA GGT G
86 TYR	5'-AG AAC ATG <u>TAT</u> TTA GGT G
184 TYR	5'-A GGT TTA TAT ATT TGG TC
184 PHE	5'-A GGT TTA <u>TTT</u> ATT TGG TC
1034 SER	5'-A TGG GGA TTC AGT CAA AG
1034 CYS	5'-A TGG GGA TTC <u>TGT</u> CAA AG
1042 ASN	5'-AA GCG CTC AAT TAT TTA T
1042 ASP	5'-AA GCG CTC <u>GAT</u> TAT TTA T

1246 ASP	5'-AC TTA AGA GAT CTT AGA A
1246 TYR	5'-AC TTA AGA <u>T</u> AT CTT AGA A

<i>Probe</i>	<i>Probe sequence</i>
<i>Series 1 (6 probes) pfdhfr Codons 50 and 51 on chromosome 4</i>	
50/51CN 50/51CN2 50/51RN 50/51RN2 50/51RI 50/51CI	TGG AAA TGT <u>A</u> AT TCC CTA TGG AAA TGT <u>A</u> AC TCC CTA TGG AAA <u>C</u> GT AAT TCC CTA TGG AAA <u>C</u> GT AAC TCC CTA TGG AAA TGT ATT TCC CTA <i>TGG AAA TGT <u>A</u>TT TCC CTA</i>
<i>pfdhfr series 2 (5 probes) 59 and 108 on chromosome 4</i>	
59C 59R 108N 108S 108T	AAT ATT TTT GTG CAG TTA AAT ATT <u>T</u> C GTG CAG TTA A AGA ACA <u>A</u> AC TGG GAA AG A AGA ACA AGC TGG GAA AG A AGA ACA <u>A</u> CC TGG GAA AG
<i>pfdhps Codons 436 and 437 (9 probes) on chromosome 8</i>	
436/437SA 436/437SG 436/437FA 436/437FG 436/437AA 436/437AG 436/437CA 540K 540E	GAA TCC <u>T</u> CT GCT CCT TTT GAA TCC <u>T</u> CT GGT CCT TTT GAA TCC <u>T</u> TT GCT CCT TTT GAA TCC <u>T</u> TT <u>G</u> GT CCT TTT GAA TCC GCT GCT CCT TTT GAA TCC GCT <u>G</u> GT CCT TTT GAA TCC <u>T</u> GT GCT CCT TTT ACA ATG GAT AAA CTA ACA <i>CAC ATG GAT <u>A</u>AA CTA CAC</i>

Annexe 7: Sequence of various primers and PCR Reaction conditions for different resistance conferring pfmdr-1 and Pfcrf genes

<i>Gene</i>	<i>Primers</i>	<i>Sequence</i>	<i>PCR Conditions and Product Size</i>
<i>Pfcrf</i> (for SNPs at 72-76)	<i>Nest 1 forward P1</i>	5'-CCGTTAATAATAAATACACGCAG-3'	94°C ^{3min} / (94°C ^{30sec} – 56°C ^{30sec} – 62°C ^{1min} x 35) / 62°C ^{5min} /15°C ^{5min} (Product Size 546 bp)
	<i>Nest 1 reverse P2</i>	5'-CGGATGTTACAAACTATAGTTACC-3'	
	<i>Nest 2 forward D3</i>	5'-AGGTTCTTGTCTTGGTAAATTTGC-3'	94°C ^{3min} / (94°C ^{30sec} – 56°C ^{30sec} – 65°C ^{1min} x 30) / 65°C ^{5min} /15°C ^{5min} (Product Size 164 bp)
	<i>Nest 2 reverse D2</i>	5'-CAAACTATAGTTACCAATTTTG-3'	
<i>Pfmdr-1 fragment 1</i> (for SNPs at 86 and 184)	<i>Nest 1 forward FN1/1</i>	5'-AGGTTGAAAAAGAGTTGAAC-3'	94°C ^{3min} / (94°C ^{30sec} – 55°C ^{30sec} – 72°C ^{1min} x30) / 72°C ^{5min} /15°C ^{5min} (Product Size 578 bp)
	<i>Nest 1 reverse REV/C1</i>	5'-ATGACACCACAAACATAAAT-3'	
	<i>Nest 2 forward mdr2/1</i>	5'-ACAAAAAGAGTACCGCTGAAT-3'	94°C ^{3min} / (94°C ^{30sec} – 60°C ^{30sec} – 72°C ^{1min} x30) / 72°C ^{5min} /15°C ^{5min} (Product Size 534bp)
	<i>Nest 2 reverse newrev1</i>	5'-AAACGCAAGTAATACATAAAGTC-3'	
<i>Pfmdr-1 fragment 2 long</i> (for SNPs at 1034 and 1042)	<i>Nest 1 forward Mdrfr2f1</i>	5'-GTGTATTTGCTGTAAGAGCT-3'	94°C ^{3min} / (94°C ^{30sec} - 55°C ^{1min} - 65°C ^{1min 30 sec} x 34) / 65°C ^{5min} /15°C ^{5min} (Product Size 958 bp)
	<i>Nest 1 reverse Mdrfr2r1</i>	5'-GACATATTAATAACATGGGTTC-3'	
	<i>Nest 2 forward Mdrfr2f2</i>	5' CAGATGATGAAATGTTTAAAGATC-3'	94°C ^{3min} / (94°C ^{30sec} - 60°C ^{30sec} - 65°C ^{1min} x29) / 65°C ^{5min} /15°C ^{5min}

	<i>Nest 2 reverse Mdrfr2r2</i>	<i>5'-TAAATAACATGGGGTTCTTGACT-3'</i>	<i>(Product Size 864 bp)</i>
<i>Pfmdr-1 fragment 2 short (for SNP at 1246)</i>	<i>Nest 1 forward Mdrfr2f1</i>	<i>5'-GTGTATTTGCTGTAAGAGCT-3'</i>	<i>94°C^{3min} / (94°C^{30sec} - 55°C^{1min} - 65°C^{1min 30 sec} x34) / 65°C^{5min}/15°C^{5min} (Product Size 958 bp)</i>
	<i>Nest 1 reverse Mdrfr2r1</i>	<i>5'-GACATATTAAATAACATGGGTTC-3'</i>	
	<i>Nest 2 forward RFLPN2F</i>	<i>5'-GAATTTTCAAACCAATCTGGA-3'</i>	<i>94°C^{3min} / (94°C^{30sec} - 60°C^{30sec} - 65°C^{1min} x29) / 65°C^{5min}/15°C^{5min} (Product Size 151 bp)</i>
	<i>Nest 2 reverse Mdrfr2r2</i>	<i>5'-TAAATAACATGGGGTTCTTGACT-3'</i>	

Annexe 8: Structural localisation of immune genes and chromosomes considered for genomics analysis

<i>Gene</i>	<i>Chromosome number</i>	<i>Chromosome location</i>	<i>rs Number</i>	<i>SNP sequence</i>	<i>SNP Location</i>
<i>DARC</i>	<i>1</i>	<i>15744130</i> <i>7</i>	<i>rs281477</i> <i>8</i>	<i>TGCTTCCAAG[A/G]TAAGAG</i> <i>CCAA</i>	<i>Less than</i> <i>10 kb</i> <i>upstream</i> <i>promoter</i>
<i>RGS2</i>	<i>1</i>	<i>19103644</i> <i>9</i>	<i>rs217965</i> <i>2</i>	<i>TCCAGCCCTG[C/T]GGCCA</i> <i>GCCTC</i>	<i>Greater</i> <i>than 10kb</i> <i>upstream</i>
<i>IL-10</i>	<i>1</i>	<i>20500745</i> <i>4</i>	<i>rs302450</i> <i>0</i>	<i>TCCTGGGGGT[A/G]GGGGG</i> <i>TAGCT</i>	<i>About</i> <i>10kb up-</i> <i>stream</i> <i>promoter.</i>
<i>IL-10</i>	<i>1</i>	<i>20501598</i> <i>8</i>	<i>rs180089</i> <i>0</i>	<i>ATTTTAAATG[A/T]ATTTTTC</i> <i>CAG</i>	<i>genic</i>
<i>IL-10</i>	<i>1</i>	<i>20501352</i> <i>0</i>	<i>rs180089</i> <i>6</i>	<i>CTACTTCCCC[C/T]TCCCAA</i> <i>ANAA</i>	<i>genic</i>
<i>CR1</i>	<i>1</i>	<i>20584947</i> <i>9</i>	<i>rs170476</i> <i>60</i>	<i>AAATGCAATT[A/G]GAGTAC</i> <i>CAGG</i>	<i>genic</i>
<i>GBP7</i>	<i>1</i>	<i>89355278</i>	<i>rs753793</i> <i>7</i>	<i>CCATT-</i> <i>GACTG[C/G]AATGCCACCT</i>	<i>genic</i>
<i>IL-1A</i>	<i>2</i>	<i>11325969</i> <i>4</i>	<i>rs174116</i> <i>97</i>	<i>GCCTAGGTCA[G/T]CACCTT</i> <i>TTAG</i>	<i>genic</i>
<i>IL-1B2</i>	<i>2</i>	<i>11330686</i> <i>1</i>	<i>rs114363</i> <i>4</i>	<i>CTATCTTCTT[C/T]GACACAT</i> <i>GGG</i>	<i>genic</i>
<i>TLR9</i>	<i>2</i>	<i>52231737</i>	<i>rs352140</i>	<i>CGGAGC-</i> <i>TACC[A/G]CGACTGGAGG</i>	<i>exonic</i>

<i>TLR9</i>	3	52236071	<i>rs187084</i>	TCAC- TGCCCT[C/T]AAGAAGCTGA	<i>exonic</i>
<i>IL-1IL-17RD</i>	3	57113459	<i>rs678099</i> 5	GCAGCTGGTC[A/G]TCTCT GTAGT	+ strand,
<i>IL-1IL-17RE</i>	3	9935070	<i>rs708567</i>	CTCCACCCCT[A/G]AGTCAG CTGC	<i>genic</i>
<i>TLR1</i>	4	38476105	<i>rs483309</i> 5	TAAGGTAA- GA[C/T]TTGATAACTT	<i>genic</i>
<i>TLR1</i>	4	38476609	<i>rs574361</i> 1	TCTCATAA- TA[C/G]AATCCAGTAT	<i>genic</i>
<i>TLR6</i>	5	38506745	<i>rs574381</i> 0	CAC- CAGAGGT[C/T]CAACCTTAC T	<i>genic</i>
<i>TLR6</i>	5	38506909	<i>rs574380</i>	CTACAAATTC[C/T]GAATGC AAAA	<i>genic</i>
<i>IRF1</i>	5	13185477 9	<i>rs270638</i> 4	CCGGGCGATC[A/C]CCTCG CCTGC	<i>genic</i>
<i>IL-1IL-13</i>	5	13202386 3	<i>rs20541</i>	TCAGTT- GAAC[C/T]GTCCCTCGCG	<i>genic</i>
<i>IL-4</i>	5	13203705 3	<i>rs224325</i> 0	AGAACATTGT[C/T]CCCCAG TGCT	<i>genic</i>
<i>C6</i>	5	41235716	<i>rs180103</i> 3	CCATGCACTG[A/C]GCCTCT GGTA	<i>genic</i>
<i>IL20RA</i>	6	13736754 0	<i>rs155549</i> 8	GTAATAGA- TA[C/T]GGGCAAAACA	<i>genic</i>
<i>LTA</i>	6	31648120	<i>rs223970</i> 4	AGGACAC- TGC[G/T]GGGCGGTAGT	<i>genic</i>

<i>LTA</i>	6	31648292	rs909253	CAGAGAG- GAA[C/T]CATGGCAGAA	<i>genic</i>
<i>TNF-alpha - 1031</i>	6	31650287	rs179996 4	GCTGAGAA- GA[C/T]GAAGGAAAAG	<i>Less than 10kb down- stream promoter, positive strand.</i>
<i>TNFalpha-376</i>	6	31650943	rs180075 0	CTGTCTG- GAA[A/G]TTAGAAGGAA	<i>Less than 10kb down- stream promoter, positive strand.</i>
<i>TNFal- p h a - 3 0 8</i>	6	31651010	rs180062 9	GAGGGG- CATG[A/G]GGACGGGGTT	<i>Less than 10kb up- stream promoter, positive strand.</i>
<i>T</i>	6	31651080	rs361525	CCT- CRGAATC[A/G]GAGCAGGG AG	<i>Less than 10kb up- stream promoter, positive strand.</i>

<i>T</i>	6	31652168	rs309366 2	GTGAATACAC[A/G]GATGAA TGGA	<i>genic</i>
<i>C</i>	6	31947288	rs224266 5	ATGGAC- CAAC[A/G]TTACTCCACC	<i>genic</i>
<i>C</i>	7	11701751 9	rs171402 29	AATAGTGT- TA[C/T]TTCAGTGAAT	<i>genic</i>
<i>N</i>	7	30458762	rs207582 0	GCGG- GACCCC[A/G]AGGAGGTGT T	<i>genic</i>
<i>h</i>	7	80138385	rs321193 8	GTACATCATA[G/T]GGTGTG CTAG	<i>genic</i>
<i>h</i>	7	80140046		TTTACAATTT[C/G]CAANNC GGCT	<i>Genic</i>

<i>h</i>	7	80140051		AATTSCAAG[- /A]CGGCTGCAGG	<i>genic</i>
<i>T</i>	9	11951512 3	rs498679 0	TAC- CTCGATG[A/G]TATTATTGA C	<i>genic</i>
<i>T</i>	9	11951542 3	rs498679 1	TTTGG- GACAA[C/T]CAGCCTAAAG	<i>genic</i>
<i>A</i>	9	13512114 3	rs817674 6	TTTC- TACTAC[A/C]TGGGGGSGTT	<i>genic</i>
<i>H</i>	11	5204749	rs339505 07	AGTTGG- TGGT[A/G/T]AGGCCCTG	<i>genic</i>

<i>h</i>	<i>11</i>	<i>5204808</i>	<i>rs334</i>	<i>CTGACTCCTR[A/T]GGAGAA GTCT</i>	<i>genic</i>
<i>T</i>	<i>11</i>	<i>5675093</i>	<i>rs793556 4</i>	<i>GA- TAAAGGAG[A/G]ATCAAGAG GC</i>	<i>genic</i>
<i>R</i>	<i>11</i>	<i>63243962</i>	<i>rs542998</i>	<i>GTGAAAGTGG[C/T]TTTACC TGAT</i>	<i>genic</i>
<i>I</i>	<i>12</i>	<i>66928914</i>	<i>rs222750 7</i>	<i>ATCTCTCCAC[C/T]CTCTCC AAGC</i>	<i>genic</i>
<i>I</i>	<i>12</i>	<i>66930885</i>	<i>rs101235 6</i>	<i>CCATTAACT[A/T]TAATAAA TCT</i>	<i>genic</i>
<i>I</i>	<i>12</i>	<i>66932788</i>	<i>rs222749 1</i>	<i>CACAC- GGCCC[C/T]GTTTCGTCACA</i>	<i>Less than or equal 10kb down- stream promoter</i>

<i>I</i>	<i>12</i>	<i>66933980</i>	<i>rs222748</i> <i>5</i>	<i>TNCTA- TA- TAGTG[A/G]CTGAGTAAGC</i>	<i>Less than 10kb down- stream</i>
<i>I</i>	<i>12</i>	<i>66934889</i>	<i>rs222747</i> <i>8</i>	<i>GTGCCAC- TGC[A/G]AAGGGTCCGA</i>	<i>Less than 10kb down- stream, near IFNG SNPs.</i>
<i>S</i>	<i>14</i>	<i>64333053</i>	<i>rs229587</i>	<i>CTGGTTTTCA[C/T]TGAGCC AGGT</i>	<i>Genic, Negative strand</i>
<i>I</i>	<i>16</i>	<i>27281681</i>	<i>rs180501</i> <i>5</i>	<i>CTTCAG- CAAC[C/T]CCCTGAGCCA</i>	<i>genic , Negative strand</i>
<i>A</i>	<i>16</i>	<i>3973437</i>	<i>rs223073</i> <i>9</i>	<i>CACAGGTCAT[A/G]AAGAAC TCCC</i>	<i>genic , Negative strand</i>

A	16	4019824	rs107753 49	G TTCAGCTTT[C/G]TTACAC ATTT	genic , Negative strand
A	17	15802057	rs253561 1	GGTACAC- TTC[C/T]TTTTTTTTT	genic
N	17	23120724	rs229751 8	CATGAA- GAGC[A/G]ATTTCTTCAG	Less than 10kb down- stream
N	17	23152636	rs180048 2	CAGAG- TTCGA[C/G]ACCAGCATGG	Less than 10kb down- stream
N	17	23152855	rs928279 9	AGTGTTGG- GA[C/T]GGTGAGATCA	Genic, Negative strand
N	17	23153339	rs807834 0	AAGGCAGAAC[C/T]AGCTAC ATCA	Genic, Negative strand
I	19	10255792	rs179996 9	TTCCCTG- GAC[A/G]GGCTGTTCCC	Genic, Negative strand

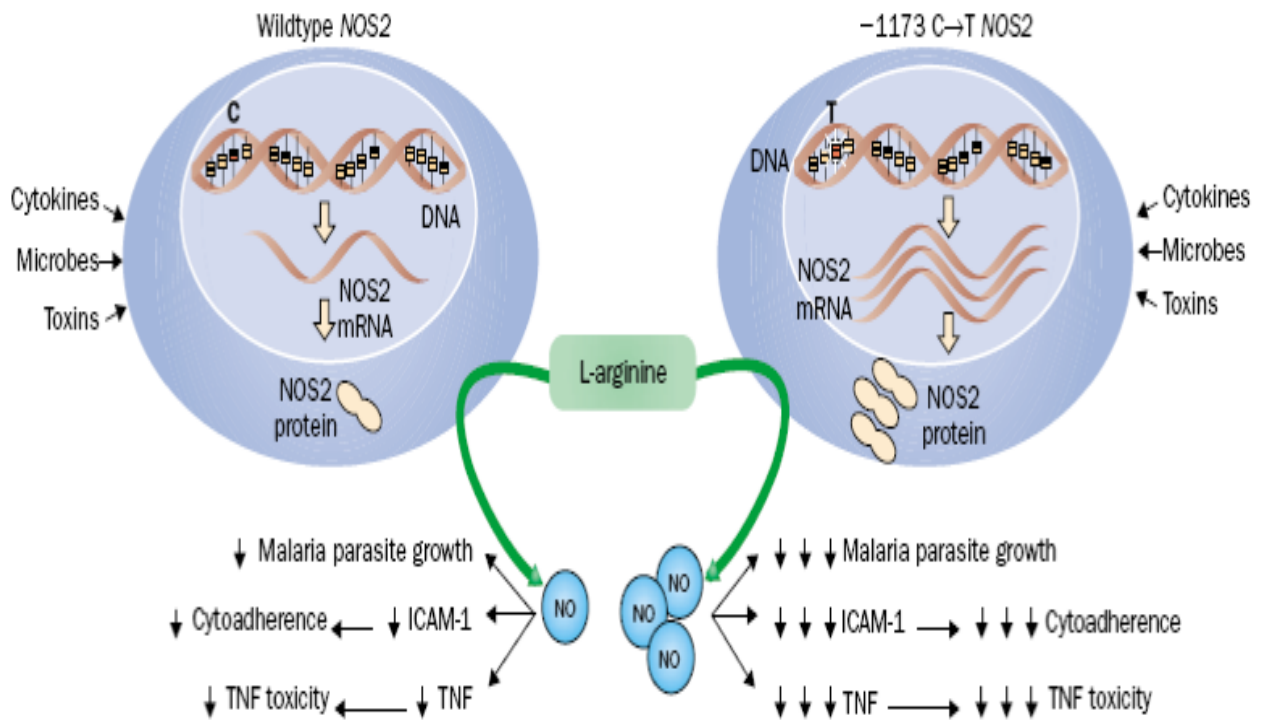
<i>I</i>	19	10256683	rs5498	GGTCACCCGC[A/G]AGGTG ACCGT	<i>Genic, Negative strand</i>
<i>E</i>	19	6870624	rs373533	TGGTGGTCTT[G/T]GAAGAA GCGC	<i>Genic, Negative strand</i>
<i>E</i>	19	6870753	rs461645	CAAAC- CTGAA[C/T]GTTCTCCAGA	<i>Genic, Negative strand</i>
<i>G</i>	20	56905438	rs205729 1	AGCTCCTTAT[A/G]GAGAAT TCTA	<i>Genic, Negative strand</i>
<i>G</i>	20	56919207	rs8386	ACACTGA- GAA[C/T]ATCCGCCGTG	<i>Genic, Negative strand</i>
<i>D</i>	22	22509132	rs317724 4	TTCTG- CAGGG[A/G]CATCCAGGAG	<i>Genic, Negative strand</i>

<i>C</i>	<i>X</i>	13555727 5	rs309294 5	TACAWCAG- CA[C/T]CAACAATTAT	Less than 10kb up- stream
<i>C</i>	<i>X</i>	13555822 1	rs112653 5	TCATA- GAAGG[C/T]TGGACAAGGT	
<i>G</i>	<i>X</i>	15341668 6	rs105082 9	TGGAGGG- CAT[C/T]CATGTGGCTG	
<i>G</i>	<i>X</i>	15341741 1	rs105082 8	GCATAGCCCA[C/T]GATGAA GGTG	
<i>a</i>		11223656		TTNCAC- NCNT[A/G]CCTCCTCTCT	

<i>a</i>		<i>11226027</i>		<i>GTGAGAG- TAA[C/T]AATACTTGCC</i>	
<i>a</i>		<i>11226571</i>		<i>GTAAAC- CTGC[A/C]TCTCTGTTTC</i>	

The chromosome, position and rs numbers were all correct up to the dbsnp build in August 2009.

Annexe 9 : A schematic representation of the effect of a gene polymorphism on the outcome of infection with malaria



-1173 C/T polymorphism in the iNOS gene increases the rate at which the gene is transcribed and the protein expressed. The result is an increase in the production of Nitric oxide and its effector functions, down-regulating ICAM and hence adherence of parasitized RBCs; as well as TNF and hence toxicity and increasing killing of parasites. (Hobbs et al, 2002).

Annexe 10: Extraction of DNA using Chelex-100 protocol

1. Required Materials and Equipment

- 1.5 ml microcentrifuge tubes
- 1-20 (1 single channel automatic pipettes
- 100-200 (1 single channel automatic pipette
- 1000 (1 single channel automatic pipette
- Filter pipette tips for the above pipettes
- Fine tip marker pens
- Ball point pen
- Paper towels or wipes
- Distilled water
- Phosphate Buffered Saline (PBS)
- Bleach (5 %) in a beaker or wash bottle
- Distilled water in a beaker or wash bottle
- Ethanol (70 %) in a beaker or wash bottle
- Chelex®-100 Resin
- Scissors or 1/8 inch hole punch (plus spare filter paper if using a punch)
- Timer
- Microcentrifuge
- Heating block or waterbath at 56 °C
- Waterbath at boiling temperature (96 °C or above)
- Vortex

Procedural steps

Important points to remember:

- Ensure the scissors are thoroughly cleaned before beginning the procedure, in between cutting filter papers and at the end of the procedure. Unclean scissors can lead to cross contamination of samples and poor quality results.
- Ensure pipette tips are of a high quality, sterile and endonuclease free.
- Do not touch pipette tips.
- Make sure pipettes are calibrated and cleaned regularly.

1. Print out a PCR worksheet and record the sample ID of each DBS to be tested on a separate numbered line.

2. Gather all required supplies.

NB. If samples have been stored at +4 °C or -20°C they must be brought to room temperature in the sample bag prior to opening.

3. Gather all required supplies.

Make Chelex reagent (20 %) by adding distilled water (e.g. 0.2 g Chelex with 10 ml distilled water).

NB. Chelex reagent should be made fresh each day it is required.

4. Clean the scissors or punch by dipping in ethanol (70%) and passing through a flame.

5. Label an appropriate number of 1.5 ml microcentrifuge tubes (label both the lid and the side of the tube) with the worksheet number and sample ID.

6. Cut 2-3 pieces of 3 mm x 3 mm or punch a 3 mm disk (holds approx. 3-5 μ l of dried blood) from the filter paper and put it into the corresponding 1.5 microcentrifuge tube or well of a 96-well microtitre plate.

NB. Clean the scissors between each sample as detailed in step 5. Clean the punch by punching clean filter paper 3 times.

7. Add 1 ml PBS.

NB. Ensure filter papers are soaked in buffer.

8. Incubate at room temperature for 10 min.

9. Centrifuge at 14,000 rpm for 2 min and discard supernatant using a clean pipette tip for each sample.

10. Add 1 ml PBS

11. Centrifuge at 14,000 rpm for 2 min and discard supernatant using a clean pipette tip for each sample.

12. Add 150 μ l of nuclease-free water.

13. Add 50 μ l of 20 % Chelex.

14. Incubate at 99 °C for 10 min.

15. Centrifuge at 14,000 rpm for 1 min.

16. Store supernatant at +4°C for use in PCR.

NB. If storing samples for longer than a day, transfer supernatant into a fresh microcentrifuge tube and stored at -20 °C.

Quality Control

Negative Control

To test for cross-contamination, each batch of 10 samples should contain at least 1 negative control. The negative control consists of a section of plain filter paper cut in the same way as the DBS samples.

5. Procedure limitations

Successful extraction of DNA is dependent upon the quality and quantity of DNA in the DBS sample, quality of laboratory reagents, equipment and supplies, and the implementation of good quality clinical laboratory practice according to this SOP.

6. Interpretation and Reporting of Results

The extracted DNA sample is used as template DNA for subsequent PCR reactions. There are no reporting requirements at this stage apart from the rejection of poor quality samples.

Source: University of Maryland Medical School, Maryland, USA.

Publications