Molecular Characterization of Severe *Plasmodium falciparum* Malaria Parasitemia and Antimalarial Drug-resistance in Eastern Sudan

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# List of Contents

Dedication.................................................................................................................................I  
This thesis is based on the work presented in the following papers .........................................II  
Acknowledgements..................................................................................................................III  
Abbreviations.............................................................................................................................V  
Definitions...................................................................................................................................VII  
Abstract......................................................................................................................................IX  
Arabic Abstract...............................................................................................................................XI  
Legends for figures.......................................................................................................................XIII  
List of tables.................................................................................................................................XVIII  
List of appendices.......................................................................................................................XIX  

## Chapter One: Introduction & literature review

1.1 Global situation of malaria.....................................................................................................1  
1.2 Epidemiology of malaria......................................................................................................3  
1.3 Clinical Manifestations........................................................................................................5  
1.4 Malaria diagnosis................................................................................................................6  
1.5 The malaria parasite...........................................................................................................8  
1.6 Malaria life cycle................................................................................................................10  
1.7 Malaria immunity................................................................................................................12  
1.7.1 Innate immunity...............................................................................................................13  
1.7.2 Acquired immunity..........................................................................................................13  
1.7.3 Strain specific immunity..................................................................................................13  
1.8 Antigenic diversity...............................................................................................................14  
1.8.1 MSP1.................................................................................................................................14  
1.8.2 MSP2.................................................................................................................................15  
1.8.3 GLURP...............................................................................................................................17  
1.8.4 Microsatellite markers.....................................................................................................17  
1.9 Genetic diversity in malaria parasites..................................................................................18  
1.10 Malaria chemotherapy and parasite resistance.................................................................22  
1.10.1 4,8-aminoquinoline drugs.............................................................................................22  
1.10.2 Antifolate drugs.............................................................................................................22  
1.10.3 Quinine...........................................................................................................................23  
1.10.4 Artemisinin and its derivatives.......................................................................................24  
1.10.5 Combination therapy to combat the spread of drug resistance...................................24  
1.10.6 Definition of parasite resistance...................................................................................25  
1.10.7 Malaria parasite resistance...........................................................................................25  
1.10.8 Evolution of antimalarial drug resistance......................................................................29  
1.10.9 Methods for monitoring parasite resistance.................................................................29
Chapter Three: Results

3.1 Severe malaria........................................................................................................64
3.1.1 Gedarif Hospital, 2000-2002...............................................................................64
3.1.1.1 Clinical pattern of severe Plasmodium falciparum malaria..............................64
3.1.1.1.1 The frequency of acute uncomplicated and complicated malaria...............64
3.1.1.1.2 The frequency of the different types of severe complications......................64
3.1.1.1.3 Comparison between the age distribution of severe and mild malaria..........67
3.1.1.1.4 Characterization of patients in the different categories of severe malaria.......67
3.1.1.1.5 Comparison between fatal and non-fatal severe malaria..............................69
3.1.1.2 Allelic polymorphism of MSP2 gene in severe P. falciparum malaria..................70
3.1.1.2.1 Clinical groups and samples........................................................................70
3.1.1.2.2 Prevalence of single clone and multi-clone malaria infections.......................70
3.1.1.2.3 Multiplicity of infection in different clinical grades of malaria infection.........72
3.1.1.2.4 The frequency of IC1 and FC27 genotype families and allele size in different........72
types of malaria infection..........................................................................................72
3.1.1.2.5 Dynamic of parasite clone numbers and types in persistent SM infections after...74
                                                         quinine treatment.........................................................................................74
3.1.1.2.6 Differential clearance of IC1 genotype in multi-clone SM infections after treatment....75
3.1.1.3 Genetic fingerprints of parasites causing severe malaria in a setting of low........76
                                                          transmission “one strain one patient”.........................................................76
3.1.1.3.1 Clinical data.............................................................................................76
3.1.1.3.2 Diversity of infection (DOI).........................................................................76
3.1.1.3.3 Genetic diversity of parasite isolates in patients with severe malaria, based on.....77
                                                          use of a single molecular marker......................................................................77
3.1.1.3.4 Genetic diversity of parasite isolates obtained from patients with uncomplicated......malaria, based on use of a single molecular marker..............................................78
3.1.1.3.5 Genetic diversity of parasite isolates obtained from malaria patients, based........78
                                                          on use of multiple molecular markers...............................................................78
3.1.1.3.6 Homogeneity of parasite isolates in patients with severe malaria................80
3.1.2 Severe malaria in New-Halfa Hospital (November 2000-February 2001)...............81
3.1.2.1 Clinical and parasitological categorization of patients.....................................81
3.1.2.2 Parasite prevalence by PCR.........................................................................82
3.1.2.3 Molecular characterization of parasite isolates.................................................83
3.1.2.4 Prevalence of antibodies against MSP Antigens..............................................84
3.2 Estimation of antimalarial drugs efficacy in Eastern Sudan.....................................87
3.2.1 Gedarif area (October-December 2003) .................................................................87
3.2.1.1 The efficacy of sulfadoxine-pyrimethamine alone and in combination with chloroquine....87
3.2.1.1.1 Characteristics of the treatment groups .................................................................87
3.2.1.1.2 Clinical and parasitological response .................................................................87
3.2.1.1.2(a) Efficacy of SP and SP plus CQ .................................................................87
3.2.1.1.2(b) Prevalence of asexual parasitemia during follow-up ........................................89
3.2.1.1.3 Drug resistance and age .....................................................................................90
3.2.1.1.4 Age and gametocyte carriage ............................................................................90
3.2.1.1.5 Gametocytogenesis and drug response .............................................................92
3.3 Implication of dhfr/dhps/pfcrt molecular markers in evolution of P. falciparum malaria parasite
   beyond the Sulfadoxine/pyrimethamine resistance: more relevance & mutual association ....94
3.3.1 Prevalence of individual pfdhfr and pfdhps mutations .................................................94
3.3.2 The linkage and the multiplicity of mutations ..............................................................95
3.3.3 Association of dhfr/dhps mutations with sulfadoxine/pyrimethamine drug response ...95
3.3.4 Multiplicity of mutations, age and the immune factor ................................................96
3.3.5 Multiplicity of mutations and pre-treatment parasitemia ............................................98
3.3.6 Multiplicity of mutations and gametocytogenesis .....................................................98
3.3.1 Submicroscopic Plasmodium falciparum infections during pregnancy .........................99

Chapter Four: Discussion:
4.1 Severe malaria ...............................................................................................................100
4.1.1 In Gedarif area during the two seasons 2000-2002 ....................................................100
4.1.1.1 Clinical pattern of severe falciparum malaria ..........................................................100
4.1.1.2 Allelic polymorphism of MSP2 gene in severe P. falciparum malaria ......................103
4.1.1.3 Genetic fingerprints of parasites causing severe malaria in a setting of low..........
   transmission “one strain one patient” .............................................................................106
4.1.2 In New Halfa area ........................................................................................................109
4.1.2.1 Cerebral malaria is frequently associated with latent parasitemia among ..........
   semi-immune population ...............................................................................................109
4.2 Estimation of antimalarial drug efficacy in Eastern Sudan ................................................111
4.2.1 In Gedarif area (October-December 2003) ...............................................................111
4.2.1.1 The efficacy of Sulfadoxine/pyrimethamine alone and in combination with chloroquine....111
4.2.1.2 Implication of the dhfr/dhps/pfcrt molecular markers in the evolution of P. falciparum
   malaria parasite beyond the SP resistance: more relevance and mutual association .....114
4.3 Submicroscopic Plasmodium falciparum infections during pregnancy .........................117
4.4 Conclusion .....................................................................................................................119
References ..........................................................................................................................121
Appendices .................................................................................................................. 147
Publications resulted from this work ........................................................................ 154
DEDICATION
TO MY FAMILY
This thesis is based on the work presented in the following Papers:


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Abs</td>
<td>Antibodies</td>
</tr>
<tr>
<td>ACPR</td>
<td>Adequate clinical and parasitological response</td>
</tr>
<tr>
<td>ACR</td>
<td>Adequate clinical response</td>
</tr>
<tr>
<td>AETF</td>
<td>Actual early treatment failure</td>
</tr>
<tr>
<td>Ags</td>
<td>Antigens</td>
</tr>
<tr>
<td>ASUM</td>
<td>Asymptomatic sub-microscopic malaria</td>
</tr>
<tr>
<td>AT</td>
<td>Artesunate</td>
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<tr>
<td>CAM</td>
<td>Convulsion associated malaria</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral Malaria</td>
</tr>
<tr>
<td>CN</td>
<td>Clone Number</td>
</tr>
<tr>
<td>COI</td>
<td>Complexity of infection</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CS</td>
<td>Clinically Suspected</td>
</tr>
<tr>
<td>csCM</td>
<td>Suspected to have Cerebral Malaria</td>
</tr>
<tr>
<td>csSMA</td>
<td>Suspected to have Severe Malarial Anemia</td>
</tr>
<tr>
<td>CT</td>
<td>Combination therapy</td>
</tr>
<tr>
<td>dH2O</td>
<td>Deionized or distilled water</td>
</tr>
<tr>
<td>dhfr</td>
<td>Dihydrofolate reductase gene</td>
</tr>
<tr>
<td>dhps</td>
<td>Dihydropteroate synthase gene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DOI</td>
<td>Diversity of infection</td>
</tr>
<tr>
<td>DPR</td>
<td>Delayed parasitological response</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra Acetate</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological Inoculation Rate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ETF</td>
<td>Early treatment failure</td>
</tr>
<tr>
<td>glurp</td>
<td>Glutamate rich protein gene</td>
</tr>
<tr>
<td>GLURP</td>
<td>Glutamate Rich Protein</td>
</tr>
<tr>
<td>GP</td>
<td>Gametocyte producing parasites</td>
</tr>
<tr>
<td>GRI</td>
<td>Genotype-resistance index</td>
</tr>
<tr>
<td>HTN</td>
<td>Hypotension associated with malaria</td>
</tr>
<tr>
<td>LTF</td>
<td>Late treatment failure</td>
</tr>
<tr>
<td>MCIF</td>
<td>Mutant clearance immunity factor</td>
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<tr>
<td>MCN</td>
<td>Maximum Clone Number</td>
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</tbody>
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MF: Malaria Free Individuals
MOI: Multiplicity of infection
MOM: Multiplicity of mutation score
MSP1: Merozoite Surface Protein1
msp1: Merozoite surface protein1 gene
MSP2: Merozoite Surface Protein2
msp2: Merozoite surface protein2 gene
PBS: Phosphate Buffer Saline
PCR: Polymerase Chain Reaction
pfcrt: Plasmodium falciparum chloroquine resistance transporter gene
pfemp1: Plasmodium falciparum erythrocyte membrane protein1 gene
PfEMP1: Plasmodium falciparum erythrocyte membrane protein1
pfmdr: Plasmodium falciparum multidrug resistance gene
SM: Severe Malaria
SMA: Severe Malarial Anemia
SNP: Single nucleotide polymorphism
SP: Sulphadoxine/Pyrimethamine
SPR: Sulphadoxine/Pyrimethamine resistance
SSOP: Sequence-specific oligonucleotide probes
Taq: DNA polymerase enzyme
TBE: Tris-Boric acid-EDTA Buffer
TMAC: Tetramethyl ammonium chloride
UM: Uncomplicated Malaria
Definitions:

Malaria definition: A patient was defined as suffering from malaria if he/she complained of fever or had body temperature measured with an oral probe of $\geq 37.5^\circ C$ plus microscopically detected asexual parasitemia.

Isolate: A sample of parasites collected at one time from an individual (not cultured or subject to any procedure which may have changed the mixture of parasites).

Prepatent period: is defined as the time from infection by mosquito bite until the time trophozoites are detectable in erythrocytes. It is fixed and constant for each species of parasites. It is associated with the length of pre-erythrocytic cycle, but is longer than it.

Incubation period: is defined as the time from infection by mosquito bite until the first appearance of clinical symptoms. It is variable but usually much longer than the prepatent period as it requires parasitemia to reach a sufficient density before symptoms occur. It is thought to be influenced by the initial sporozoite load, and subsequently by the load of merozoites invading the blood stream: the higher the load the shorter the incubation period.

Entomological inoculation rate (EIR): vector density, human blood index and sporozoite rate allow the entomological inoculation rate to be calculated. The EIR is given by the density (number of vectors per inhabitant) multiplied by the human blood index and by the sporozoite rate. If we assume for instance that the catches provided a number of 15 anoph eles per inhabitant and per night, that 78% of them were human fed and that 10% of the vectors showed to be sporozoite infected, the EIR will be $15 \times 0.78 \times 0.1 = 1.17$.

Recrudescence: is the renewed clinical manifestation of the infection due to persistence of erythrocytic forms into the circulation. This is typically observed in P. falciparum infection exposed to subcurative drug treatment (latency of few days to few weeks) and in P. malariae (latency as long as several years).

Relapse: is a renewed clinical manifestation of the infection started by persistent liver merozoites (hypnozoites) which start an exo-erythrocytic cycle months after the invasion of the hepatocyte. This is typically observed in P. vivax and P. ovale infection treated with drugs which have no action on the parasite liver stages.

Clone: A set of genetically identical parasites derived from one parasite by asexual reproduction.
**Line:** Parasites which have been passaged in vitro or in vivo, initially starting from one isolate. If parasites have been cloned in the laboratory, they are referred to as cloned lines.

**Genotype:** A genetic characteristic of a parasite, the type of allele found at a polymorphic locus in an individual.

**Allele:** One of several alternative forms of a gene.

**Allelic type:** Alleles of a gene can be grouped with regard to similar characteristics, e.g. the allelic types of MSP1 and MSP2, also referred to as allelic families.

**Multiplicity of infection:** number of infecting genotypes in an isolate.
Abstract

A hospital-based study was carried out in Gedarif town, an area of markedly unstable malaria transmission in eastern Sudan during the two malaria transmission seasons (2000-2002). Among the 2488 diagnosed malaria patients, 110 fulfilled the WHO (2000) criteria for severe malaria (SM), and seven died of cerebral malaria (CM). The predominant complication was severe malarial anemia (SMA, 45.45%), followed by convulsions associated malaria (CAM, 20.9%), cerebral malaria (16.4%) and hypotension (HTN, 11.8%).

DNA was extracted from 616 parasites obtained from 231 donors with asymptomatic submicroscopic malaria (ASUM), uncomplicated malaria (UM) and SM. The MSP2 locus was exploited for determination of parasite genotype. 51 parasite genotypes were detected. There was no correlation between the number of infectious clones and initial parasite count or patient age. In all infections, the mean clone number (MCN) was 1.5 clone/infection, and it was comparable between SM and UM. However detailed analysis revealed that; individuals with ASUM had significantly lower MCN compared with UM, SMA, and non-fatal CM. The frequency of IC1 and FC27 allele families was comparable between SM and UM and the distribution of the individual allele sizes was correlated. Further analysis showed that, 1. FC27 was associated with the mildest form of malaria and was not recognized in fatal CM, 2. Faster clearance of IC1 genotype in the multi-clone infections after quinine treatment 3. Multi-clone infections with different genotype families (IC1 and FC27) were far more frequent than that with the same genotype family, suggesting stronger cross-immunity within rather than between MSP2 gene families.

The diversity of infection (DOI) was estimated by using different molecular markers (pfcr76, pfmdr1 86, glurp and MSP2) separately and by considering them together to generate a multi-locus genetic profile for each isolate. pfcr7 and pfmdr1, are not usually used for parasite genotyping, here they revealed DOI of 0.043 and 0.064, respectively. However, when used in combination with the most polymorphic markers (MSP2 and GLURP) they disclosed a hidden diversity that would have not been detected. The DOI as estimated by MSP2 and GLURP was, 0.553 and 0.435, respectively. However, combining all 4 molecular markers (multi-locus genetic profile), had revealed a finger print pattern of diversity, DOI of 0.936, which almost indicated that there were one unique strain for each patient, and that was comparable between SM and UM (although for UM, GLURP was not analyzed). Not only, the genetic make up of parasites were different, but clinical data suggest that the virulence markers should also be extremely diverse.
Another hospital-based study was carried out in New Halfa town, where 120 individuals were enrolled in the study, including febrile patients with and without microscopically detectable parasitemia, and apparently healthy individuals. PCR for parasite detection and ELISA tests for measuring serum antibody levels were carried out on all blood samples. A majority of the febrile patients, who were parasite negative by microscopy, showed the presence of a *P. falciparum* infection by PCR. The occurrence of *P. falciparum* infection with parasitemia below the detection level of microscopy was recognized more often in patients with CM symptoms than SMA, and in older rather than younger patients. Patients clinically suspected to have cerebral malaria (*CS*CM) mostly had a single clone infection and a large proportion of them acquired antibodies against MSP antigens. The therapeutic response to quinine treatment was comparable between patients with *CS*CM and CM.

In a 28-days in-vivo study of pyrimethamine/sulfadoxine (SP) and SP plus chloroquine (CQ) efficacy, 260 patients with uncomplicated *P. falciparum* malaria were enrolled in rural Eastern Sudan. The results revealed a comparable treatment failure (TF) for SP alone (32.99%) or with CQ (31.7%). Patients who achieved adequate clinical response (ACR), were significantly older than patients who had TF. Regarding gametocytogenesis; a. the microscopic gametocyte productivity was significantly higher in patients with TF when compared to ACR. Thus, gametocytemia was more associated with younger age b. gametocyte counts were comparable between TF and ACR groups of patients until Day7 of follow up, thereafter, at D14, D21 and D28, gametocytes count was significantly higher in patients with TF. However, the longevity of gametocytes in patients with TF and ACR were comparable.

Parasite isolates from 168 patients (including all patients with TF) were genotyped at the *dhfr*, *dhps* and *pfcr* loci. The molecular image of the in-vivo phenotype, revealed the following findings: the prevalence of *dhfr* mutations c51-I, c108-N, and double mutant c51I/108N, were estimated to be 91.9%, 92.3% and 79.7%, respectively, while only one isolate (0.6%) was found carrying c59R and no c164L mutation was recognized. The *dhps* mutations; c437G, c540E and double mutant c437/c540 had a prevalence rate of 90.2%, 79.3% and 58.5%, respectively, while those of c436F and c581G were, 0.6% and 13%, respectively. For *pfcr* gene at c72-76, 92.26% were the mutant type CVIET, only 6.55% were the wild type CVMNK and 2 patients harbored mixed infection. Based on the multiplicity of mutation score (MOM) ranging from 1 to 5, the prevalence according to the MOM score were; 0.97, 0.931, 0.866, 0.719, 0.121, respectively.
The text is in Arabic and does not translate into natural language as it contains scientific and medical content that cannot be accurately translated. It seems to discuss medical conditions and research methods.
لا يوجد نص يمكن قراءته بشكل طبيعي من الصورة المقدمة.
Legends for figures:

Figure (1.1): Global malaria distribution and endemicity, 2003 (microbiology syllabus on malaria 2004)

Figure (1.2): The Malaria Life Cycle

Figure (1.3): Malaria endemicity level in Sudan 2001.

Figure (1.4): Sudan major malaria strata 2001.

Figure 2.1: Map for the study area

Figure 2.2: Flow chart of the dihydrofolate reductase, dihydropteroate synthase, and *Plasmodium falciparum* chloroquine resistance transporter sequence-specific oligonucleotide probe–enzymelinked immunosorbent assay (SSOP-ELISA) method.

Figure 3.1: Symptoms in the 110 patients admitted to Gederif Hospital, Eastern Sudan and fulfilling the WHO criteria for severe malaria, the patients were admitted over a period of two years.

Figure 3.2: The age distribution of patients with severe malaria and with uncomplicated malaria in Gederif Hospital (period 2000 – 2002).

Figure 3.3: Comparison between four groups of patients with severe malaria (anemia, convulsions, cerebral malaria and hypotension) admitted to Gederif Hospital.

Figure 3.4: Shows the MSP2 allele-size polymorphism in uncomplicated and severe malaria.

Figure 3.5: Shows the genetic diversity of infection in different severe malaria patients at the time of malaria diagnosis.

Figure 3.6: Shows the differential and faster clearance of IC1 genotypes in multi-clone infections with IC1 and FC27 parasite clones, in SM patients.

The Figure 3.7: The multi-locus genetic profiles of parasites isolated from patients with severe malaria.

Figure (3.8): The proportions of anti-body responders to any of the MSP fragments (MSP119, MSP2GF and MSP2T9), in the different study groups in New Halfa area.

Figure (3.9): A representative data from the three most diverse study groups; the clinically suspected cerebral malaria, microscopically confirmed uncomplicated malaria and malaria free donors In New Halfa area.

Figure (3.10): Shows the clinical and parasitological response of 260 *P. falciparum* malaria patients treated with CQ plus SP or SP alone in Gederif area.

Figure (3.11): Shows the proportion of patients who had microscopically detectable asexual parasitemia during the days of follow up and the others were treated with SP alone.

Figure (3.12): (A) Shows the age of patients who had asexual parasitemia during the days of follow up, after treatment with either SP alone or in combination with CQ.

Figure (3.13): The gametocyte carriage rate (%) during the days of the follow up, a comparison between two different treatment regimes, SP alone and SP plus CQ.

Figure (3.14): The gametocytes count (median) in the different days of follow up, for patients achieved adequate clinical response and in patients with treatment failure.

Figure (3.15): In the this figure, parasite isolates obtained from patients with uncomplicated malaria were genotyped at *dhfr/dhps* loci, and grouped into five groups based on the number of mutations in the previous loci (Max. 5 mutations) per each parasite.

Figure (3.16): Comparison between isolates obtained from patients attended adequate clinical response and those had treatment failure.
List of tables:

**Table 2.1:** Sequences of *P. falciparum* allele specific primers used in genotyping

**Table 2.2:** Distribution of the probes used for the *dhfr*, *dhps*, and *Pfcrtr* ELISA haplotyping assay* SSOP sequence† Probe

**Table 3.1:** Clinical categorization of patients presented with malaria-like symptoms to the malaria clinic at Gedarif Hospital, in the period, from September 2000 to January 2002. The patients died of severe malaria were all had cerebral malaria.

**Table 3.2:** Shows levels of variable parameters (parasitological, hematological and biochemical) in individuals died of severe malaria, and comparison of the mean values of these parameters between fatal and non-fatal severe malaria groups.

**Table 3.3:** The frequency of single clone and multi-clone infections in patients with severe malaria (SM), uncomplicated malaria (UM) and malaria free donors (MF).

**Table 3.4:** The mean clone number and the frequency of infection with FC27 allele family alone in; MF donors and individual complications of SM, including fatal and non-fatal CM

**Table 3.5:** Estimation of the diversity of infection (DOI) in severe and uncomplicated malaria, by using different molecular markers (*pfcrt*, *pfmdr1*, *msp2* and *glurp*) and by compiling all markers together.

**Table 3.6:** Characteristics of three pairs of patients infected with parasites of the same multi-locus genetic profile in each pair, but differ between pairs.

**Table 3.7** The table shows, number of individuals, number of patients with coma and mortality rate of comatose patients in the three study groups. Grouping of study individuals was based on presence of malaria symptoms and microscopic detection of parasites.

**Table 3.8** Parasitemia detected by microscopy and by polymerase chain reaction and the mean number of clones, in different study groups or subgroups in New Halfa area.

**Table 3.9** Characteristics of the two treatment groups (sulfadoxine/pyrimethamine alone or in combination with chloroquine in Daraweesh and Kajara villages, Gedarif area.

**Table (3.10):** The prevalence of individual and multiple molecular markers of SP resistance, the *dhfr* and *dhps* mutations, in parasite isolates obtained from patients from Eastern Sudan.

**Table (3.11):** The period prevalence of gametocytemia and gametocytogenesis in patients infected with parasites bearing the wild-type variant of *dhfr/dhps* genes and others with various numbers of mutations.

**Table (3.12):** some investigations for pregnant women.
List of appendices:

Appendix 1: The questionnaire for the severe malaria patients

Appendix 2: The questionnaire for the drug resistance study

Appendix 3: Schematic representation of MSP-1 of *P. falciparum* and of recombinant MSP-1 antigens. The division into 17 blocks is as outlined by Tanabe et al., (1987); blocks of conserved sequences are denoted by open boxes.

Appendix 4: Schematic representation of MSP-2 of *P. falciparum*

Appendix 5: Schematic representation of GLURP of *P. falciparum*

Appendix 6: The consent
Chapter One
Introduction and Literature Review

1.1 Global situation of malaria:

Malaria is among the oldest diseases, in one form or another; that has infected and affected our ancestors since a long time ago. During our recent evolution, its influence has probably been greater than that of any other infectious agent. As shown in fig. (1.1) the true magnitude of the mortality and morbidity attributable to malaria worldwide is at best a scientific guess, although it is not disputable that the greatest burden is in sub-Saharan Africa.

Figure (1.1): Global malaria distribution and endemicity, 2003

At the end of 2004, 107 countries and territories had areas at risk of malaria transmission, where some 3.2 billion people lived at risk of malaria transmission. An estimated 350-500 million clinical malaria episodes occur annually; most of these caused by \textit{P. falciparum} and \textit{P. vivax}. \textit{P. falciparum} causes more than 1 million deaths each year. It also contributes indirectly to many additional deaths mainly in young children, through synergy with other infections and illnesses.

The pattern of malaria transmission and disease vary markedly between regions and even within individual countries. This diversity results from variations between malaria parasites and mosquito vectors, ecological conditions that affect malaria transmission and
socioeconomic factors, such as poverty and access to effective health care and prevention services. About 60% of the cases of malaria worldwide, 75% of global *falciparum* malaria cases and more than 80% of malaria deaths occur in Africa south of the Sahara. *P. falciparum* causes the vast majority of infections in this region and about 18% of deaths in children under five years of age. Malaria is also a major cause of anemia in children and pregnant women, low birth weight, premature birth and infant mortality. In endemic African countries, malaria accounts for 25-35% of all outpatient visits, 20-45% of hospital admissions and 15-35% of hospital deaths, imposing a great burden on already fragile health-care systems. Malaria remains a major global problem, exacting an unacceptable toll on the health and economic welfare of the world’s poorest communities.

Outside tropical Africa malaria occurs mainly among individuals who lack immunity and are infected with *P. falciparum* in areas where appropriate diagnosis and treatment are not available. It is estimated that malaria claims more than 100000 lives per year where it is not endemic in the world and these deaths occur in all age groups. The total number of cases recorded in these regions is approximately five million per year. About 80% of these cases are found in Asia, where extremely severe parasite resistance to the drugs has developed.

Malaria is an impediment to social and economic development, being responsible for lost days at school and work, draining limited health care budget and rendering some areas uninhabitable for non-immune populations. In countries of South America, where malaria is endemic, the number of days lost each year because of malaria has been shown to vary between 1.5 and 14.3 days per person. Studies in rural areas of Africa reveal that over one third of primary school children had malaria during school term, more than half of this group had two or more attacks, missing a week or more of school days with each attack.

Malaria is considered as one of the main health problem in Sudan, it is estimated to account for 20-40% of the total hospital out-patients visits and 10-40%, from the in-patients admissions, and it is the cause of death for 10-15% of the in-patients.

### 1.2 Epidemiology of malaria:

The “many epidemiologies” of malaria have been characterized by degrees of endemicity. Malaria is described as endemic when there is a constant incidence of cases over a period of many successive years. At the other extreme malaria transmission may be epidemic when there is a periodic or occasional increase in the incidence of cases. A more general classification into stable and unstable malaria has been introduced. Stable malaria refers to
high transmission without any marked fluctuations over the years, although seasonal fluctuations may exist. Unstable malaria describes transmission that varies from year to year with the possibility of epidemics. The former situation is characterized by high degrees of collective immunity whereas the latter is not. These terms describe extremes of a wide range of situations.

Malaria is transmitted primarily by the bite of infected anopheline mosquitoes. The source of infection can be either a sick person or an otherwise symptomless carrier of parasites. It can also be transmitted by inoculation of infected blood and congenitally. Anophelines feed at night and their breeding sites are primarily in rural areas. The greatest risk of malaria is therefore from dusk to dawn in rural areas. In many malaria-endemic areas, there is little or no risk in urban areas. However, urban transmission is common in some parts of the world, especially Africa.

Most infected children in malaria endemic areas have asymptomatic parasitemia and only a minority of the infection progress to become life threatening. The incidence of severe clinical manifestations varies seasonally within endemic areas and it is not possible at present to predict which asymptomatic individual will develop severe disease. The observation that host genetic factors can modify disease outcome is also of importance in preventing severe disease.

The epidemiological profile and clinical pattern of severe malaria in Africa has been shown to vary according to the intensity of exposure in children living in rural areas with different levels of transmission. The degree of endemicity varies between countries and even between different areas in the same country. In Sub-Saharan Africa cerebral malaria and severe malarial anemia are leading causes of mortality.

The pattern of pathology also differs with changes in the degree of endemicity. In areas of high endemicity, although individuals after 5 years of age continue to harbour malaria parasites, the frequency of disease is greatly reduced. This protection from disease in older children and the development of clinical immunity usually is never reached in regions where there is low or seasonal exposure to parasite.

Morbidity and mortality due to malaria will be substantially greater upon exposure of individuals who are innately susceptible to this disease. Such groups may be selectively targeted for interventions.

Malaria has reinvaded areas in which it has previously been well under control such as Azerbaijan, Tajikistan, Iraq and Turkey. Epidemics in these areas resulted from rapid
deterioration of malaria prevention and control operations brought about by military conflicts followed by economic crisis. Migration from rural to urban areas can lead to the movement of infected people to towns with consequent enhancement of malaria transmission within the town. The converse may also be true where none-immune migrants arrive in an urban area where malaria transmission is occurring. Urban migration can also increase malaria apart from increasing transmission in peri-urban areas.

Persons who normally should not be at risk of serious life threatening disease are put in danger when they have no access to early diagnosis and adequate treatment of malaria disease. This can be due to inadequate health infrastructure, inadequately trained health staff, or scarcity of resources (of the patients and/or health services). Wars, political unrest and famines may cause increased risk of malaria. This may be due to either disruption of existing health care structures in endemic areas or the movement of people to new geographic locations creating new risk for migrants or the communities they cohabit. Malaria epidemics have been associated with military conflicts, social unrest and natural catastrophes.

The impact of human-induced global climate change poses an obvious threat to human health. The insect-vectors of *Plasmodium* spp. thrive in warm climates of tropical countries. Global warming leading to increased temperature in temperate areas, could provide a habitat suitable for the increased distribution of anopheline vectors. Whether the potential increase in vector populations will lead to a concomitant increase in malaria transmission is not clear. Increased temperature can both increase the mortality of the vector and the biting rate as well as effect the duration of the sporogonic cycle.

Malaria is unstable in the semi-arid savannah of central and northern Sudan and the great majority of infective bites take place in September and October, immediately prior to the seasonal peak of malaria cases during these months. Malaria morbidity and mortality, with special reference to central Sudan, affects all age groups. In Sudan it has been found that, despite variation between districts, urbanization tends to lead to reduced human malaria transmission. Moreover these results are similar to those of Trape and Zoulani in the Cango.

### 1.3 Clinical Manifestations:

The most characteristic symptom of malaria is fever. Other common symptoms include chills, headache, nausea, and vomiting. Diarrhea, abdominal pain, and cough are occasionally seen. As the disease progresses, some patients may develop the classic malaria paroxysm with
bouts of illness alternating with symptom free periods. The malaria paroxysm comprises three successive stages. The first is a 15 to 60 minute cold stage characterized by shivering and a feeling of cold. Next comes the 2 to 6 hours hot stage, in which there is fever, sometimes reaching 41°C, flushed, dry skin, and often headache, nausea, and vomiting. Finally, there is the 2 to 4 hours sweating stage during which the fever drops rapidly and the patient sweats. In all types of malaria the periodic febrile response is caused by rupture of mature schizonts. In *P. vivax* and *P. ovale* malaria, a brood of schizonts matures every 48 hours, so the periodicity of fever is tertian ("tertian malaria"), whereas in *P. malariae* disease, fever occurs every 72 hours ("quartan malaria").

The fever in *falciparum* malaria may occur every 48 hours, but is usually irregular, showing no distinct periodicity. These classic fever patterns are usually not seen early in the course of malaria, and therefore the absence of periodic, synchronized fevers does not rule out a diagnosis of malaria.

Physical findings in malaria are nonspecific and offer little aid in diagnosis. In many cases there may be no positive findings other than fever. Splenomegaly is common but may not be apparent early in disease. Hepatomegaly, jaundice, hypotension and abdominal tenderness may also be seen.

A variety of laboratory abnormalities may be seen in a case of uncomplicated malaria. These include normochromic, normocytic anemia, thrombocytopenia, leukocytosis or leukopenia, hypoglycemia, hyponatremia, elevated liver and renal function tests, proteinuria, and laboratory evidence of disseminated intravascular coagulation (although clinically important, bleeding is rare). Patients with complicated malaria occasionally show evidence of massive intravascular hemolysis with hemoglobinemia and hemoglobinuria.

If the diagnosis of malaria is missed or delayed, especially with *P. falciparum* infection, potentially fatal complicated malaria may develop. The most frequent and serious complications of malaria are cerebral malaria and severe anemia. Cerebral malaria is defined as any abnormality of mental status in a person with malaria and has a case fatality rate of 15 to 50 percent. Other complications include: hyperparasitemia (more than 3 to 5 percent of the erythrocytes parasitized); severe hypoglycemia; lactic acidosis; prolonged hyperthermia; shock; pulmonary, cardiac, hepatic, or renal dysfunction; seizures; spontaneous bleeding; or high output diarrhea or vomiting. These manifestations are associated with poor prognosis. Persons at increased risk of severe disease from malaria include older persons, children, pregnant women, nonimmune persons and those with underlying chronic illness. Other
complications of malaria infection include gram-negative sepsis, aspiration pneumonia and splenic rupture19.

1.4 Malaria Diagnosis:

The standard method for detection of *Plasmodium* in human blood is by microscopical examination of Romanovsky-stained thick and thin blood films8. This technique can, when used in optimal conditions by a competent microscopist, detect a parasitemia as low as 0.001% (10-40 parasites per µl of blood), but it is a time-consuming technique for the detection of scanty parasites and often difficult to use accurately to identify mixed infections.

Several alternative diagnostic methods have been developed in order to reduce the time spent examining slides or to enable less-trained personnel to achieve equally reliable results. These include fluorescence microscopy20, concentration-techniques such as the Quantitative Buffy Coat method (QBC®)21, or immunological ‘methods for the detection of *P. falciparum* antigens such as ParaSight F™22. These methods have somewhat improved the speed and accuracy of individual diagnosis, but at the expense of increased reliance on sophisticated equipment and/or much increased cost. Various techniques have been developed, based on the detection of malarial deoxyribonucleic acid (DNA), including DNA probes23,24, the polymerase chain reaction (PCR)25, and combinations of the PCR and DNA probes26. The DNA techniques have the potential advantages of greater sensitivity and the fact that they may be used for the detection and identification of the parasite in the vector as well as the human host27. While their use for individual diagnosis may be limited, even in reference laboratories in the industrialized world, molecular methods have enormous potential for epidemiological applications, since they are capable of detecting not only the presence of parasites but also of identifying isolate-specific type characteristics28, and they may be used to examine the association of mutant genes with anti-malarial drug resistance29.

PCR is a molecular tool, which revolutionized the epidemiology of malaria, but for case diagnosis it is neither practical nor diagnostic in areas where malaria is hyper-endemic, where parasite detection in symptomatic cases is not a problem. Parasite detection in early symptomatic malaria may be a problem in malaria non-endemic countries or areas of low malaria transmission, where low parasitemia is associated with disease manifestations. On the other hand the serological diagnosis of current malaria infection by detection of generally long-lasting antibodies (Abs) against malaria antigens (Ags) is unreliable30 but it has been tried as a crude marker for recent exposure31.
The ribosomal ribonucleic acid (RNA) genes (rLLX4) have generally proved very useful in molecular systematic because they contain a mixture of regions which evolve at different rates and hence can be used at different taxonomic levels. Their structure and function have been reviewed in relation to *Plasmodium* by Waters. Part of the small subunit ribosomal DNA (ssrDNA) was used by Lal et al., to develop oligonucleotide diagnostic probes for the 4 species of human malaria, but the method involved working with RNA (which is less stable than DNA) and radioactively labeled probes, further developed the method for a PCR identification assay, but their approach involved a separate PCR amplification reaction for each species-diagnostic pair of primers and there was no control reaction.

Detection of mixed infections by microscopy is difficult, especially when one of the species of *Plasmodium* is under-represented or if there are more than 2 species involved in the infection. In mixed infections of *P. malariae* and *P. falciparum* (the most common mixed infection in Africa), even when one of the species was under-represented in the sample, the SnM-PCR method could detect the mixed infection. When dilutions were made, the double infection was still correctly diagnosed even when the species under-represented was reduced to the calculated level of 0.01 parasite/µL. In natural infections, it shows that it is possible to detect double and triple mixed infection, even in cases where some of the *Plasmodium* species were present at less than one parasite per 100 thick blood film fields. The SnM-PCR method is able to detect DNA from considerably less than one parasite per µl of blood, which is the validation limit reported for the 4 tubes method. However, it is likely that the 4 tubes method is more sensitive, because it uses a multicopy DNA sequence (ssrDNA), and it is possible to detect the merozoite surface protein 1 gene (MSP-1, a single copy gene) at levels at least as low as 0.1 parasite per µL of blood.

1.5 The Malaria Parasite:

The malaria parasite was first discovered and described in 1880 by Laveran in Algeria. The life cycle and transmission of malaria by mosquitoes was later described by Ross and Grassi in 1897-8. Deployment of electron microscopy had revealed more detailed observations of the various stages and species of malaria parasites and enriched the knowledge about the life-cycle and the fine structure of malaria parasites. With further developments, and the use of the immunoelectron microscopy information about the subcellular localization of malaria antigens and their functions in specific parasite organelles has increased rapidly. The various stages of the life cycle of malaria parasites show many common ultrastructural features.
Plasmodia have arisen from the coccidian stem and share most of the typical features of the Apicomplexa. $P. \text{falciparum}$ belongs to the subgenus Laverania, while the remainder belongs to the subgenus Plasmodium. The genus Plasmodium consist nearly of 200 named species that parasitized reptiles, birds and mammals. Plasmodium belongs to the Apicomplexa, a large and complex phylum with about 5000 known species, which are all parasites characterized by eponym structure, the apical complex. The microorganisms causing malaria are commonly referred to as malaria parasites. This term is restricted to the family Plasmodiidae within the order Coccidiida, sub order haemoporidae.

Over 25 distinct species of malaria parasites of primates have been named; four are the recognized malaria parasites of humans, namely: $P. \text{falciparum}$, $P. \text{vivax}$, $P. \text{ovale}$, and $P. \text{malariae}$. The two which almost certainly achieved the widest global distribution are Plasmodium vivax and Plasmodium malariae. To the Europeans, these have been known and characterized long time ago as reviewed by as the "benign tertian" ($P. \text{vivax}$) and "quartan" ($P. \text{malariae}$) periodic fevers. "Benign tertian" fevers were so named because they were not associated with the severe and often fatal manifestations of the "subtertian, malignant" periodic fevers ($P. \text{falciparum}$). "Tertian" and "quartan" refers to their characteristic feature of an acute febrile episode, or paroxysm, that returns respectively every third ($P. \text{vivax}$) or fourth ($P. \text{malariae}$) day. Tertian and quartan fevers are referred to with similar frequency in writings from northern Europe through much of the past millennium and from around the shores of the Mediterranean Sea from about the 5th century B.C. onward reviewed by.

Today, $P. \text{malariae}$ has lost whatever predominance it may once have had and $P. \text{vivax}$ and $P. \text{falciparum}$ are the most commonly encountered malaria parasites. $P. \text{vivax}$ is still found sporadically in some temperate regions, where in the past it was widely prevalent. It remains, however, very common throughout much of the tropics and subtropics. Because of the temperature limitations on its transmission by its mosquito vectors, $P. \text{falciparum}$ is normally present only in tropical, subtropical, and warm temperate regions. In the tropics today, $P. \text{falciparum}$ remains widely prevalent.

The fourth human malaria parasite is Plasmodium ovale, which, like $P. \text{vivax}$, is the agent of tertian malaria and which, also like $P. \text{vivax}$ malaria today, carries a very low risk of fatal outcome. $P. \text{ovale}$ has the most limited distribution of all the malaria parasites of humans. While it is prevalent throughout most of sub-Saharan Africa, it is otherwise known to be endemic only in New Guinea and the Philippines reviewed by.
In *P. falciparum* malaria, severe and life-threatening conditions commonly arise, it causes the most deadly and severe infections, it infects all ages of RBCs, leading to a higher parasitemia, mature trophozoites and schizonts are sequestered in the microvascular system leading to tissue ischemia and there is widespread drug resistance for it. It associated sometimes with serious complications such as, cerebral malaria, severe anemia and others. It is characterized by the tendency of producing a very high density parasitemia, numerous clinical manifestations and liability to severe pernicious course of fatal outcome as it accounts for 98% of all lethal cases. The outcome of *P. falciparum* infection depends on several factors. One such factor may be a parasite virulence factor, which determines the course of infection.

Of the insects that serve as vectors for parasitic diseases, Anopheles species is arguably the most important. There are approximately 400 species of Anopheles, about two dozen serve as vectors for malaria (*Plasmodium* spp.) in humans. Mosquitoes also serve as the vector for canine heart worm (*Dirofilaria immitis*). Six of the seven recognized species in the *An. gambiae* complex occur in Eastern and Southern Africa. *Anopheles gambiae* and *An. arabiensis* are the major vectors, with *An. Arabiensis* occurring throughout the region from South Africa to Sudan and Egypt, while *An. gambiae* is prevalent in the tropical belt only. The saltwater breeder *An. merus* is found in mainly coastal areas of Kenya and Tanzania, but also far inland in Zimbabwe and South Africa.

Female Anopheles mosquitoes seek a blood meal as a protein source for egg production. Usually, to lay the first batch of eggs a female mosquito requires 2 or even 3 blood meals, but subsequently blood taking and oviposition alternate regular, the duration of the gonotrophic cycle, defined as the period between the blood meal and the subsequent oviposition is temperature dependent. Under favorable environmental conditions, an anopheles mosquito can live for more than 3-4 weeks, but the mean survival in nature is much shorter (6-9 days).

### 1.6 Malaria Life Cycle:

Malaria parasites are lower eukaryotes, with a genomic complexity which is five times greater than that of bacteria, and a complex life-cycle involving both mammalian and invertebrate hosts. The parasite has to invade three different types of cells; erythrocytes,
hepatocytes and mosquito midgut epithelial cells, and need to survive in very different hostile environments\textsuperscript{13}.

A female Anopheles mosquito injects saliva before taking a blood meal. If the salivary glands contain malaria sporozoites, these are then injected into the human blood (figure 1.2). After approximately 30 minutes in the circulation, the sporozoites reach the liver and invade hepatocytes in which they mature during 6-16 days, depending on species. One sporozoite develops into approximately 10-30000 merozoites which are released into the blood, where they invade red blood cells, grow and multiply asexually into 8-24 merozoites each. When mature, the schizonts rupture and the merozoites can then invade new erythrocytes. The erythrocytic cycle takes 48 hours for the three common human species, but 72 hours for \textit{P. malariae}. All blood stages are normally found in the peripheral blood, but for \textit{P. falciparum}, the parasites sequester into the deep vascular system during the second half of the cycle. Parasites adhere to endothelial cells to escape clearance by the spleen and therefore usually not detected in the peripheral blood.

In the course of the schizogonic cycle (within a red blood cell) some of the merozoites become differentiated into sexual forms (the gametocytes); the mechanisms at the basis of this differential development are unknown. Gametocytes appear early (approximately from the third generation) in infections caused by \textit{P. vivax}, \textit{P. ovale} and \textit{P. malariae} while at least 10 generation are thought to be required before \textit{P. falciparum} gametocytes appears in the blood, which probably reflect the slow maturation and the sequestration of the immature stages in this species\textsuperscript{13}.

Sexual forms i.e. gametocytes are produced sometimes during the asexual cycle. When a new mosquito takes a blood meal containing female and male gametocytes. Sexual reproduction commence in the mosquito gut with the development of zygote, ookinete and then oocysts, the later can generates up to 1000 sporozoites, which are harbored in the salivary glands and can be transmitted at the time of the next blood meal. The life cycle in the mosquito takes about 9-16 days and is dependent on conditions such as temperature.

\textit{P. vivax}, \textit{P. ovale} and \textit{P. cynomologi} have a dormant stage, the hypnosoite\textsuperscript{53,54} that may remain in the liver for weeks or many years before the development of exoerythrocytic schizogony. This results in relapse of infection. \textit{P. falciparum} and \textit{P. malariae} have no persistent phase\textsuperscript{9}. \textit{P. malariae} can stay undetectable for many years, but it is unclear how and where in the host.
The ring form is the only asexual stage usually identifiable in the peripheral blood of patients with *P. falciparum* infection (gametocytes may be present after the second week of patent asexual parasitemia) because more mature stages of this species adhere to the endothelium of post-capillary venules in the tissues. In *P. vivax* and *P. ovale*, the ring forms have similar features, but the cytoplasm may early present amoeboid tendency and traces of stippling start to appear in the parasitized erythrocyte.

**Fig (1.2) Malaria Life Cycle**

In these species trophozoites are seen in the peripheral circulation. *P. malariae* trophozoites are regular in shape (except those assuming a characteristic band form across the erythrocyte), with a small vacuole, early and abundant pigment, and no stippling. Two types of gametocytes are found in the peripheral blood: the female macro-gametocytes and the male micro-gametocytes. They can be differentiated by the fact that in the male parasite nuclear material is dispersed (preparing to exflagellation) while in the female parasite it is condensed.
1.7 Malaria Immunity:

There are two types of clinical immunity against malaria, one which reduces the risk of death from malaria and another which reduces the intensity of clinical symptoms. A third type of protective antimalarial immunity is antiparasitic immunity, which directly reduces the numbers of parasites in an infected individual. These are epidemiological definitions of immunity. The actual cellular and molecular mechanisms of immunity may overlap to a considerable degree between these categories. The number of malarial inoculations experienced, and the intervals between them, are all-important to the malaria immune status of an individual\(^1\). In the case of acute attacks of \(P\).\textit{falciparum} malaria, it is possible that a degree of immunity to some aspects of severe, life-threatening disease may be achieved after only one or two infections\(^55\). However, clinical immunity to other, non-life-threatening clinical effects of malaria requires more and frequent inoculations of malaria\(^56,57\). Effective antiparasitic immunity is achieved only after many and more frequent infections\(^58\), reviewed by\(^1,59\).

1.7.1 Innate immunity: Some individuals are either naturally resistant to malaria infection or less likely to develop a severe form of the disease. Innate resistance to infection is generally only a partial resistance, and may be linked to the fact that malaria parasites find it harder to invade certain types of human erythrocytes. Also, it could be due to the fact that certain host erythrocytes have a reduced ability to sustain the growth of parasites\(^13\).

1.7.2 Acquired immunity: The host-parasite relationship has been likened to a “molecular arm race”\(^60\). In this analogy, the parasite is constantly developing new and improved strategies to obliterate the human race. Those individuals who survive this battle supposedly produce effective counter-measures that destroy the parasite. In endemic areas, most individuals develop an immune response that controls parasite replication but does not eliminate parasite from blood. Acquired immunity to malaria develops after repeated exposure to the parasite, in individuals living in malaria endemic areas. Immunity is rarely sterilizing but protects against high parasite density and clinical disease.

1.7.3 Strain Specific immunity:

The long time required to develop protective immunity against malaria may be explained by poor immunogenicity of the parasite and/or that immune responses are strain specific. A large number of infections would then be required to encounter the whole local repertoire of different antigens.
Early studies of induced malaria indicated that malaria immunity is strain specific. The malaria infections were induced to treat neurosyphilis. The natural course of infection and transmission could then be studied, and patients were reinfected with homogeneous or heterogeneous strains. When homogeneous strains were given, clinical and parasitological characteristics were less severe than with heterogeneous strains. If immunity is strain specific, clinical episodes should then be caused by new strains not previously encountered by the individual. This may however be difficult to study since the repertoire of different antigens that an individual has met in endemic areas cannot be identified. New genotypes were however detected during clinical episodes in a longitudinal study in Senegal. Another way to approach this issue might be serological analyses of polymorphic antigens. However, antibodies to two merozoite surface antigens, MSP1 and MSP2, where relatively short lived and represented only the ongoing or recent infections37.

1.8 Antigenic diversity:

Since the malaria parasites invade three different types of cells (human erythrocytes and hepatocytes and mosquito midgut epithelial cells) they need to survive in very different hostile environments. In the circumstances, it is not surprising that the antigenic structure of the parasite will be radically different from one stage of the life cycle to another. There are about 40 major antigens of *P. falciparum* in sporozoites, pre-erythrocytic schizonts, merozoites, erythrocytic schizonts, gametocytes and gametes.

Many *P. falciparum* antigens have now been studied by gene cloning and sequencing. Comparison of these sequences has shown that there is a high degree of diversity among different parasite clones. A major cause of antigenic polymorphisms is variation in the sequence of the short tandem repeats that are a prominent characteristic of many malaria antigens. More conventional polymorphisms resulting from point mutations have been described in antigens with or without repetitive regions. Some examples of the particular polymorphisms that characterize the leading asexual blood-stage vaccine candidates are summarized below:

1.8.1 Merozoite Surface Protein-1 (MSP1):

The merozoite surface protein-1 (MSP1), a large polypeptide 200 KDa, is expressed at the surface of the merozoite (Appendix 3). The function of MSP1 is related to invasion of erythrocytes since antibodies against MSP1 can inhibit invasion61. The *msp1* gene, located on chromosome 9, contain 17 discrete blocks, classified as conserved, semi conserved and
variable depending on the homology at the amino acid level\textsuperscript{62}. The two most highly conserved regions, block 1 and 17, encode the N- and C-terminal sequences respectively. The variable blocks, except four blocks, can all be assigned to two allelic types, K1 and MAD20, but through intra-genic recombination other forms have been found. The block 2 which is the most polymorphic region of \textit{msp1} contains tripeptide repeats, which vary in number and sequence between different isolates\textsuperscript{63}. It can be one of three allelic types/families: K1-, MAD20- and RO33- types. Block 2 of RO33 form, unlike that of K1 and MAD20, lacks the N-terminal tripeptide repeats and was previously described as a rare type in culture collection\textsuperscript{64}. The variable blocks are flanked by highly conserved regions. The \textit{msp1} gene has high sequence diversity. MSP1\textsubscript{19} (corresponding to block 17) and MSP1-block 2 has been found to targets for protective immunity in many studies and are therefore considered potential vaccine candidate antigens. A fragment of MSP1 was included in the Spf66 vaccine\textsuperscript{37}.

The sequence polymorphism is true of the molecule as a whole, however, in the block two region, extensive sequence diversity exists. Over 50 different sequences have been identified in block 2, which fall into three main types represented by variants of the K1, MAD20 and RO33 isolates. Block 2 sequences of the K1–like and MAD20–like types contain variable tri– or hexa peptide repeats, whereas block 2 of the RO33 type contain a non-repetitive sequence, which varies only little. The 19 KDa fragment appears to confer immunity in monkeys and in some epidemiological studies were found to be associated with protective immunity in humans.

\textbf{1.8.2 Mezoite Surface Protein-2 (MSP2):}

The merozoite surface protein 2 (MSP2), denoted as MSA-2 or GP3 by different researchers. It is a smaller polypeptide, with a molecular weight of 45Kda, which is not processed during parasite maturation (Appendix 4), but like MSP1, appeared to be anchored in the merozoite membrane by a glycoylphosphatidylinositol moiety\textsuperscript{65}. The \textit{msp2} gene is located on chromosome 2 and is composed of 5 blocks of which the central block 3 is the most polymorphic. The \textit{msp2} alleles can be grouped according to two allelic types/families, FC27 and IC1/3D7 according to the dimorphic structure of the central variable non-repetitive region\textsuperscript{66,67}. The extensive diversity is due to the allele-specific sequences in the central
region, which comprises tandem repeats of varying size. Difference in the number of copies of these repeats will result in length polymorphism. These characteristics of msp2 have been exploited by several genotyping methods. Common to all methods of msp2 genotyping is a polymerase chain reaction (PCR) amplification of a central part of the msp2 gene which comprises the allele-specific repetitive region and conserved flanking sequences. The major methods used for msp2 genotyping are: (i) analysis of size polymorphism with subsequent hybridization with family-specific probes, or (ii) restriction fragment polymorphism (RFLP) of the amplified product. The msp2 gene has been used extensively as polymorphic marker gene in field studies showed extensive polymorphism at this locus, even in areas of low endemicity.

The use of msp2 as marker has proven to be of great use when individual Plasmodium falciparum infections need to be identified. PCR-RFLP genotyping of parasites for msp2 makes it possible to distinguish the individual parasite infections concurrently present in a blood sample. The possibility of tracing individual parasite clones over time allows detailed studies of infection dynamics. In immunological studies, genotyping can also provide important information on the diversity of antigenic challenge. Most importantly, genotyping makes it possible to determine the multiplicity of infection, which can be used as an outcome measurement of interventions such as drug trials, vaccine trials, or exposure-reducing interventions.

The msp2 is not only an extremely polymorphic marker gene, but its product is also a vaccine candidate. MSP2, as part of a subunit vaccine, is currently under field trial in Papua New Guinea (PNG). Two genotyping studies from PNG have already shown that the two allelic families of msp2 are differently associated with morbidity. Hybrids between FC27 and IC/3D7 have been found in the field isolates but intra-genic recombination between the two allelic families appears to be rare. In four epidemiological studies, with about 3500 infections analyzed by PCR-RFLP, 82 different msp2 alleles were found, of which 41 were FC27-types and 41 were 3D7-types.

The MSP2 protein exhibits considerable sequence diversity and this diversity translates into antigenic polymorphism. The single copy gene encoding this antigen contains highly conserved N- and C-terminal regions which flank a central polymorphic region. This central region contains two segments of tandem repeats, which vary in number, length and sequence in different alleles. The blocks of repeats are flanked by non-repetitive variable regions, which can be used to define two allelic families designated the IC1 type (serogroup
A) and the FC27 type (or serogroup B)\textsuperscript{66,79,80}. These two groups, which are defined by distinct sequences flanking the central repeat region, also differ with respect to their central repeats. The central repetitive region consists of one or more 32 amino-acid repeats, which occur as two tandemly repeated units in FC27 and as a single copy in the \textit{P. falciparum} strain IC1\textsuperscript{66,83}, followed by one or more copies of a 12 amino-acid repeat. Members of the IC1/3D7 CAMP (serogroup A) allelic family are characterized by shorter sequence repeats (4-8 residues) and can be sub grouped both according to repeat type and number and also according to the presence or absence of short sequence stretches within the 3 non repetitive variable region\textsuperscript{84}. 21 alleles of MSA-2 have been derived from isolates of different geographic regions and have been sequenced\textsuperscript{85}.

### 1.8.3 Glutamate Rich Protein (GLURP):

The glutamate rich protein (GLURP), of 220 KDa, is a soluble antigen which is released during schizont rupture but can be detected in all stages of the parasite i.e. the hepatic, asexual and sexual stages (Appendix 5)\textsuperscript{86}. The gene is located on chromosome 10 and has two repeat regions, which are moderately polymorphic. High level of IgG antibodies in older children has been associated with clinical protection\textsuperscript{87}. It contains 22\% glutamate residues and includes two repeat regions, R1 and R2. The R1 region consist of 6 repeat units, each contain 15 or 16 amino-acid residues and 2 repeat units of 50 amino–acid residues. These repeat units of R1 are poorly conserved along the gene. R2 region consists of 14 repeat units, each composed of 19 or 20 residues, and 11 of which are well conserved along the gene. At the N-terminal end of \textit{glurp} there is a 32 amino-acid residue segments that may represent a single sequence. PCR analysis of R2 region has shown considerable size polymorphism of different alleles in different \textit{P. falciparum} isolates\textsuperscript{86}.

### 1.8.4 Microsatellite markers:

Microsatellite (MS) markers are simple sequence repeats that have been found in every eukaryotic study\textsuperscript{88}, although their density varies among species. In \textit{P. falciparum}, markers generated from these repeats are often polymorphic with variable length in different parasite clones. Consequently MS markers exhibit multiple alleles in general populations and are very informative for genetic studies\textsuperscript{89}. The advantages of MS markers include their general omnipresence and the ease with which they can be scored. MS typing is based on PCR and requires only small amount of DNA. Moreover the process of genotyping can be automated,
and multiple markers can be assayed by multiplexing PCR and labeling PCR products with different fluorescent dyes\textsuperscript{90}.

The \textit{P. falciparum} genome is rich with microsatellites mostly \((TA)_n\) or \((T\text{ or } A)_n\), where \(n\) is typically between 10 and 30, occurring at a frequency of about one per kilobase genome-wide\textsuperscript{91,92}. The abundance of microsatellite markers in \textit{P. falciparum} genome has facilitated the development of linkage map, laying the foundation for identification and characterization of \textit{P. falciparum} functional genes.

\textbf{1.9 Genetic diversity in malaria parasites:}

All the evidences are indicating the enormous genetic diversity of \textit{P. falciparum} in a given parasite population in malaria hyperendemic regions\textsuperscript{77,93,94,95,96}, however, low parasite diversity was reported, mainly from areas of low transmission\textsuperscript{97,98,99,100}. Thus, the extensive polymorphism of the parasite antigens is not surprisingly; however, it is accused for the delay in the naturally acquired immunity and for the redundancy in the vaccine development. The differences between parasites with regard to; enzymes\textsuperscript{101} antigens\textsuperscript{102}, other proteins\textsuperscript{103}, and response to drugs\textsuperscript{104}, is well documented.

Molecular characterization of different \textit{P. falciparum} antigens has shown extensive polymorphism\textsuperscript{94}. In high-transmission area, parasite population diversity is highly complex and dynamic. Recombination during the sexual stage of the parasite in the anopheles mosquito is believed to generate this diversity\textsuperscript{105,106}.

The genetic diversity of \textit{P. falciparum} may be limited by two variables in a natural population: Firstly by the number of frequencies of different alleles present in the population, and secondly by the frequency of genetically mixed infections since this only offer the possibility of cross fertilization in the mosquito vector\textsuperscript{81}. The role of age and acquired immunity were highlighted in many studies, but indirectly are indices for transmission intensity. The phenomenon of clone fluctuation, which consists of kinetic changes in the relative load of each parasite subpopulation present in blood\textsuperscript{107,108}, is another aspect of genetic diversity. Serotyping with monoclonal antibodies was used to estimate the number and frequencies of allelic variants of two merozoite surface proteins (MSP1 and MSP2) and the exported protein Exp1 in \textit{P. falciparum} in an urban region of the Gambia, results showed that the combination among unlike loci is a common consequence of sexual reproduction of \textit{P. falciparum}. The use of the polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA), for the detection of the genetic polymorphism in malaria
parasite, has expanded the understanding of the parasites natural genetic diversity originally characterized by protein electrophoresis\textsuperscript{109,110}.

High genetic diversity has been found in the population of the malaria parasite, \textit{P. falciparum}\textsuperscript{77,93,95,111}. High diversity increases the risk that an individual is infected with different parasite genotypes. Such infections are generally described as complex or multiple infections. The phenomenon of clone fluctuation, which consists of kinetic changes in the relative load of each parasite subpopulation present in blood\textsuperscript{107,108}.

Studies in Tanzania\textsuperscript{112,113} and in Papua New Guinea\textsuperscript{76} have suggested that multiple infections can protect against clinical malaria attacks in partially immune children, presumably as a result of immunological cross-reaction against superinfecting parasites. This protection is not evident in very young; immunological relatively naive hosts\textsuperscript{68}.

In areas highly endemic for malaria, many individuals are infected simultaneously with several \textit{P. falciparum} clones. The term multiplicity of infection (MOI) has been used to describe the number of concurrent infections per carrier. Multiplicity of infection, also termed as complexity of infection (COI), is determined by genotyping of a highly polymorphic marker genes and it reflects the minimal number of different genotypes detected\textsuperscript{17}. In areas of high endemicity, multiplicity depends on the age of the host\textsuperscript{71,114} and on the transmission intensity\textsuperscript{115}. In Idete, Tanzania, mean MOI in asymptomatic children aged 3-7 years was five infections per carrier\textsuperscript{116}. In contrast, in children aged less than one year in the same village, mean multiplicity was only two infections per child\textsuperscript{117}.

A number of studies have found relationships between MOI and the risk of malaria morbidity. Several studies of partially immune children have found that multiple malaria infections are associated with reduced incidence of clinical episodes of \textit{P. falciparum}\textsuperscript{76,116,118}. A study in Sao Tome found that the apparent protection extended also to non-malaria fever episodes\textsuperscript{119}. Other studies, such as those of infants in Idete\textsuperscript{114,117}, of children aged less than three years in western Kenya\textsuperscript{120}, of children in Ghana\textsuperscript{121} and of individuals in relatively low transmission settings in the Sudan\textsuperscript{15} and Mozambique (Mayor et al., 2003)\textsuperscript{122} have found that multiple infections were a risk factor for clinical malaria attacks.

A series of epidemiological studies in Senegal, Tanzania and Papua New Guinea has indicated the importance of multiplicity of infection\textsuperscript{36,71,76,116}. These studies established that: firstly, the multiplicity of infection within a host appears to depend not only on exposure but also on age; secondly, multiplicity can reach high levels; thirdly, multiplicity of infection can
be positively associated with protection against mild episodes of malaria. In older children, a
high multiplicity of *P. falciparum* infection is the feature of chronic, low level parasitemia.
This in turn appears to confer cross-protection against newly inoculated parasites based on
partially genotype-specific response, that might last only a little longer than the infection itself\(^{123}\).

Current molecular studies on malaria parasite have been directed towards identifying
functional genes, such as genes controlling response to drugs and malaria vaccines candidate
genes. Most of these genes encode polypeptides that are found to be polymorphic, and their
alleles can be distinguished by regions containing variable numbers of tandemly repeated
codons as well as sequence variations. One of the most commonly used markers for the
genotyping of *P. falciparum* populations is the gene coding for the merozoite surface protein 2
(*msp2*). This has been considered a suitable marker as it exhibits a large degree of
polymorphism, both in length and sequence\(^{71,85}\).

Molecular techniques have proven to be powerful in revealing such polymorphism. This
includes relative differences in mobilities of PCR-amplified fragments, following
electrophoresis. This is in addition to the sequence specific amplification by use of restriction
fragment length polymorphism (RFLP) in the amplified product, blotting and hybridization of
PCR products with allele-specific probes and direct sequencing of the amplified products.

The recombination during the sexual life of the parasite in the mosquito is believed to be a
major source for diversity\(^{105,106}\). Heterozygous recombination, which occurs during meiosis,
is one mechanism for generating genetic diversity. The rate, at which such recombination
occurs, defines the genetic structure of the parasite population and can influence the ability of
the parasite to respond to selection pressure\(^{124}\). Population dynamic studies, which consider
genetic heterogeneity of *P. falciparum*, have shown fluctuations of different serotypes in
space and time. The host immune response appears to play an important role in generating
these dynamics. Children are often subject to concurrent infections with numerous diverse
parasite strains\(^{71,125}\). Longitudinal variations in parasite populations within individuals have
been demonstrated when observed over weeks\(^{107,126}\).

The malaria genome has shown extensive polymorphism, the arrangement and composition
of the subtelomeric blocks suggests frequent recombination between the telomeres. Many
genes coding for antigens are located near the chromosome ends, i.e. the most labile regions,
this might be a way to generate new antigenic variants and thereby evading immunity. The
crossing experiments have shown that intragenic recombination occurs between different alleles during meiosis\textsuperscript{127}.

The daily dynamics of \textit{P. falciparum} subpopulations were investigated in asymptomatic children in Tanzania. In children harboring chronic parasitemia, infections were found to be highly complex with daily changes in both parasite density and genotypic pattern suggesting that these infections consist of inherently synchronous subpopulations of parasites\textsuperscript{108}. \textit{P. falciparum} has a number of polymorphic antigens, only single copies of genes encoding these antigens have been found in the haploid genome\textsuperscript{94,128}.

As most polymorphic antigens lie on different chromosomes, novel genotypes can be created by independent assortment of chromosomes, as well as by crossing over during meiosis\textsuperscript{124}. Two distinct terms were brought into the discussion of resistance to malaria, firstly, semi" immunity, referring to a situation of acquired immunity as consequence of continual exposure controlling infection and leading to low level chronic parasitemia, but clearing the infections\textsuperscript{129}. The second is, premunition describing protection against superinfection results from existing infection\textsuperscript{130}.

1.10 Malaria chemotherapy and parasite resistance:

The use of chemotherapy is important in alleviating the suffering and reducing the mortality caused by the disease. The action of antimalarial drugs lies in their effect on the metabolic process or the vital structure of the parasite which lead to the disruption of its activity and then to death. However the efficacy of the drug depends also on the species and strain of the parasite and its sensitivity towards a given drug, as well as the stage of the immunity and the specific protective forces of the body\textsuperscript{131}.

Patients successfully treated with antimalarial drugs may thus be healthy but may infective for up to two months until the \textit{P. falciparum} gametocytes die off naturally, or until another drug such as primaquine is given to eliminate the mature gametocytes\textsuperscript{13}. Hereunder are an example of drugs commonly used in malaria chemotherapy, they are also in common use in our study sites, and we used them in this study.

1.10.1 4,8-aminoquinoline drugs:

Such as chloroquine, amodiaquine, and primaquine. Chloroquine is a 4-aminoquinoline that has marked and rapid schizonticidal activity against all infections of \textit{P. malariae} and \textit{P. ovale} and against chloroquine-sensitive infections of \textit{P. falciparum} and \textit{P. vivax}. It is also gametocytocidal against \textit{P. vivax}, \textit{P. malariae} and \textit{P. ovale} as well as immature gametocytes
of *P. falciparum*. It is not active against intrahepatic forms, and should therefore be used with primaquine to effect radical cure of *P. vivax* and *P. ovale*. It was the most important antimalarial for more than 40 years because of its activity against the four plasmodia that infect human. In addition, chloroquine is the only antimalarial known to be safe for children and pregnant women. However, the value of chloroquine has been severely compromised by the spread of chloroquine–resistant strains of *P. falciparum*, which are now prevalent in Southeast Asia, South and Central America, and sub-Saharan Africa. Thus although chloroquine remains safe, chloroquine resistance has increased the morbidity and mortality of malaria and has led to the wide spread use of alternative antimalarial drugs.

### 1.10.2 Antifolate drugs:

The only useful combinations of antifolate drugs for the treatment of malaria are synergistic mixtures that act against the parasite-specific enzymes, dihydropteroate synthetase and dihydrofolate reductase. Available combinations include the sulfad drug-pyrimethamine combinations sulfadoxine-pyrimethamine and sulfalene-pyrimethamine, the former being more widely available. Cotrimoxazole, the co-formulated combination of sulfamethoxazole and trimethoprim, has weak antimalarial properties because trimethoprim has a much lower affinity than pyrimethamine for the parasite dihydrofolate reductase enzyme. Cotrimoxazole should not be used for the treatment of malaria. The use of sulfad drug pyrimethamine combinations for chemoprophylaxis is no longer recommended because of the risk of severe skin reactions.

Antifolate drugs such as pyrimethamine and proguanil are slow blood schizonticides but they have the advantage that they are effective in smaller doses, safe and free from side effects. The ability of sulfas (sulfonamides and sulfones) to show potentiating effect with proguanil or pyrimethamine suggested that combination of these two groups of drugs would not only be more effective antimalarial agent, but would also delay, if not avoid, the development of resistance. The most commonly used combination is that of sulfonamide, sulfadoxine, and pyrimethamine (fansidar), which has been particularly effective in areas such as Africa where chloroquine resistance is now widespread. However, resistance to the pyrimethamine sulfadoxine combination became widespread and the successive failure of these two drugs in Africa was described in 1998 as malaria disaster.

### 1.10.3 Quinine:


It is normally effective against falciparum infections that are resistant to chloroquine and sulfa drug-pyrimethamine combinations. Decreasing sensitivity to quinine has been detected in areas of South-East Asia where it has been extensively used for malaria therapy. This has occurred particularly when therapy was given in an unsupervised and ambulatory setting with regimens longer than 3 days. In these settings, patient adherence to therapy is low, leading to incomplete treatment; this may have led to the selection of resistant parasites. There is some cross-resistance between quinine and mefloquine, suggesting that the wide use of quinine in Thailand might have influenced the development of resistance to mefloquine in that country\textsuperscript{144}. Strains of \textit{P. falciparum} from Africa are generally highly sensitive to quinine. Quinine, for various reasons is not in use for treatment of mild to moderate malaria\textsuperscript{145}. There are other chemically related drugs to quinine such as mefloquine, lumefantrine, halofantrine, quinidime and quininmax.

\subsection*{1.10.4 Artemisinin and its derivatives:}
Artemisinin is poorly soluble in oils or water but the parent compound has yielded dihydroartemisinin, the oil-soluble derivatives artemether and arteether, and the more water-soluble derivatives sodium artesunate and artelinic acid. These derivatives have more potent blood schizonticidal activity than the parent compound and are the most rapidly effective antimalarial drugs known. They are used for the treatment of severe and uncomplicated malaria\textsuperscript{146}. They are not hypnozoiticidal but gametocytocidal activity has been observed\textsuperscript{147}. These compounds are not recommended for use in the treatment of malaria due to \textit{P. vivax}, \textit{P. malariae} or \textit{P. ovale} since other effective antimalarial drugs are available for this purpose.

\subsection*{1.10.5 Combination therapy to combat the spread of drug resistance:}
Combination therapy (CT) is defined as “the simultaneous use of two or more antimalarial drugs with different biochemical targets in the parasite or in tissue hosting the parasite, which are synergistic, additive or complementary in their effect”. Using this definition, two or more drugs which have the same biochemical target in the parasite such as SP, chlorproguanil-dapsone, atovaquone-proguanil are not considered CT. Similarly, an antimalarial which is coadministered with a non-antimalarial drug which enhances its action is also not classified as CT. Suffice to say that CT therefore can be either fixed combinations, where all components are coformulated in a single tablet/capsule or free combinations where the components are in separate tablets/capsules but are coadministered\textsuperscript{148}. 
Due to the rapid emergence and spread of resistance to almost all available antimalarial drugs, much of interest has been focused upon the use of drugs in combination. The underlying science behind the therapeutic effect of the combinations that include an artemisinin derivative is that the artemisinin rapidly kills most of the parasites and then those that remain are killed by the companion drug149.

In sub-Saharan Africa, the first two combinations to be considered on a larger scale were pyrimethamine/sulfadoxine plus chloroquine and pyrimethamine/sulfadoxine plus amodiaquine150. Recently interest has been directed towards the use of drugs in combination with artesunate in purpose of delaying the emergence of resistance151. The antifolate product proguanil has been formulated together with a new type of inhibitor, atovaquone, to yield malarone, recently licensed for clinical use152. Several other combination regimens are undergoing field testing, especially in Africa to find out which would be the most effective and appropriate for a given endemic region153.

1.10.6 Definition of Parasite Resistance:

The term “parasite resistance” has been used with different meanings. It has been defined as “the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject”154,155. This definition was subsequently modified to specify that “the drug must gain access to the parasite or the infected red blood cell for the duration of the time necessary for the normal action of the drug”156. Despite this modification, the term “parasite resistance” has been used interchangeably in the published literature when referring to the parasite phenotype characterized in vitro by the confirmed ability to survive a threshold (nanomolar) concentration of the drug under standard conditions of continuous culture. Resistance has also been used when referring to therapeutic failure after administration of a standard dose of a drug; this definition is used in the WHO standard in vivo test protocol157. However, in this test, serum drug levels are not normally measured and the observed therapeutic failure might be due to malabsorption, rapid or abnormal metabolism, or the presence of latent infections other than malaria. Therefore, it is important to define the terms being used in order to avoid confusion.

Although the definition of resistance in vitro more accurately reflects biological resistance to the drug, true parasite resistance requires a demonstration of the ability of parasites to survive in vivo in the presence of an adequate therapeutic concentration of the drug in serum. When serum drug concentrations are not measured, the in vivo therapeutic failure data should be
interpreted with caution since these might overestimate the true parasite resistance; this is particularly true for slowly acting or long-acting drugs such as SP and mefloquine.

1.10.7 Malaria Parasite Resistance:

The escalation in *P. falciparum* malaria morbidity and mortality in recent years is attributed to the parasite resistance to the affordable drugs, chloroquine (CQ) and sulfadoxine/pyrimethamine (SP)\(^\text{158,159,160}\). The resistance has propagated and upsurge in the last 20 to 25 years in sub-Saharan Africa\(^\text{143,161}\).

Although, the increment in malaria morbidity is beyond doubt, we believe that the rise in mortality due to resistance is doubtful, as the resistance-virulence relationship is not harmonized, and it is possibly in an opposing relation\(^\text{162}\). As yet, no solid clinical data, or appropriate laboratory investigation confirmed the increased virulence of mutant isolates. The fitness of mutant parasites was brought to light by a series of recent publications, discussed the evolution of drug resistance and parasite population genetics\(^\text{163,164,165,166}\).

Despite the relatively recent introduction of SP as a first line for malaria treatment it is efficacy has been eroded dramatically in many malaria endemic regions, for example in east Africa, alarming levels of *P. falciparum* treatment failures to SP have been recorded\(^\text{167}\). However, investigation of SP resistance is important for many reasons; it is still in wide use as monotherapy or as a component of combination therapy (in the Sudan SP is used with artesunate), the clinical and molecular link between CQ and SP resistance\(^\text{168,169}\) and the unique association between SP treatment and resistance with gametogenesis\(^\text{170,171}\). Furthermore, historically, it was mentioned that SP resistance has evolved more rapidly in areas of low transmission\(^\text{172}\), which mimic the situation in eastern Sudan.

The sulfadoxine-pyrimethamine drug combination is widely used in malaria-endemic countries in sub-Saharan Africa as a first-line drug to treat uncomplicated *Plasmodium falciparum* malaria and has replaced chloroquine in many endemic countries due to widespread resistance. Despite the relatively recent introduction of SP into, for example, east Africa, alarming levels of *P. falciparum* treatment failures to SP have been recorded in the region. The continuous monitoring of resistance to SP and other antimalarial drugs is therefore of major importance for rational decisions regarding future treatment guidelines.

The reduction of malaria-attributable mortality depends on accurate and early diagnosis followed by prompt treatment with effective drugs (the choice of which depends on knowledge of the antimalarial sensitivity profile of the local strains of *P. falciparum*). During
the last 50 years, the development and spread of resistance in *P. falciparum*, to most of the drugs used in the first-line prevention and treatment of malaria, has given reason for great concern, especially in the continued absence of very effective vaccines\(^{173,174}\).

Various factors relating to drug, parasite and human host interactions contribute to the development and spread of drug resistance. The molecular mechanism of drug action is a critical element in the speed at which resistance develops. In addition, drugs with a long terminal elimination half-life enhance the development of resistance, particularly in areas of high transmission. Similarly, increased drug pressure is a significant contributor to drug resistance. As increased amounts of a drug are used, the likelihood that parasites will be exposed to inadequate drug levels rises and resistant mutants are more readily selected\(^{175}\). Parasite factors associated with resistance include the *Plasmodium* species concerned, the intensity of transmission and the widespread and/or irrational use of antimalarial drugs. The role of host immunity in propagating resistance is unclear. However, immunity acts synergistically with chemotherapy and can enhance therapeutic effects and even parasite clearance of drug-resistant infections. The role of immunity in clearance of drug resistant parasites was shown before in Mali, an area hyperendemic for malaria, where age was used as a surrogate marker for immunity\(^{176}\). However, it is tempting to know the efficacy of immunity in drug-resistant parasite clearance in semi-immune population living in an area of moderate to low malaria transmission. Previously, study in eastern Sudan showed that risk of clinical malaria infection reduced to the half by the age of twenty years, while the disease affects all age groups\(^1\), that indicate acquisition of some sort of immunity after the age of twenty.

Resistance to antimalarial drugs arises as a result of spontaneously-occurring mutations that affect the structure and activity at the molecular level of the drug target in the malaria parasite or affect the access of the drug to that target\(^{104}\). Mutant parasites are selected if antimalarial drug concentrations are sufficient to inhibit multiplication of susceptible parasites but are inadequate to inhibit the mutants, a phenomenon known as "drug selection"\(^{177,178}\). This selection is thought to be enhanced by subtherapeutic plasma drug levels and by a flat dose-response curve to the drug.

Combinations of single nucleotide polymorphisms (SNPs) in the dihydrofolate reductase (*dhfr*) gene at codons 51, 59, 108 and 164 and the dihydropteroate synthetase (*dhps*) gene at codons 436, 437, 540, 581, and 613 correlate with *P. falciparum* resistance to SP *in vivo*\(^{179,180}\). There are evidences that the *dhfr* triple mutations with or without the *dhps*
mutations can predict SP resistance (SPR), studies conducted in East Africa have showed that the triple (*dhfr* Asn-108, Ile-51, and Arg-59) mutant genotype was associated with SPR. However, in another study conducted in Malawi, it was found that the quintuple mutant genotype (*dhfr* Asn-108, Ile-51, and Arg-59 plus *dhps* Gly-437 and Glu-540) was more strongly associated with SP treatment failure than was the *dhfr* triple mutant.

Mutant parasites are selected if anti-malarial drug concentrations are sufficient to inhibit multiplication of susceptible parasites but are inadequate to inhibit the mutants, a phenomenon known as "drug selection". This selection is thought to be enhanced by sub-therapeutic plasma drug levels and by a flat dose-response curve to the drug.

Although elimination of the asexual stages of *Plasmodium falciparum* is the focus of treatment of individual symptomatic patients, at a population level, reducing the carriage of gametocytes - the sexual stage responsible for infection of the mosquito vector - is necessary to limit the transmission of malaria parasites and the spread of anti-malarial resistance. The factors that trigger and regulate the switch from asexual to sexual development of the malaria parasite remain largely mysterious, but may involve both genetic mechanisms, and environmental mechanisms. In relation to SP treatment, the gametocyte carriage and infectivity to mosquitoes was found to be consistently higher in patients infected with drug resistant compared with drug sensitive malaria parasites.

The ambiguity in the association between the in-vivo response and the prevalence of the molecular markers, as the later is always higher, is attributed to many factors of which the most important is the host immunity. However, other factors include micronutrients, bioavailability and pharmacokinetics, in addition to the complexity of data analysis need to be considered. The term, genotype-resistance index (GRI) was suggested to encounter for the confounding factors mainly immunity, it is calculated by dividing the prevalence of resistant genotype by prevalence of in-vivo resistance and it was found to be varying from place to another. In Mali, for CQ resistance and *pfcr* the GRI values felled between 1.6 and 2.8 in different sites over 3 years.

In this study, we planed to estimate the prevalence of and the linkage between the individual *dhfr* and *dhps* mutations and the association between the mutations and the response to SP treatment (in-vivo). Furthermore, we wanted to investigate the relation between these mutations and the parasite fitness in term of, in-vivo parasite growth and gametocytogenesis. It is worth to mention that we are benefiting from a unique setting that well suits our objectives, of the credits of this study are; been a village-based whole population study, low
transmission rate with minimal overlap of infections, and the relative homogeneity of the study populations.

**1.10.8 Evolution of antimalarial drug resistance:**

Drug resistance in malaria is considered to result from spontaneous gene point mutations, a process though to be independent from drug pressure, followed by the selection of the more resistant mutants under drug pressure\(^{174}\). Evidence from field and theoretical studies indicate that resistance continues to spread as long as there is any drug pressure, and that beyond a certain frequency, the rate of spread is very rapid\(^{188,189,190}\).

A number of models were suggested for the evolution of drug resistance, except for the role of the drug selective pressure, these models are not consistent and the predictions obtained are contradictory. Various factors have been considered in these models include the degree of drug use, the drug elimination half-life, and host heterogeneity\(^{191}\), parasite biomass\(^{192,193}\). That in addition to the malaria transmission intensity\(^{194}\) and its proxy factors such as the number of parasite clones in a single infection (clone multiplicity), the immunity of hosts, and intrahost dynamics\(^{195}\).

**1.10.9 Methods for Monitoring Parasite Resistance:**

Several methods for monitoring antimalarial drug resistance exist; they include in vivo and in vitro tests and, more recently, molecular markers.

**1.10.9.1 The in-vivo tests:**

Are traditionally the “gold standard” methods for detecting drug resistance\(^{196}\). The advantage of the in vivo tests over the in vitro assays is that they can be conducted in the field with little equipment and personnel and the results are easy to interpret. They reflect the true biological nature of treatment response, which involves a complex interaction between the parasites, the drugs, and the host response, while in vitro tests measure only the interaction between the parasites and the drugs.

**1.10.9.2 The in-vitro tests:**

Are based on the inhibition of the growth and development of malaria parasites by different concentrations of a given drug relative to drug-free controls. The WHO in vitro microtest is based on counting the parasites developing into schizonts, while the isotopic microtest is based on measurement of the quantity of radiolabeled hypoxanthine, a DNA precursor, incorporated into the parasites\(^{197}\). There are newer colorimetric tests that can test low
parasitemias taken directly from patients in the field: the parasite lactate dehydrogenase enzymatic assay\textsuperscript{198}, the parasite lactate dehydrogenase double-site enzyme-linked immunodetection assay\textsuperscript{199} and the histidine-rich protein II assay\textsuperscript{200}. However, because of the limited amount of data published, it is still unclear whether these new tests will replace the traditional (micro and isotopic) tests. In the latter, drug resistance is identified when parasite growth occurs above a threshold concentration.

1.10.9.3 Drug resistant genes:
1.10.9.3.1 Genes implicated in resistance to chloroquine:
There has been a prolonged search for the genetic and biochemical basis of resistance to chloroquine. Several genes encoding candidate proteins, suggested being involved in the transport of chloroquine into or out of the digestive vacuole, have been proposed over the past decade. \textit{P. falciparum} multi-drug resistance gene (\textit{pfmdr1})\textsuperscript{201,202} a candidate gene (Cg2)\textsuperscript{136} and the chloroquine resistance transporter gene (\textit{pfcrt})\textsuperscript{203}.

\textbf{pfmdr1:} Two genes \textit{pfmdr1} and \textit{pfmdr2}, homologue of human mdr-type gene were initially identified\textsuperscript{202,204}. Later \textit{pfmdr2} gene expression levels were found to be unchanged between resistant and sensitive \textit{P. falciparum} parasites\textsuperscript{205,206} and no polymorphism has been observed between them\textsuperscript{207}. So \textit{pfmdr2} was excluded as candidate gene. However, polymorphism in \textit{pfmdr1} gene was found to be strongly linked to chloroquine resistance in a number of \textit{P. falciparum} parasites\textsuperscript{201}. \textit{pfmdr1} is localized in chromosome 5 in \textit{P. falciparum} and it consists of continuous open reading frame of 4257 nucleotides\textsuperscript{204}. It encodes a 162 KDa polypeptide, which has been named P-glycoprotein homologue1 (\textit{Pgh1})\textsuperscript{208}. \textit{Pgh1} is expressed in the membrane of the parasites digestive food vacuole throughout all sexual stages in the life cycle\textsuperscript{208}. It has been suggested that, resistance could be due to amplification of \textit{pfmdr1} allowing more gene product to be expressed\textsuperscript{202} or to mutation in \textit{pfmdr1} causing alteration in transport by the encoded P-glycoprotein\textsuperscript{201}. A key amino acid substitution (Tyrosine to Asparagine) at position 86 has been suggested to be involved in chloroquine resistance\textsuperscript{201}. However this was not always evident in subsequent surveys\textsuperscript{208,209,210}.

A clear evidence against the involvement of this gene in resistance was shown in genetic cross experiment between the resistant clone Dd2 and the sensitive one HB3, in which the Dd2 allele of the \textit{pfmdr1} did not co segregate with resistance phenotype\textsuperscript{211}. More field studies have found no significant association between allelic variants or level of expression of \textit{pfmdr1} and chloroquine resistance\textsuperscript{210,212,213,214,215} suggesting that there can be no simple association between mutations in the \textit{pfmdr1} gene and the degree of chloroquine susceptibility.
However, transfection work indicated that one allele of the *pfmdr1* plays a role in modulating the level of chloroquine response. The resistant *pfmdr1* allele of the *P. falciparum* 7G8 was replaced by a sensitive form containing codons determining amino acids Ser 1034, Asn 1042 and Asp 1246. Results showed that the transfectants accumulated nearly twice the level of chloroquine of that of the original 7G8 isolate.

In general, work on *pfmdr1* showed that mutations or increased expression of this gene might be important for the modulation of chloroquine response observed in some parasite isolates and laboratory clones.

**Cg2:** The analysis of a genetic cross between two cloned parasite populations, Dd2 (chloroquine resistant parasite) and HB3 (chloroquine sensitive parasite) had shown that a region of 400 Kb locus on chromosome 7 segregated with chloroquine resistance in *P. falciparum*. Subsequent genetic mapping on this 400 Kb segment using RFLP and microsatellite markers has localized a key gene to a region of about 36 Kb on chromosome 7. Analysis of polymorphism within this region indicated an association of a gene candidate (Cg2) with chloroquine resistance. Few field studies have been done in the involvement of Cg2 in chloroquine resistance. It appears that polymorphism in this gene are represented differentially in chloroquine sensitive and chloroquine-resistant *P. falciparum* parasites. However, transfection studies, replacing alleles of this gene in resistant parasites by their counterparts from sensitive parasites, showed that they had no effect on chloroquine response of the transformed parasites.

**pfcrt:** Further transcriptional analysis of the DNA sequences near the Cg2 gene has identified the *pfcrt* gene. The coding region of this gene spans 3.1 Kb in 13 exons ranging in size from 45-269 bp. This gene encodes a transmembrane protein in the digestive vacuoles of malaria parasite. *pfcrt* gene was found to segregate perfectly with chloroquine resistance among field isolate of *P. falciparum* polymorphism in this gene showed complete linkage to the chloroquine resistant phenotype in 40 strains examined. Sets of point mutations in *pfcrt* were associated with chloroquine resistance in vitro in laboratory lines of *P. falciparum* from Africa, South America and Southeast Asia. Chloroquine resistant parasites from Africa and Southeast Asia carry point mutations at codons 74, 75, 76, 220, 271, 326, and 371, whereas South America parasites carry mutations at codons 76, 220 and either 72, 326 and 356, or 75, 97 and 371. Only one mutation, the substitution of threonine for lysine at position 76 (K76T) was present in all resistant isolates and absent from all sensitive isolates tested in vitro. Furthermore, genetic transformation experiments with plasmids expressing mutant forms of
pfcr conferred chloroquine resistance on three different chloroquine-sensitive clones. These studies point to key role for pfcr K76T mutation in conferring in vitro chloroquine resistance. Numerous recent field studies have provided additional evidence for the central role of pfcr in determining response to chloroquine: Clinical studies in Mali suggested that, the key pfcr amino acid substitution, pfcr K76T, is selected after chloroquine treatment and is a reliable genetic marker for chloroquine resistance. This pfcr mutation was found to be significantly associated with in vivo response to chloroquine in Mozambique and with both in vivo and in vitro response in Cameroon and in Sudan. Other studies from Brazil, Uganda, Laos and Papua New Guinea have described the role of pfcr K76T mutation in chloroquine treatment failures. These studies provide substantial support for a sweeping association of pfcr K76T mutation with different foci of chloroquine resistance.

1.10.9.3.2 Genes implicated in resistance to pyrimethamine/sulfadoxine:

The combination of pyrimethamine and sulfadoxine has widely been used in malaria chemotherapy. The target molecule of pyrimethamine is dihydrofolate reductase (DHFR) and for sulfadoxine is dihydropetroate synthase (DHPS), both enzymes being components of the folate biosynthesis pathway.

dhfr: The dhfr in P. falciparum exist as a bi-functional enzyme together with thymidylate synthase (TS), which forms d-TMP from d-UMP methylenetetrahydrofolate as methylating agent. DHFR activity is essential in maintaining a constant supply of folate factors for key one-carbon transfer reactions, in which only the fully reduced (terahydro) forms are functional. The dhfr gene was cloned from P. falciparum in 1987. P. falciparum dhfr gene, unlike its human counterpart, is found to be very sensitive to inhibition by pyrimethamine.

DHFR sequences from various P. falciparum parasites were examined extensively. These studies showed that, a single intronless open reading frame of about 1.8 kb coding sequence was observed, with the DHFR domain residing at the amino terminus and TS at the carboxyl terminus, and a peptide junction of approximately 100 amino acid residues separates the DHFR and TS domains. The findings of these studies have revealed correlation between pyrimethamine resistance and point mutations in the dhfr gene. Mutations in P. falciparum dhfr involving amino acid residues 16, 51, 59, 108 and 164 have been reported across a number of geographical areas. Asn-108 mutation is critical point mutation found in all pyrimethamine-resistant isolates, and is believed to be the
prime point mutation necessary for the development of mutants with higher degree of pyrimethamine resistance in malaria.

**dhps:** dhps genes codes for an enzyme involved in the de novo folate biosynthesis pathway located upstream dhfr. This enzyme is responsible for the synthesis of 7,8-dihydropteroate diphosphate and p-amo no benzoic acid (PABA), forming dihydropteroate in the step preceding dihydrofolate synthesis. The gene encoding dhps enzyme has been cloned and shown to be a bi-functional enzyme with 6-hydroxymethyl-7,8-dihydroprotein pyrophosphate (PPPK) at the N- terminus of the protein. Expression and purification of the functional PPPK-DHPS enzyme from P. falciparum in E. coli shows that the activity purifies as an 83-Kda protein and molecular mass range from 204 to 246 Kda, suggesting that the enzyme is multimer of two or three subunits. The drugs of sulfa group are PABA analogues and have been shown to be competitive inhibitors of DHPS enzyme in P. berghei. Characterization of P. falciparum dhps gene revealed mutations in codons, 436, 437, 540, 581 and 613, which could be associated with sulfadoxine resistance. Mutations in these five codons have been observed in large number of field samples of diverse geographical origins.

1.10.9.3.3 Drug resistant genes as molecular markers:

The increased understanding of the molecular mechanisms for parasite resistance to the antifolates and, to some extent, to CQ has led to a proliferation of field studies investigating the role of molecular markers in detecting drug resistance. Some studies have demonstrated a causal relationship between discrete polymorphism in the candidate genes and parasite resistance to either CQ or SP in vitro and in vivo, but others have not.

The precise role of molecular markers in detecting and estimating P. falciparum resistance still needs to be established because the genetic mutations that predict parasite resistance remain unclear. A genotype resistance index (GRI) and genotype failure index (GFI), derived by computing the ratio between the age-adjusted frequency of the pfcr mutation at position 76 and the prevalence of parasitologic and clinical CQ resistance, respectively, have been proposed for the surveillance of CQ resistance. Such indices consistently gave a stable value of 2 to 3 for several sites or different years, indicating that the prevalence of clinical or parasitologic failure was two to three times lower than the prevalence of the gene mutation.

The association of the pfcr codon 76 mutations with in vivo CQ treatment failure has been observed in several settings but not in others, especially in areas where CQ
resistance was very high. In these situations, the GRI and GFI might not predict CQ treatment failure because the prevalence of the gene mutation in these populations has reached a plateau\textsuperscript{248}. In such settings, other indices are needed. In Uganda, a strong positive correlation between the \textit{pfcr}t codon 76 mutant/wild (M/W) ratio and the late stages of high-grade CQ treatment failure (i.e., ETF and RIII parasitologic failure) has been observed\textsuperscript{249}. Such a ratio could be useful in estimating high-grade CQ resistance in cases where the prevalence of the \textit{pfcr}t mutation is already high.

For SP resistance, there is growing evidence that the \textit{dhfr} triple mutations with or without the \textit{dhps} mutations can predict SP treatment failure (SP-TF). Studies conducted in East Africa have observed that the triple (\textit{dhfr} Asn-108, Ile-51, and Arg-59) mutant genotype was associated with SP treatment failure\textsuperscript{181,182}. However, a study in Malawi observed that the quintuple mutant genotype (\textit{dhfr} Asn-108, Ile-51, and Arg-59 plus \textit{dhps} Gly-437 and Glu-540) was more strongly associated with SP-TF than was the \textit{dhfr} triple mutant\textsuperscript{180}. Lack of consistency in the results is probably a consequence of differences in the laboratory methods used to identify the mutations and in the estimation of the frequency of the gene mutations.

In most studies, the prevalence of the gene mutations in host infections, i.e., the number of infected individuals carrying the mutations divided by the total number of infected individuals sampled has often been used. However, the prevalence of infections with the mutant genotype might differ from the frequency of the mutant allele in the parasite population, and the latter is likely to be a more appropriate measure for estimating the number of circulating mutant parasites. In Ugandan sentinel sites, the \textit{dhfr} codon 59 M/W ratio, but not the prevalence of infections with the \textit{dhfr} codon 59 mutation, was positively correlated with SP treatment failure\textsuperscript{250}.

1.11 The malaria parasite genome:

Evolution of both the anopheles species and \textit{Plasmodium} species in the face of natural and man-made selection will inevitably occur. The developments of molecular epidemiological approaches to monitor changes in the parasite and vector biology at a level of detection are able to identify rare variants in populations. They may allow us to respond more rapidly to potential failure of drugs, vaccines and pesticides. A fundamental understanding of the population biology of \textit{P. falciparum} may also help design innovative control strategies in the face of social change and human migration.
Genome analysis in several laboratories revealed unique features of *P. falciparum* chromosome organization which appear to allow rapid evolution of genes involved in parasite/host interaction. *Plasmodium* has, in addition to the nuclear genome, two unrelated organellar genomes, one mitochondrial (6kb) and the other probably of plasmid origin (35kb).9

In the analysis of the parasite *Plasmodium falciparum* genome sequence of clone 3D7, there is a 23-megabase nuclear genome consists of 14 chromosomes, encodes about 5,300 genes, and is the most (A + T)-rich genome sequenced to date. Introns were predicted in 54% of *P. falciparum* genes. Excluding introns, the mean length of *P. falciparum* genes was 2.3 kb. Genes involved in antigenic variation are concentrated in the subtelomeric regions of the chromosomes. Compared to the genomes of free-living eukaryotic microbes, the genome of this intracellular parasite encodes fewer enzymes and transporters, but a large proportion of genes are devoted to immune evasion and host–parasite interactions. Many nuclear-encoded proteins are targeted to the apicoplast, an organelle involved in fatty-acid and isoprenoid metabolism. The genome sequence provides the foundation for future studies of this organism, and is being exploited in the search for new drugs and vaccines to fight malaria.251

The genome of *P. falciparum* contains a number of simple sequence repeats (microsatellite DNA). Microsatellite DNA has been found to occur at an average rate of one in every 2-3kb. These repeats were predominantly of the forms ATₙ, Tₙ and TAAₙ while the CAₙ repeat forms that frequent mammalian genomes were not found.91 The size polymorphism associated with these repeats and their regular distribution in the genome make them a useful tool for genetic linkage analysis.

*Plasmodium falciparum* chromosomes vary considerably in length, with most of the variation occurring in the subtelomeric regions. Field isolates, even those from individuals residing in a single village,252 exhibit extensive size polymorphism that is thought to be due to recombination events between different parasite clones during meiosis in the mosquito.253 Chromosome size variation is also observed in cultures of erythrocytic parasites, but is due to chromosome breakage and healing events and not to meiotic recombination.254,255 Subtelomeric deletions often extend well into the chromosome, and in some cases alter the cell adhesion properties of the parasite owing to the loss of the gene(s) encoding adhesion molecules.256,257 Because many genes involved in antigenic variation are located in the subtelomeric regions, an understanding of subtelomere structure and functional properties is
essential for the elucidation of the mechanisms underlying the generation of antigenic diversity.

Centromeres have not been identified experimentally in malaria parasites. However, putative centromeres were identified by comparison of the sequences of chromosomes 2 and 3. Eleven of the 14 chromosomes contained a single region of 2–3 kb with extremely high (A + T) content (>97%) and imperfect short tandem repeats, features resembling the regional *S. pombe* centromeres; the 3 chromosomes lacking such regions were incomplete251.

Meiosis is followed by mitotic division to haploid sporozoites. Crossing experiments have shown that intragenic recombination occurs between different alleles during meiosis127,258. Many genes coding for antigens are located near the chromosome ends, i.e. the most labile regions. This might be a way to generate new antigenic variants and thereby evading immunity.

The issue of sexual mating has been debated. One view is that natural infections are derived from one or few genotypes, which undergo mating infrequency, implying mainly clonal reproduction259. Recombinations however do occur frequently in nature30,260 but with higher selfing rate in areas of low transmission105. In strain theory, it was suggested that malaria parasites only exist as a limited number of stable antigenic types, called strains261 and that cross-immunity between mild and virulent strains is slow51.

1.12 Malaria prevention and control strategies:

Strategy is the art of planning and managing different activities in a coherent design for the attainment of specific objectives. The aim of any malaria control strategy is to prevent mortality and to reduce morbidity and the social and economic losses due to disease262. In areas where the malaria transmission has been interrupted, the goal is to avoid the re-introduction of malaria13.

Malaria is preventable, treatable and curable. Its epidemiology, however, is highly variable, and control strategies must therefore be adapted according to local biological, social and health system factors. Despite this complexity, in most countries, especially those in Africa with the highest burden, three approaches to reduce mortality and morbidity based on effective and low-cost interventions can and should be applied to give full coverage of all populations at risk. These are: (i) prompt access to treatment, especially for young children; (ii) prevention and control in pregnant women; and (iii) use of insecticide-treated nets and other methods of vector control. Prompt access to treatment160.
A large proportion of deaths from malaria results from delayed administration of effective antimalarial treatment, as death can supervene within days or even hours of onset of the disease. It is essential that all people who develop the disease, especially young children and pregnant women, have prompt access to effective treatment.

After the widespread failure of malaria eradication campaigns in the 1950’s and 1960’s, malaria has returned with renewed vigor to most tropical countries where its impact is increased with the spread of multidrug resistant parasites and insecticide resistant vector. Many methods have been proposed for intervening in parasite transmission and thus reducing malaria incidence:

1.12.1 Vector Control:

Attempts to control malaria by targeting the vector started as early as the beginning of this century. The advent of DDT as an immagociding agent in the early 1940’s was a breakthrough in the fight for malaria control, before that vector control relied mainly on reducing larval populations by drainage and using larvicides. This programme saw initial success, as malaria was eliminated or reduced from 77 countries, however soon the number of reported cases recovered and then quickly exceeded the previous levels. The failure was due to development of insecticide-resistant mosquitoes. Nevertheless in most cases insecticide-based approaches are relatively cheap and still very effective in reducing the impact of malaria in highly endemic areas. To overcome insecticides resistance, new synthetic insecticides were developed. For example, pyrethrum impregnated bed nets were used for malaria control in African villages and have achieved encouraging results.

Recently great attention has been given to the use of environmental management measures for vector control. This approach aims at intensifying and extending the natural factors involved in limiting vector breeding or survival, and contact with man. Many biological agents ranging from viruses to invertebrate predators are under investigations and some bacterial, fungal and protozoan pathogens nematodes and invertebrate predators have reached the field of entomological evaluation.

1.12.2 Malaria vaccine:

The prevention of malaria by vaccination is a conceivable approach, which may eventually have a place in malaria control. Attempts to develop a malaria vaccine began early in the twentieth century, and in spite of advances in biomedical technology and periodic bouts of
unsubstantiated optimism in the field, no effective vaccine is available for widespread use. There are indeed formidable reasons for pessimism. There is as yet no precedent for a broadly applicable human vaccine against an organism’s complex as a protozoan an even a lifetime of repeated exposure to malaria rarely, if ever, induces sterile immunity. *Plasmodium* species have evolved multiple mechanisms of immune evasion at the individual and population level, including stage specific antigen expression, allelic variation, “hot spots” of variability within T cell epitope sequence, and antigenic variation. The ability of the malaria parasite to adapt against and survive environmental changes, drug pressure and the host immune responses, has hindered malaria control programs. For these reasons vaccination against malaria has now greatly increased in importance, although it is potentially hindered by the fact that *Plasmodium falciparum* exhibits extensive polymorphism. One of the major obstacles to the design of an efficient malaria vaccine is the large genetic diversity of *P. falciparum* parasite.

### 1.12.3 Chemoprophylaxis:

Several factors must be considered when choosing the chemoprophylactic regimen for a patient: (i) Whether the patient will actually be at risk of acquiring malaria. (ii) Whether the patient is allergic to or has other contraindications to the antimalarial drug of choice. (iii) Whether the patient is a risk of acquiring drug-resistant *P. falciparum* malaria. Resistance of *P. falciparum* to chloroquine has been confirmed or is probable in all countries with *P. falciparum* malaria except the Dominican Republic, Haiti, and Central America north of the Panama Canal, Egypt, and most countries in the Middle East. (iv) Chemoprophylaxis should preferably begin 1-2 weeks before travel to endemic areas. This allows any potential side effects to be evaluated and treated before departure. (v) Chemoprophylaxis should continue during travel in the endemic areas and for 4 weeks after leaving the endemic areas.

Chemoprophylactic Regimens as follows: Regimen A: For travel to areas of risk where chloroquine-resistant *P. falciparum* has not been reported: Once-weekly use of chloroquine Regimen B: For travel to areas of risk where chloroquine-resistant *P. falciparum* exists: Mefloquine

### 1.12.4 Prevention and control of malaria epidemics:

Areas prone to epidemics can be identified by epidemiological stratification that takes account of vectorial transmission capacity, environmental (including meteorological) conditions, social and economic conditions, population migration patterns and other factors.
The entomological inoculation rate has been proposed as a comprehensive indicator of epidemic risk on which to base forecasting. It is defined as the mean daily number of bites inflicted on an individual by mosquitoes infected with sporozoites, and therefore requires the determination of the man-biting and sporozoite rates which, particularly the latter, may be practically impossible to determine in many epidemic-prone areas. Epidemics may occur with very low sporozoite rates, often undetectable by common practices of entomological services, particularly in areas where the vectors are only partially anthropophilic, such as most areas of the Americas and the Indian subcontinent.

Local health services have much to contribute to this process, and should be the first to report a suspicious increase in the number of patients with fever. On the basis of this stratification, a limited set of indicators of epidemic potential or risk factors can be prepared that can be monitored by local health personnel and used to build up community preparedness and prevention.

1.13 Malaria during pregnancy:
Malarial infection during pregnancy is a major public health problem in tropical and subtropical regions throughout the world. In most endemic areas of the world, pregnant women are the main adult risk group for malaria. Malaria in pregnant women has been most widely evaluated in sub-Saharan Africa, where most malaria is due to *Plasmodium falciparum* infection, the most deadly form of the disease. Africa south of the Sahara bears 90% of the global malaria burden. Every year at least 24 million pregnancies occur among women in malarious areas of Africa, yet less than 5% of pregnant women have access to effective interventions.

Of the 50 million pregnancies that occur in malaria-endemic areas every year, approximately half are to be found in sub-Saharan Africa. The problem posed by, and epidemiology of, malaria during pregnancy depends on the local level of transmission. In areas where the intensity of transmission is low, malaria can cause severe materno–foetal syndromes, such as cerebral malaria, abortion, stillbirth and low birth weight. In contrast, those women who live in areas of stable and intense transmission enjoy considerable immunity and generally experience few symptoms during episodes of malarial infection, although some may develop severe anemia.

A paradoxical situation prevails during pregnancy in women with lifelong residence in a malarious area (example having reached a state of semi-immunity): an apparent loss of immunity occurs during pregnancy, while at the same time, these women are able to transfer
a fully effective protective immunity to their infant. This phenomenon is particularly pronounced in primigravidae and becomes less of a problem during later pregnancies. The features of falciparum malaria in a primigravida are the following: infection takes place and the parasite develops in the placenta, while remaining at a low level in the peripheral circulation; in the absence of treatment, an infection acquired during the early stages of pregnancy will be maintained throughout pregnancy in the placenta, often with a heavy parasite load.

The classical explanation of what happens during malaria in first pregnancies is a reduction of acquire immunity, consistent with the immunosuppression generally observed in all pregnancies; this is in line with the observation of reduced lymphoproliferative responses in pregnant women compared to age-matched non pregnant individuals.

Two alternative explanations for malaria in pregnancy have been proposed. The first takes into account of the fact that the placenta is a “new” origin in primigravidae. Which could bypass existing immune responses in the host (without implying an actual loss of immunity) or allow for the development of unusual phenotypes of \textit{P. falciparum}; the acquisition of a placenta-specific immunity or the previous exposure to new malaria phenotypes (in an earlier pregnancy), would explain the decrease susceptibility to infection in multigravidae. The second alternative suggests that what happens in pregnancy is a change in the balance of the local placental environment from an essential TH2 environment (prevailing in normal placenta) to a TH1 environment during acute malaria; this is consistent with the presence of a large number of monocytes in the infected placenta\textsuperscript{13}.

\textit{Plasmodium falciparum} infection during pregnancy increases the chance of maternal anemia, abortion, stillbirth, prematurity, intrauterine growth retardation, and infant low birth weight (defined as less than 2500 grams), which is the greatest single risk factor for death in the first month of life. Malaria has been estimated to cause 8% to 14% of all low birth weight babies and 3% to 8% of all infant deaths in areas of Africa with stable malaria transmission. In terms of its effect on mothers, severe anemia increases the risk for maternal mortality, and malarial anemia is estimated to cause as many as 10,000 maternal deaths each year in Africa. The impact of the other three human malaria parasites (\textit{P. vivax}, \textit{P. malariae}, and \textit{P. ovale}) is less clear\textsuperscript{266}.

In programmes for the prevention or treatment of malaria in pregnant women, two major issues are the safety and effectiveness of the antimalarial drug regimen. The programmatic effectiveness of a given drug is determined by the efficacy of that drug against the parasite
and by the drug’s characteristics, including affordability, availability, acceptability to the target population, and deliverability in terms of dosing requirements and incorporation into existing antenatal care delivery systems.

1.14 Malaria in Sudan:

Excluding a narrow band in Northern Sudan, all the Sudanese people are prone to have malaria.

Figure (1.3)

Taking into consideration the endemicity of malaria (fig. 1.3) and other factors that lead to malaria infection (inbreeding sites, parasite, vector and others.) we can divide Sudan into five parts (high perineal transmission, irrigated malaria, seasonal malaria, urban malaria and riverine malaria (fig. 1.4).
In the same report, malaria death cases have also been reported for the same period in in-patient clinics as ranging between 10-15% from total hospital in-patients.

As shown in fig. (1.3 & 1.4) the whole country is now endemic with varying degrees. Generally speaking, the endemicity increases from north to south. Certain geographical and topographical features like the annual fluctuations of the Nile floods, the winter rains of the Red Sea Hills and the irrigated areas in the central regions, alter this transition of endemicity creating pockets of elevated levels of endemicity. Thus malaria is hypoendemic in the north with mesoendemic areas determined by the cultural schemes in the area and the Nile floods. It is mesoendemic in the central areas with hyperendemic spots resulting from agricultural practices mainly, and the intensity of the annual rains. In the southern part hyper- to holoendemic malaria prevails and transmission is perennial.

All four types of human malaria have been reported in Sudan but the predominant parasite species is *P. falciparum* which is responsible for >95% of reported cases, followed in descending order by *P. vivax, P. malariae* and the relatively rare *P. ovale*. As a result of migration the parasite prevalence map in Khartoum State was changed from that in 30 years ago since the infection with *P. vivax* and *P. ovale* increased.

Chloroquine is the first line drug for malaria treatment in Sudan until 2004, when the Ministry of Health replaced it with combination of artesunate plus fansidar. The possible emergence of chloroquine resistance in Sudan was first suggested in 1978 in Central Sudan. However, confirmation by in vivo test came in 1982. High grade chloroquine resistance was noted in eastern Sudan later. Chloroquine failure rate was estimated by in vitro tests to be 35.2% in study carried in the Khartoum the capital in 1997. A subsequent study in different area in Khartoum in 1998 revealed high rate (55.2%) of all grades of chloroquine resistance.

The deterioration of malaria situation in Sudan is due to appearance of parasites resistant to antimalarial drugs, and vector resistance to some insecticides in use and the vast extension in agriculture and irrigation schemes. Other contributing factors are inadequate financial recourses, poor community participation and the lack of expert staff due to brain drain. These are coupled with poor health coverage and weak information system in a large country like Sudan. The mosquito vector, *Anopheles arabiensis* occurring throughout the country and An. *gambiae* and *An. Funistus* increases towards the south.
1.14.1 Malaria in Eastern Sudan:

Malaria field research has been established since 1986 in Gedarif, eastern Sudan, aiming to study the population dynamics of *P. falciparum* malaria and the human immune responses to this infection. In this area, malaria transmission is seasonal and unstable. The dominant influence on malaria transmission in the Sahel and savannah belts of Sudan is the short rainy season in which 80-90% of precipitation falls in July and August.

The epidemiology of malaria in eastern and central Sudan is considered to be at the unstable end of the spectrum described by MacDonald. The brief exposure of the semi-immune inhabitants to mosquitoes after the short rainy season leads to a major health problem during October and November each year. In this area malaria incidence is not confined to young children, but older children and young adults are at great risk. No obvious source of Anopheline mosquitoes was observed during the lengthy dry season.

During the 1991 and 1992 there was no rain in the area which led to severe drought all over the country. Less than ten malaria cases were detected throughout the whole transmission season in a study site of about 450 inhabitants, but epidemics and outbreaks occurring during the transmission season of 1993. It is puzzling, the dynamics by which the parasite, the host immune responses and the Anopheles vector committed to survive this long period of severe drought when malaria transmission had actually ceased.

Studies in Daraweesh village have demonstrated that a significant proportion of the population harbour asymptomatic infections detectable by transmission season associated rises in anti-malaria antibody titers, despite the absence of clinical disease. Parasitological confirmation of the presence of persistently microscopically negative but PCR positive individuals had been reported. These results were unexpected since malaria in Daraweesh did not show the pattern of age stratification of clinical malaria incidence characteristic of areas of holo-endemic transmission. Individuals living in areas of unstable and low intensity transmission have not traditionally been considered to acquire significant immunity to the disease.

In the past 15 years, regular surveys have been carried in Asar village. These studies have revealed remarkable amount of allelic polymorphism and great genetic diversity among *P. falciparum* parasites that raise the annual malaria epidemics in the village. The parasite population of this village was compared to that of a community with a similar population size in Tanzania. In Asar village there were fewer alleles of each gene studied...
than in Tanzania, and the average number of clones was less. These differences reflect the differences in transmission intensities in each region, which can be expected to have an impact on the prevailing mode of reproduction and responses to locally selective forces.

Surveys carried in Asar village have also shown that, some patients do not clear their acute *P. falciparum* infections following treatment, but remain harboring sub-patent parasitemia throughout the dry season. Thus it seems clear that the malaria epidemics following rains, most probably originates from parasites surviving the dry season among the inhabitants rather than that from infections brought into the village by human or mosquito immigration.

### 1.15 Rationale:

Molecular techniques, more specifically the most simple, popular and affordable technique, the polymerase chain reaction (PCR), have revolutionized the knowledge about disease, including the tropical diseases. *Plasmodium falciparum* malaria is the most important tropical
infection and it is the real hurdle to development that challenged mankind over the centuries. The mortality is mainly due to the severe and complicated form of the disease, and the rest of the malaria burden is attributed to the drug resistance of the parasite (morbidity). Pathogenesis and mechanisms of severe disease and the drug resistance are both crucial issues which are not yet well understood. But what is not beyond the limits of doubt, is that the parasite factors are an important element in both conditions. However, for living organisms including malaria, the control of the vital functions and the phenotypic characteristics can be mirrored through the information centre, which is the DNA. In this study we are aiming to deploy the polymerase chain reaction (PCR) for exploration at molecular level of the parasites that cause the severe disease in general and the individual complications in specific. Further more, in Eastern Sudan, the semi-immune populations are exposed during the annual short window of malaria transmission to markedly variable degrees of malaria infections but on average were less than one infectious bite per person per season. That have many consequences including: a. the low complexity of infection in the area, with minimal overlap of infections, which generate a favorable condition for studying the parasite dynamics in relation to the clinical outcome of the malaria infection b. very low parasitemia (sub-microscopic) could result in symptomatic or even severe and fatal malaria, that creates a dilemma with regard to malaria diagnosis with critical consequences. Again molecular techniques can ideally be used in this setting and can reveal very valuable clinical information. The above is also applied; if we want to study the molecular aspects of drug resistance.

1.16 The overall objective of this study:

This study was done to validate the use of modern molecular techniques in diagnosis and determination of the parasite factors associated with severe malaria morbidity and mortality. Furthermore, to deploy these techniques in; evaluation of the anti-malarial drug efficacy and
study of the molecular marker of parasite resistance, with special reference to chloroquine and sulfadoxine/pyrimethamine combination therapy.

1.16.1 Detailed objectives:

1. To study the molecular characterization of severe malarial parasitemia in terms of parasite genotypes and multiplicity of infection.

2. To validate the use of molecular techniques (PCR) in detection of submicroscopic parasitemia in severe malaria and pregnancy associated malaria.

3. To estimate the frequency of drug-resistance associated mutations in severe and uncomplicated malaria in the study area.

4. To validate the use of the new molecular techniques, PCR-ELISA, in detection of single nucleotide polymorphisms (SNPs) in \textit{P. falciparum} chloroquine resistant transporter, dihydrofolate reductase and dihydropetroate synthase genes.

Chapter two
Materials and Methods

2.1 Study area:
2.1.1 Gedarif area:
The severe malaria study was carried out in Gedarif Teaching Hospital, Gedarif state/ town in Eastern Sudan (350 to 400 thousand inhabitants) during two consecutive malaria seasons (September to January) over the years 2000 to 2002. Gedarif town is situated on the Sudanese
savannah, at an altitude of about 600 meters above sea level; it is 450 Km from the Capital, Khartoum (fig. 2.1). The epidemiology of febrile uncomplicated malaria episodes in the area has been reported before. Malaria in this area is highly seasonal, and follows the annual June - October rains and accordingly the incidence of malaria varies considerably from year to year. The Plasmodium falciparum is the predominant species (98%), and the Anophiles arabiensis is the sole malaria vector in this area. The entomological inoculation rate (EIR) in Gedarif has not been measured, but was found to be very low and difficult to measure by conventional methods in the surrounding villages.

For anti-malarial drugs study, samples were collected from the Daraweesh and Kajara villages, 16 km from Gedarif town, from October 2003 to December 2003. Daraweesh is inhabited by approximately 550 individuals, of a Fulani origin who settled in Sudan a century ago. Kajara inhabited by approximately 2000 individuals including different ethnic groups; it is one or two km from Daraweesh. The villages’ economy is based on subsistence agriculture of sesame and dura.

2.1.2 New Halfa area:

Data and samples were also collected from New Halfa Teaching Hospital, the Sugar Cane Health Center and Alhara Aloula Health Center, New Halfa town, Kassala state, Eastern Sudan, about 550 Km from Khartoum (fig. 2.1). New Halfa is 450 m above sea level, located between 15-19 Latitude, North and 35-36 Longitude, East. It is characterized by average annual rainfall of 238 mm and average annual relative humidity of 35%. New Halfa is a relatively large town center surrounded by more than 100 small villages on the outskirts of the town; it is inhabited by around 250000 individuals with approximately 2000-3000 individuals in each village. Several ethnic groups including Afroarab tribes were settled in the area in 1964. A large sugar cane industry and associated agricultural schemes represent the backbone of the economy in the area. The agriculture depends on rainfall and permanent irrigation system from Atabra River and many water canals found around and between the villages.
The malaria infections in New Halfa region are predominantly (95%) caused by *Plasmodium falciparum* and the transmission is seasonal and unstable. Although malaria is mesoendemic in this area, with the peak of transmission occurs between September-January following the rainy season, severe epidemics are occasionally recognized. The health facilities of the area constitute of one referral hospital, where severely ill patients are brought, one single-doctor hospital and twenty dispensaries.

### 2.2 Study design:

This study is part of a large project funded by the Multilateral Initiative on Malaria in Africa (MIM/TDR/WHO) about pathogenesis of severe malaria in Eastern Sudan. Collaborators from different disciplines (physicians, scientists, technicians and others) and from different countries (Sudan, Denmark and Sweden) were involved in this study. The study received ethical clearance and national endorsement by the Sudan Ministry of Health.

We aimed in part of the project to deploy the molecular techniques to study the malaria parasite at a molecular level in relation to: a/ severe malaria development b/ sub-patent malaria in clinically diagnosed cases of severe malaria or pregnancy associated malaria with negative blood smears. c/ drug resistant parasites. Each of these studies was conducted in a separate cohort of patients for variable period of times. Each cohort was selected based on specific criteria.
a. For severe malaria in Gedarif: The selection of severe malaria patient was based on the WHO criteria for severe malaria, which includes: 1. Cerebral malaria, an unarousable coma persisting for at least 30 minutes, it should be differentiated from transient postictal phenomenon 2. Severe malarial anemia, normocytic anemia with haematocrit <15% or hemoglobin <5g/dl, in presence of parasitemia 3. Respiratory distress 4. Hypoglycemia, whole blood glucose concentration of <2.2mmol/l (40mg/dl) 5. Circulatory collapse or shock, hypotension (systolic blood pressure <50 mmHg in children (1-5 years) or <70mmHg in adults), with cold clammy skin. 6. Renal failure, urine output of <400ml/24 hours in adults or 12ml/kg in children inspite of rehydration, and elevated serum creatinine level, >265µmol/l (>3mg/dl) 7. Spontaneous bleeding from gums, nose, gastrointestinal tract, etc. and/or laboratory evidence of desminated intravascular coagulation (DIC) 8. Repeated generalized convulsions at least two per 24 hours despite cooling; febrile convulsions in children should be considered 9. Acidosis, an arterial pH <7.25 or a plasma bicarbonate concentration <15 mmol/l 10. Macroscopic hemoglobinuria. Other supportive (non-defining) manifestations of severe malaria include: a) Impairment of consciousness. b) Jaundice. c) Prostration or weakness. d) Hyperpyrexia and e) Hyperparasitaemia. Patients will be characterized clinically and grouped

A matched control group (age, sex, tribe and etc.), which included; patients presenting with mild uncomplicated *falciparum* malaria, and malaria free individuals, were enrolled as positive and negative controls, respectively. This control group case was selected mostly from among the severe malaria patient’s relatives or from the same area. Full history and detailed symptoms of illness were obtained from the malaria patients or their accompanying relatives. (questionnaire and informed consent are attached as annex).

b. For severe malaria in New-Halfa: The data was obtained in the period from November 2000 to February 2001. A medical doctor examined patients presenting with malaria-like symptoms in New-Halfa Hospital, in an outpatient malaria clinic. The malaria diagnosis was done by examination of thick and thin blood smears under light microscopy. Patients with confirmed malaria infection were treated with chloroquine, fansidar or quinine, as mentioned before. During the study period, all patients admitted to hospital in coma and clinically suspected to have cerebral malaria ($^{cS}$CM), but with negative blood smear for malaria parasites, were included in the study after the consent of their guardians was obtained. The enrolled patients were treated according to the health policy and management protocol adopted by the local health authorities. They were treated initially by quinine infusion and,
after suitable clinical improvement, by quinine tablets (10mg/kg body weight, 8-hourly, for a total duration of 7 days). Two patients were further treated with benzyl penicillin (10⁶ IU/6-hourly, for 5 days) and one of them was in addition given gentamycin (80mg/8-hourly for 5 days) all were given in parallel with quinine from the first day. Only a small fraction from other groups of individuals with and without microscopically confirmed *P. falciparum* malaria were included in the study, as controls in addition to apparently healthy donors.

c. For drug resistance in Daraweesh/Kajara (Gedarif area): A clinic was set in each village, and inhabitants were informed to come to the clinic if they or their relatives had fever or other malaria like symptoms. A health team consisting of; a medical doctor, nurse, microscopist and the investigator were informed to cover both villages. They were available in the villages on daily basis. Most of the patients who had malaria were included in the study, and only small number of patients were excluded from the study based on the exclusion criteria mentioned before. However, other remaining patients were examined, and were either treated or prescribed a drug not available in the clinic or referred to Gedarif Hospital.

In this study the 28-days in-vivo drug efficacy protocols of the WHO¹⁹⁶ which are used in malaria hyperendemic regions was modified. The main modification was that malaria diagnosis does not depend on parasite in this setting i.e. malaria was diagnosed when parasitemia of any count was detected in a febrile (≥ 37.5°C) patient or a patient had a recent history of fever. The SP was given as a single dose of 1.25 mg P/kg body weight, and CQ was given in a dose of; 10-10-5 mg/Kg/day, over 3 days. The treatment was supervised; in the first day (D0), the patient was given SP alone or with CQ simultaneously in the clinic and the dose is repeated if the patient vomited during his/her stay (half an hour), then, CQ was given in D2 and D3 in the clinic as in D0. Patients were examined clinically and parasitologically by taking blood smears and blood in filter paper for parasite detection, during the follow up on days; 3, 7, 14, 21 and 28. Finally, the treatment outcome was to be classified as; early treatment failure (ETF), late treatment failure (LTF), and adequate clinical and parasitological response (ACPR).

The alternative treatment (quinine) was given to patients in D3 if they developed severe malaria, and was given thereafter if parasitemia associated with symptoms was detected.

Parasite genotyping was done to distinguish recrudescence and re-infection and molecular markers were used to investigate the relationship between in-vivo drug resistance and the mutations in drug resistance genes.
d. For pregnancy associated malaria in New-Halfa: All the pregnant women visiting the center’s antenatal clinic for the first time were enrolled. After verbal consent was obtained, each woman’s obstetric history was recorded in details, including the date of her last menstrual period, gravidity, and parity. Each woman was then asked specifically if she had any symptoms indicative of malaria: fever, headache, sweating, joint pain and/or vomiting. Body temperature was recorded, obstetric and physical examinations were conducted, and blood pressure, pallor and fundal level were recorded. Pregnancy was confirmed by ultrasound and its duration was calculated from the date of the last menstrual period. The blood concentration of hemoglobin was estimated using a colorimetric method. The main aims were to assess the prevalences of submicroscopic and multiple *P. falciparum* infections in pregnant women (using the *P. falciparum* merozoite surface protein-2 as a polymorphic marker in PCR-based assays) and to determine the effects of such infections on anemia during pregnancy.

2.3 Study population:

a. Severe malaria in Gedarif Hospital: All patients in all age groups with a primary diagnosis of malaria confirmed by a positive blood smear for *P. falciparum* and no other cause for the clinical presentation, in Gedarif Hospital during the malaria seasons of 2000 to 2002, were enrolled in this study after informed consent from the patient or guardians.

More than 16600 patients were screened for malaria infection, 2488 were slide positive and among them 103 individuals had fulfilled the WHO (2000) criteria of severe malaria and were enrolled in a longitudinal study. Samples were taken at day0 (D0) before treatment, D3, D7 (during treatment) and D28 (after treatment). Blood spots were taken onto filter paper (Whatman 3MM chromatography paper) from finger prick, allowed to air-dry, then placed individually in plastic bags and stored in refrigerator for further analysis. All severe patients were treated with quinine infusion for 48 hours followed by quinine tablets (10mg/kg) for total of seven days. For uncomplicated falciparum malaria, adults and older children were treated with chloroquine tablets, and for children chloroquine syrup was used (10, 10 & 5mg/kg body weight for three days). In case of treatment failure, patients were given fansidar tablets.

**Follow-up:** The patients were discharged from hospital after clinical improvement and the ability to continue the treatment orally at home under medical supervision. Patients were
followed up, clinically and parasitologically up to day 28 after diagnosis, and were advised to visit our malaria clinic if symptoms recurred thereafter.

b. Severe malaria in New-Halfa: For the study of the sub-patent severe malaria in New-Halfa, patients with malaria symptoms with and without microscopically detectable parasitemia were included. The case definition for severe malaria was mentioned previously. Patients clinically suspected to have severe malaria (CSSM) had the above symptoms, but parasitemia was not detectable by microscopy, and the other possible diagnoses were excluded. The latter was done by meticulous clinical examination, clinical consultancy of the clinical experts and by doing basic routine investigations like; urine/stool general, complete hemogram, chest x-ray, lumber puncture and etc. The details about the patients included in the study and their characteristics are shown in the result section.

c. Drug combination in Daraweesh/Kajara: In this total population study, all individuals in Daraweesh and Kajara villages during 2003 malaria transmission season, who had confirmed malaria infection (clinical and parasitological) and accepted to be seen and examined during the follow up, were included in study of efficacy of sulphadoxine/pyrimethamine (SP) alone and in combination with chloroquine (CQ) in treatment of uncomplicated P. falciparum malaria. The total villagers were estimated to be more than 2500 individuals, of them one quarter were living in Daraweesh. The estimated sample size was between 50 and 100 individuals for each of the treatment arms respectively. All individuals in both villages, who had confirmed malaria infection (clinical and parasitological) and accepted to be seen and examined during the follow up, were included in the study. Pregnant women, severely ill patients, patients co-infected with other organism, children too small to swallow tablets (mostly less than 4 years), or patients known to be allergic to the antimalarial drugs were excluded from the study. In addition, patients with severe malaria or with mixed malaria infections were also excluded. Patients and/or their guardian's verbal consent were obtained, as well as both village leaders and patients were informed about the study and the objectives.

d. Pregnancy associated malaria in New-Halfa: A third group in New Halfa city including the pregnant women attending Alhara Aloula health center from August 2003 to July 2004 were enrolled in a separate study. 142 pregnant women were enrolled as they attended antenatal clinic for the first time.

2.4 Blood sampling:
Venous blood samples (5-10 ml) were taken into EDTA or heparinised vacutainer at D0 and D28. Red blood cells were separated by centrifugation of the blood samples, re-suspended with glycerol freezing solution and stored in liquid nitrogen until used (mainly for immunological studies). The buffy coats (mainly leucocytes) were removed and plasma was transformed into cryotubes and stored at -20°C, also plasma was used mainly for immunological studies. Few drops were used for preparation of blood smears and parasite DNA. Finger pricks were used to obtain few drops of blood to be used for preparation of blood smears for parasite detection and to be blotted in filter paper for parasite DNA extraction. The finger prick samples were collected during the follow up days; D3, D7, D14, D21 and D28.

2.5 Malaria diagnosis and parasite count:

The malaria diagnosis was carried out as described by the WHO, in brief; thick and thin blood smears were prepared, stained with Giemsa and examined under light microscopy at a magnification of x1000, by an expert malaria technician. Films were considered negative after examination of 200 thick smear fields without detection of parasites. Another two expert malaria microscopists using quality control-evaluated microscopes at the reference malaria laboratory rechecked all slides. Parasites were counted per 200 leukocytes and standardized to 1 micro liter of blood assuming that the number of WBCs in Sudanese individuals is 6000/µl of blood.

2.6 Other investigations:

General routine investigations were done for severe malaria patients such as: complete hemogram (Hematocrit, reticulocytes, total and differential white blood cell count-TWBC-thrombocyte count, ESR-1hour) and urine general. Other investigations were done on special condition and upon request from the clinician e.g. blood glucose level (electronic glucometer, accu trend™), blood insulin measured by radioimmunoassay-kit IMK-414, Beijing, China, and renal function test (blood urea, serum creatinine, plasma bicarbonate). However, other investigations were done for exclusion of other febrile conditions e.g. Widal test for typhoid and brucellosis, sickling test, spinal fluid culture and Chest X-ray. Then the results and the clinical data were entered into a data base program, Microsoft Access.

2.7 In vivo study:

Patients were allocated randomly to either of the two treatment regime (SP alone and SP plus CQ) with consideration of the possible similarity between the two groups with regard to age,
gender and village. The first treatment group was given SP single dose of 1.25 mg P/kg body weight, and the second treatment group (SP plus CQ) was given SP single dose of 1.25 mg P/kg body weight and CQ (dose; 10-10-5 mg/Kg/day) over 3 days. All patients were first weighed, the two drugs in the first treatment group were administered simultaneously under our supervision, and repeated if vomiting occurred within half an hour, and the same was applied for SP treatment.

During the follow up, patients were seen and investigated for malaria (clinical and parasitological) on day 0, 3, 7, 14, 21 and 28, guided by the major steps of the WHO protocols for in-vivo studies, with some modifications which were mentioned before. Clinical information was recorded in follow up sheets and later entered into data base programme; in addition blood smears and blood in filter papers were taken in all the days of the follow up.

Classification of treatment outcome: After the in-vivo test was performed, the treatment outcome had been classified into three categories; early treatment failure (ETF), late treatment failure (LTF) both clinical and parasitological failure, and adequate clinical and parasitological response (ACPR)\textsuperscript{196}. However, a number of patients had parasitemia at day 3 with or without symptoms, resolved by D7, those were categorized as having delayed parasitological response (DPR).

2.8 Polymerase chain reaction – PCR:

The polymerase chain reaction (PCR) was used for; a. parasite detection b. parasite genotyping c. and detection of single nucleotide polymorphism (SNP), the mutations associated with chloroquine resistance and sulphadoxine/pyrimethamine resistance

2.8.1 DNA extraction:

Extraction of DNA from bloodspots on filter papers was carried out by the chelex-100 method described by Wooden et al.\textsuperscript{284} with some modifications described by Pearce et al.\textsuperscript{285}. DNA extraction from bloodspots on filter paper was carried out in a 96-well plate format. A segment of the bloodspot was first soaked in 0.5% saponin–1X phosphate-buffered saline overnight and was then washed twice in 1 ml of 1X phosphate-buffered saline. The segment was then boiled for 8 min in 100 µl of PCR-quality water–50 µl of 20% Chelex suspension in distilled water (pH 9.5)\textsuperscript{285}. 
Another approach was also used for DNA extraction by using also chelex-100. Samples were extracted as follows: 50µl of 10% saponin as a detergent were added to the sector of 10–30µm of the blood spotted on filter paper and 1ml phosphate buffer saline (PBS) pH 7.4 was added, and incubated at 4°C for 4-6 hours or overnight, spined for 1 minute at 13000 rpm., the supernatant was aspirated from the tube and washed with 1ml 1x PBS, incubate for 1-2h in 4°C, spined for 1 minute at 13000 rpm and the supernatant was removed. The filter paper was boiled at 95°C with 100µl highly qualified distilled water and 50µl 20% chelex-100 solution for 10 minutes, vortexed vigorously at least twice during boiling time. The DNA was separated after centrifugation and stored in -20°C till used.

2.8.2 PCR amplification:

The polymerase chain reaction (PCR) was used to complement microscopical monitoring, for the detection of low level parasitemia and for genotyping. From the extracted DNA 2µl were amplified in a 20µl reaction volume containing 0.2mM of each dNTP (0.4µl), 50mM Mgcl₂ (0.6µl), 10µM of each primer (0.4µl), 1U Taq (DNA polymerase enzyme), 2µl of 10X PCR buffer, and the volume was completed to 20µl using autoclaved or highly qualified distilled water.

Positive and negative controls were run parallel to each test sample (positive controls; 3D7 and HB3 for IC1 and Fc27 MSP2 allelic families respectively) and negative control containing no target DNA.

2.8.3 Parasite genotyping:

The parasite isolates were genotyped at the allelic variants in the polymorphic regions of MSP2 and GLURP marker genes

2.8.3.1 Genotyping at the MSP2 locus:

The amplification at the MSP2 locus was carried out as a nested reaction. The outer PCR primers were used in the initial amplification reaction for 30 cycles at 94°C for 25sec., 42°C for 1min., and 65°C for 2min. One µl of the outer reaction was then used as template for a second inner reaction for amplification using the primers (Table 2.1) and conditions of 94°C for 25sec., 55°C for 1min., and 70°C for 1min., for 30 cycles for IC1 and FC27 alleles. Both alleles were amplified using S1 primer and either Fc27 or IC1 primers (Table 2.1).

2.8.3.2 Genotyping at the GLURP locus:
Alleles at the GLURP gene locus were detected in nested PCR reactions using outer and inner primers derived from the published gene sequence. Outer primers, G4 and G5 (see Table 2.1 for sequence), were used for initial amplification for 30 cycles under the following conditions; 94°C for 25 sec., 45°C for 1 min. and 68°C for 2 min. One µl of the outer reaction was used as template in an inner reaction using primers G1 and G3 (Table 2.1) in a reaction of 30 cycles with parameters of 94°C for 1 min., 55°C for 2 min. and 70°C for 2 min.

2.8.4 Detection of PCR products using Electrophoresis Techniques:

The PCR products were analyzed by gel electrophoresis, 8 µl of PCR products were loaded onto 1.5% agarose gel (Sigma agarose type II: Medium EEO) in 1X Tris-borate EDTA (TBE) buffer (pH 8.3), then were visualized using UV transillumination after staining with 0.5 µg/ml ethidium bromide.

2.9 PCR-ELISA for detection of drug-resistance-associated mutations:

This new approach is used for detection of SNP (mutations) of genes associated with drug resistance; in this study we used it for detection of chloroquine and sulphadoxine/pyrimethamine resistance genes. It is characterized as being a fast and reliable approach by combining the advantages of both PCR and ELISA in sensitivity and amplification. This method was carried out in several steps.

2.9.1 Plasmodium falciparum clones and field isolates, as control:

Five *P. falciparum* laboratory clones (3D7, FCR3, 7G8, K1, and DD2) were used to verify the specificity of PCR-ELISA technique and were used as a standard control panel in subsequent experiments. In addition three *P. falciparum* parasite isolates from Daraweesh/Kajara, were used in the control panel since they were found to expressing the 436/437AA, 436/437AG and 540E genotypes of *dhps*. Almost all known SNPs of *dhfr*, *dhps*, and *pfcrt* (codons 72-76) are represented in this panel of parasites.

**Table 2.1: Sequences of *P. falciparum* allele specific primers used in genotyping**

<table>
<thead>
<tr>
<th>Primer used</th>
<th>Primer sequence (5’ to 3’ direction)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-2 S2</td>
<td>GAGGGATGTTGCTGCTCCACAG</td>
<td>MSP-2 Outer</td>
</tr>
<tr>
<td>MSP-2 S3</td>
<td>GAAGGTAATTAAACATTGTC</td>
<td>MSP-2 Outer</td>
</tr>
<tr>
<td>IC1/3D7 S1</td>
<td>GAGTATAAGGAGAAGTATG</td>
<td>MSP-2 IC1 allele specific</td>
</tr>
<tr>
<td>IC1/3D7-2</td>
<td>CAGGTGTACAGTCAGTGGAC</td>
<td>MSP-2 IC1 allele specific</td>
</tr>
<tr>
<td>FC27 S1</td>
<td>GAGTATAAGGAGAAGTATG</td>
<td>MSP-2 FC27 allele specific</td>
</tr>
</tbody>
</table>
### 2.9.2 Polymerase chain reactions for *dhfr/dhps* and *pfcrt*:

A nested PCR described by Pearce et al.\(^{285}\) was used to amplify fragments of the *dhfr* and *dhps* genes. A nested PCR described by Djimde et al.\(^{187}\) was used to amplify fragments of the *pfcrt* gene (described in details at [http://medschool.umaryland.edu/CVD/nejm2001djimde.htm](http://medschool.umaryland.edu/CVD/nejm2001djimde.htm)). The only modification in our procedure was that, the M9, R/ and TCRD2 primers for the *dhfr*, *dhps*, and *pfcrt* nested PCRs were biotinylated at the 5’-end by the supplier (MWG Biotech, Riskov, Denmark). We used 20-µL *dhfr/dhps* outer PCR mixture consisted of 0.3 mM of each dNTP, 0.25 µM of either primer set M1/M7 (*dhfr*) or N1/N2 (*dhps*), one unit of DNA HotStart polymerase (Qiagen, Albertslund, Denmark), buffer containing 1.5 mM MgCl\(_2\) and 1 µL of extracted DNA.

1 µL of the outer *dhps* and *dhfr* PCR products were used in the nested PCRs. The nested *dhfr* and *dhps* PCR reaction mixture was the same as the outer PCR mixture using primer sets M3b/M9 and R2/R/ for the *dhfr* and *dhps* PCR, respectively. The outer and nested *dhfr/dhps* PCR conditions were as previously described\(^{285}\). We used 20-µL *pfcrt* outer PCR mixture consisted of 0.2 mM of each dNTP, 1 µM of the primer set TCRP1/TCRP2, 1.25 units of DNA Qiagen HotStart polymerase, buffer containing 2.5 mM MgCl\(_2\), and 1µL of extracted DNA. The reaction mixture of the nested *pfcrt* PCR was identical to that of the outer PCR and the primer set TCRD1/TCRD2 was used. The conditions of the outer and nested *pfcrt* PCR were as previously described\(^{187}\).

Amplifications were performed in 96-well PCR plates. The nested PCR products were confirmed by electrophoresis on a 1.5% agarose gel along with a set of controls.

### 2.9.3 SSOP-ELISA:

Single nucleotide polymorphism (SNP) in the SP resistance associated genes; *dhfr* and *dhps* for (SP), and *pfcrt* for chloroquine were analysed by a recently developed method, the PCR-ELISA\(^{289}\). The ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with streptavidin in phosphate buffered saline (PBS) (1µg/mL), covered, and left overnight at 4°C.
The plates could be kept for at least two weeks at 4°C without loss of reactivity. Prior to use, the plates were washed three times in washing buffer (1× PBS containing 0.05% Tween 20). The nested PCR products were diluted 1:10 in water in a 96-well PCR plate, denatured at 95°C for 5 minutes, and thereafter cooled to 4°C, until used. One hundred microliters of cold dilution buffer (1× PBS with 0.05% Tween 20) and 2 µL of the diluted PCR products were then added to each well of the ELISA plate. Replicate ELISA plates were made to enable simultaneous probing with sequence specific oligonucleotide probes (SSOPs) targeting the full panel of \textit{dhfr}, \textit{dhps}, and \textit{pfcrt} SNP/haplotypes. The plates were incubated at room temperature for one hour and washed three times in washing buffer (1× PBS with 0.05% Tween 20). The 3´-end digoxigenin- conjugated SSOPs (MWG Biotech) were diluted in tetramethyl ammonium chloride (TMAC; Sigma Aldrich Chemie, Seelze, Germany) solution (3 M TMAC, 50 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 2 mM EDTA, pH 8.0), heated to 53°C, and 100 µL was then added to each well at the concentrations indicated in Table 2.2. The plates were then incubated in a hybridization oven (AH Diagnostics, Aarhus, Denmark) at 53°C on a shaking device for one hour and washed three times in washing buffer. This was followed by two rounds of washing and incubation (12 minutes per round) in TMAC solution at the temperatures indicated in Table 2.2. To remove TMAC, the plates were then washed three times in washing buffer and peroxidase-conjugated anti-digoxigenin antibody in dilution buffer (1:1,000) (Roche Diagnostics, Mannheim, Germany) was added to each well. After incubation for one hour at room temperature, the plates were washed three times in washing buffer and an o-phenylenediamine solution of 1.5 mg/mL of 1,2-phenyldiamine dihydrochloride (Dako, Glostrup, Denmark) dissolved in water containing 0.015% H$_2$O$_2$ was added to the plates. After 30 minutes, the reaction was stopped by adding 1.25 M H$_2$SO$_4$ and the optical density (OD) at 492 nm was measured in an ELISA reader. A flow chart of the method is shown in Figure 2.3.
**SSOP-ELISA PROCEDURE**

Nested PCR product, diluted 1:10

\[ \downarrow \]

Denature (95°C) 5 min
Cool (4°C) until use

\[ \downarrow \]

Add PCR product to
Streptavidin coated ELISA plates
Incubate 1 h, room temp

\[ \downarrow \]

Add SNP probes in TMAC
Incubate 1 h, 53°C

\[ \downarrow \]

3 x wash

\[ \downarrow \]

High stringency TMAC wash
2x12 min, at temperatures stated
in table 1

\[ \downarrow \]

3 x wash

\[ \downarrow \]

Add anti-DIG-antibody
Incubate 1 h, room temp

\[ \downarrow \]

3 x wash

\[ \downarrow \]

Add OPD-solution
Incubate ½ h, room temp

\[ \downarrow \]

Add H₂SO₄

---

**Figure 2.3:** Flow chart of the dihydrofolate reductase, dihydropteroate synthase, and *Plasmodium falciparum* chloroquine resistance transporter sequence-specific oligonucleotide probe–enzymelinked immunosorbent assay (SSOP-ELISA) method.
TABLE 2.2: Distribution of the probes used for the *dhfr, dhps*, and *pfcrt* ELISA haplotyping assay* SSOP sequence† Probe

<table>
<thead>
<tr>
<th>SSOP</th>
<th>SSOP sequence†</th>
<th>Probe Conct.‡</th>
<th>Washing Temp.§</th>
<th>Isolate(s) ¶</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dhfr</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50/51CN</td>
<td>TGG AAA TGT AAT TCC CTA</td>
<td>nM 20</td>
<td>58</td>
<td>3D7, FCR3, K1</td>
</tr>
<tr>
<td>50/51CI</td>
<td>TGG AAA TGT ATT TCC CTA</td>
<td></td>
<td></td>
<td>DD2</td>
</tr>
<tr>
<td>50/51CN2</td>
<td>TGG AAA TGT AAC TCC CTA</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>50/51RN</td>
<td>TGG AAA CGT AAT TCC CTA</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>50/51RN2</td>
<td>TGG AAA CGT AAC TCC CTA</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>50/51RI</td>
<td>TGG AAA CGT ATT TCC CTA</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>59C</td>
<td>AAT ATT TTT GTG CAG TTA</td>
<td>4</td>
<td>60</td>
<td>3D7, FCR3</td>
</tr>
<tr>
<td>59R</td>
<td>AAT ATT TTT GTG CAG TTA</td>
<td></td>
<td></td>
<td>DD2, K1</td>
</tr>
<tr>
<td>108S</td>
<td>A AGA ACA AGC TGG GAA AG</td>
<td>4</td>
<td>62</td>
<td>3D7</td>
</tr>
<tr>
<td>108N</td>
<td>A AGA ACA AAC TGG GAA AG</td>
<td></td>
<td></td>
<td>DD2, K1</td>
</tr>
<tr>
<td>108T</td>
<td>A AGA ACA ACC TGG GAA AG</td>
<td></td>
<td></td>
<td>FCR3</td>
</tr>
<tr>
<td>164I</td>
<td>GT TTT ATT ATA GGA GGT T</td>
<td>4</td>
<td>60</td>
<td>–</td>
</tr>
<tr>
<td>164L</td>
<td>GT TTT ATT TTA GGA GGT T</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td><strong>dhps</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>436/437AA</td>
<td>GAA TCC GCT GCT CCT TTT</td>
<td>4</td>
<td>60</td>
<td>DK1</td>
</tr>
<tr>
<td>436/437AG</td>
<td>GAA TCC GGT GCT CCT TTT</td>
<td></td>
<td></td>
<td>DK4</td>
</tr>
<tr>
<td>436/437SA</td>
<td>GAA TCC TCT GCT CCT TTT</td>
<td></td>
<td></td>
<td>FCR3</td>
</tr>
<tr>
<td>436/437SG</td>
<td>GAA TCC TCT GGT CCT TTT</td>
<td></td>
<td></td>
<td>3D7, K1</td>
</tr>
<tr>
<td>436/437FA</td>
<td>GAA TCC TTG GCT CCT TTT</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>436/437FG</td>
<td>GAA TCC TTG GGT CCT TTT</td>
<td></td>
<td></td>
<td>DD2</td>
</tr>
<tr>
<td>436/437CA</td>
<td>GAA TCC TGT GCT CCT TTT</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>540K</td>
<td>ACA ATG GAT AAA CTA ACA</td>
<td>4</td>
<td>60</td>
<td>3D7, FCR3, DD2, K1, DK1, DK4, SP70</td>
</tr>
<tr>
<td>540E</td>
<td>ACA ATG GAT GAA CTA ACA</td>
<td></td>
<td></td>
<td>SP70</td>
</tr>
<tr>
<td>581A</td>
<td>A GGA TTT GCC GGC AAA CA</td>
<td>4</td>
<td>60</td>
<td>3D7, FCR3, DD2, DK1, DK4, SP70</td>
</tr>
<tr>
<td>581G</td>
<td>A GGA TTT GGG GAC AAA CA</td>
<td></td>
<td></td>
<td>K1</td>
</tr>
<tr>
<td>613A</td>
<td>GA TTT ATT GCC CAT TGC</td>
<td>4</td>
<td>60</td>
<td>3D7, FCR3, K1, DK1, DK4, SP70</td>
</tr>
<tr>
<td>613S</td>
<td>GA TTT ATT TCC CAT TGC</td>
<td></td>
<td></td>
<td>DD2</td>
</tr>
<tr>
<td>613T</td>
<td>GA TTT ATT ACC CAT TGC</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td><strong>Pfcrt c72-76</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVMNK</td>
<td>TA TGT GTA ATG AAT AAAA</td>
<td>4</td>
<td>60</td>
<td>3D7</td>
</tr>
<tr>
<td>CVIET</td>
<td>TA TGT GTA ATT GAA ACA A</td>
<td></td>
<td></td>
<td>FCR3</td>
</tr>
<tr>
<td>SVMNT</td>
<td>TA AGT GTA ATG AAT ACA A</td>
<td></td>
<td></td>
<td>7G8</td>
</tr>
<tr>
<td>CVIEK</td>
<td>TA TGT GTA ATT GAA AAA A</td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>S-VMNT</td>
<td>TA TCT GTA ATG AAT ACA A</td>
<td></td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>
2.10 Measurement of Antibodies against MSP Antigens by ELISA:

The measurement of antibodies (Abs) against MSP1 antigens (Ags) in some of the samples used in this study was done. However, hereunder there was a brief description for the methodology.

Anti-MSP1 antibodies were measured by indirect enzyme linked immunosorbent assay (ELISA), using a protocol described by Cavanagh et al.\textsuperscript{277}. In brief, 96-well, microtitre plates were coated at 4°C for 3 days with MSP1\textsubscript{19} (fused with Glutathione-S Transferase, GST) or with GST (50ng, pH 9.4). Plates were washed with washing buffer (PBS-Tween), and were blocked by skimmed milk. One hundred µl of diluted plasma (1:500) was added in duplicates to coated plates and the latter incubated overnight at 4°C. After washings, plates were incubated sequentially with; 100µl horseradish peroxidase-conjugated rabbit antihuman IgG Abs (1:5000 dilution) and 100µl substrate buffer, then the reaction was stopped with 2M H\textsubscript{2}SO\textsubscript{4}. The optical density (OD) was measured using an ELISA reader at 492nm, and the mean value of each pair of wells (after subtraction of that of GST), was calculated. A pool of adult African hyperimmune sera and sera obtained from 17 Danish blood donors known not to have been exposed to malaria, were used as positive and negative controls in each plate. The same steps were followed for measurement of Abs against MSP2 Ags, GF and T9\textsuperscript{290}. The cut-off level for positive plasma was considered as the mean plus 3xSD of OD readings of the Danish blood donors. The OD readings of serial dilution of the hyperimmune sera were used as a reference for grading of Ab levels into; high, intermediate and low levels.

2.11 Statistical Analysis:

For statistical analysis we used the Statistical Package for Social Sciences (SPSS) software. For comparison of groups, first the distribution of data was tested for normality by descriptive statistics, normally distributed data analyzed by independent samples T-test, and when normal distribution was violated data transformed by computing, and then analyzed. Uncorrected distributions were analyzed using non-parametric tests. Kruskal-Wallis test was used for comparison between clinical subgroups of severe malaria. The quoted levels of significance allow for post-hoc testing using Scheffe's method (comparison of age, hemoglobin and blood glucose level between individual complications) or the Least Significant Difference (LSD) method (parasite count between individual complications).

SigmaStat software was used also for the statistical analysis. For example when comparing between any two sub-groups we used Mann Whitney Rank Sum Test. The Fisher Exact Test
was used for comparison of proportions of antibody responders and prevalence of MSP alleles between the study groups.

Chapter Three

Results

3.1 Severe malaria:

3.1.1 Gedarif Hospital, 2000-2002

3.1.1.1 Clinical pattern of severe *Plasmodium falciparum* malaria:

3.1.1.1.1 The frequency of acute uncomplicated and complicated malaria:
The total number of patients clinically suspected to have malaria and for whom blood smears were taken and examined was 16606; among them 2488 patients (15%) were found to be parasitemic (Table 3.1). *P. falciparum* was the causative agent in 98.88% (2460/2488), while *P. vivax* accounted for 0.64% (16/2488), *P. malariae* 0.44% (11/2488) and *P. ovale* 0.04% (1/2488) of all malaria infections. No mixed infections were detected. Based on the clinician’s decision 252 malaria patients were hospitalized, and 110 (4.4%) fulfilled the WHO criteria for severe malaria were included in the study. The remaining hospitalized patients were admitted, because they had early treatment failure (chloroquine or fansidar), were vomiting or prostrated. The mean admission period was 2.8 days (min 0.5, max 4). Seven patients died, all had cerebral malaria. The mortality rate was 0.3% of all malaria infections.

**Table 3.1:** Clinical categorization of patients presented with malaria-like symptoms to the malaria clinic at Gedarif Hospital

<table>
<thead>
<tr>
<th>All Patients</th>
<th>Rate of malaria infection</th>
<th>Uncomplicated malaria</th>
<th>Proportion of severe malaria</th>
<th>Severe malaria death rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>16606</strong></td>
<td>15% (2488/16606)</td>
<td>95.6% (2378/2488)</td>
<td>4.4% (110/2488)</td>
<td>6.4% (7/110)</td>
</tr>
</tbody>
</table>

3.1.1.1.2 The frequency of the different types of severe complications:

The WHO criteria for severe malaria (SM) were used to classify malaria patients as uncomplicated and severe. The clinical presentation of patients categorized as having SM is shown in figure 3.1. Severe malarial anemia (SMA) was the most commonly recognized complication as it accounted for 45.46% of all severe malaria cases. Cerebral malaria was found in 16.36% of the patients. The rest had convulsions (20.91%), hypotension (11.82%), or a mixture of above manifestations (5.45%). There was a tendency that a higher percentage of patients had anemia during the second malaria season, but the difference between clinical presentations in the two years was not statistically significant.
Fig. 3.1: Symptoms in the 110 patients admitted to Gedarif Hospital, and fulfilling the WHO criteria for severe malaria, the patients were admitted over a period of two years.

The six patients who had more than one type of complication were; two patients with cerebral malaria were also anemic, while four severely anemic patients also presented with convulsions. Other complications, like respiratory distress syndrome, renal failure, and bleeding abnormalities (thrombocytopenia) were not recognized during the study. There was no difference in the clinical presentations of females and males.

3.1.1.1.3 Comparison between the age distribution of severe and mild malaria:

Uncomplicated and severe malaria affected both children and adults, while small children between two and four years constituted a much higher percentage among patients with severe malaria compared to patients with uncomplicated disease. Of the 110 severe malaria patients
27 (14 females and 13 males) were above 12 years of age. The incidence of severe malaria in males and females was (1:1) (figure 3.2).

![Diagram showing age distribution of patients with severe malaria and uncomplicated malaria in Gedarif Hospital (period 2000 – 2002).](image)

**Figure 3.2:** The age distribution of patients with severe malaria and uncomplicated malaria in Gedarif Hospital (period 2000 – 2002).

### 3.1.1.1.4 Characterisation of patients in the different categories of severe malaria:

The mean age varied markedly between the different categories of patients with severe malaria. Patients with anemia or convulsions were infants and small children, patients with cerebral malaria were older children and young adults, whereas hypotension was found in the adults (figure 3.3a).
Figure 3.3: Comparison between four groups of patients with severe malaria

The mean parasite count was highest in patients with convulsions and in this group the parasitemia was significantly higher than in patients with hypotension or anemia (figure
3.3b). The group of patients with severe anemia by definition had low levels of haemoglobin, but the other groups did differ with respect to this parameter (figure 3.3c). The mean blood glucose levels at diagnosis and before treatment, was higher in patients with cerebral malaria than in patients with anemia (figure 3.3d), but did not differ significantly between the other groups. The white blood cell (WBC) count was highest in patients with cerebral malaria (6876.5 ± 4556, mean ± SD), but the differences between the groups were not significant.

### 3.1.1.1.5 Comparison between fatal and non-fatal severe malaria:

Seven patients with cerebral malaria died despite treatment. The characteristics of these patients are shown in (Table 3.2). Two of the patients were adults and the remaining were children between 5-11 years.

**Table 3.2:** Shows levels of variable blood parameters in individuals who died of severe malaria, and comparison of the mean values of these parameters between fatal and non-fatal severe malaria groups

<table>
<thead>
<tr>
<th>Age (years) / sex</th>
<th>Symptoms duration (BA)</th>
<th>Parasite count (parasite/µl)</th>
<th>Hb level (%)</th>
<th>TWBC (cell/µl)</th>
<th>Blood glucose (mg/dl)</th>
<th>Insulin level (mIU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/male</td>
<td>3 days</td>
<td>4350</td>
<td>ND</td>
<td>ND</td>
<td>219.6</td>
<td>32.6</td>
</tr>
<tr>
<td>35/male</td>
<td>UK</td>
<td>180000</td>
<td>92</td>
<td>1900</td>
<td>122.0</td>
<td>9.0</td>
</tr>
<tr>
<td>09/female*</td>
<td>4 days</td>
<td>96000</td>
<td>60</td>
<td>4000</td>
<td>246.6</td>
<td>20.3</td>
</tr>
<tr>
<td>05/male</td>
<td>3 days</td>
<td>114000</td>
<td>65</td>
<td>11600</td>
<td>102.6</td>
<td>4.1</td>
</tr>
<tr>
<td>55/male</td>
<td>5 days</td>
<td>4800</td>
<td>46</td>
<td>4600</td>
<td>311.0</td>
<td>25.0</td>
</tr>
<tr>
<td>10/male</td>
<td>7 days</td>
<td>3150</td>
<td>ND</td>
<td>3500</td>
<td>149.4</td>
<td>ND</td>
</tr>
<tr>
<td>06/female</td>
<td>2 days</td>
<td>42000</td>
<td>40</td>
<td>11000</td>
<td>111.6</td>
<td>ND</td>
</tr>
<tr>
<td>R1 18.7</td>
<td></td>
<td>63471±</td>
<td>60.6±</td>
<td>6100±</td>
<td>180.4±</td>
<td>18.2±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68590</td>
<td>20.3</td>
<td>4131</td>
<td>79.7</td>
<td>11.6</td>
</tr>
<tr>
<td>RII 10.5</td>
<td></td>
<td>37940±</td>
<td>42.18±</td>
<td>5790±</td>
<td>94.3±</td>
<td>13.8±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66741</td>
<td>18.3</td>
<td>2962</td>
<td>29.9</td>
<td>12.7</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.226</td>
<td>0.035</td>
<td>0.885</td>
<td>0.000</td>
<td>0.406</td>
</tr>
</tbody>
</table>

The duration of symptoms before admission varied between two to seven days. The mean (±SD) hemoglobin and the mean blood glucose levels were higher in patients who died of severe malaria than in the survivors (p=0.035 and p<0.001, respectively, T-test).
The difference in glucose levels were not reflected in the insulin levels, which were comparable between the groups, although insulin level in fatal malaria was above the normal physiological range (4.0 to 16.8 mIU/L).

3.1.1.2 Allelic polymorphism of MSP2 gene in severe *P. falciparum* malaria:

3.1.1.2.1 Clinical groups and samples:

A total of 616 blood samples were obtained from 325 donors; 116 were apparently healthy malaria-free (MF) volunteers, 106 patients with UM and 103 patients with SM. The patients with SM were had; SMA (48), CAM (23), CM (17), HTN (9) or multiple complications (6). From patients with SM blood samples were taken at D0 (n=103), D3 (n=97), D7 (n=97) and D28 (n=97). All blood samples were screened by PCR for *P. falciparum* parasite detection and genotyping into IC1 and FC27 families of MSP2 locus. At the time of malaria diagnosis and before treatment (D0), the parasite DNA was detected in all samples obtained from malaria patients (UM and SM) and 22 of the MF donors (n=231), see Table (3.3). The group of MF donors who had PCR detectable parasitemia (n=22) in fact had asymptomatic sub-microscopic (ASUM) infection. The number of clones in the bloods obtained at diagnosis and before treatment (D0) in individual infections was not correlated with the initial parasite count (CC=0.051, P=0.48, Pearson Product Moment Correlation). Similarly, when we correlated the clone number in individual infections with age in all study groups (SM, UM, and MF) there was no correlation neither (CC=-0.016, p=0.818, Pearson Product Moment Correlation).

3.1.1.2.2 Prevalence of single clone and multi-clone malaria infections:

As seen in Table 3.3, the single clone infections in SM accounts for 61.2% [63/103], which was not significantly different from UM, 54.7% [56/106], neither in the frequency nor in the genotypes (IC1, 45.6%; FC27, 15.5%) and (IC1, 33%; FC27, 21.7%), respectively. In all clinical malaria infections (UM and SM) the multi-clone infections (MCI) accounted for 42.1% (88/209). The MCI in SM (38.8%, 40/103) and UM (45.3%, 48/106) was similar and basically of two types; either composed of clones of one allele family, IC1 or FC27 (homogenic), or of both allele families, IC1 and FC27 (heterogenic). Interestingly, while only 6 SM (5 IC1 and 1 FC27) and 7 UM (5 IC1 and 2 FC27) infections were homogenic, 34 SM and 41 UM infections, were heterogenic.

Table 3.3: The frequency of single clone and multi-clone infections in patients with severe malaria (SM), uncomplicated malaria (UM) and malaria free donors (MF)
<table>
<thead>
<tr>
<th>Donors</th>
<th>Single clone infection</th>
<th>Multi-clone infection</th>
<th>Total CN IC1/FC27</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC1</td>
<td>FC27</td>
<td>HO-MTCI IC1</td>
</tr>
<tr>
<td>SM (n=103)</td>
<td>47 (45.6%)</td>
<td>16 (15.5%)</td>
<td>6</td>
</tr>
<tr>
<td>UM (n=106)</td>
<td>35 (33%)</td>
<td>23 (21.7%)</td>
<td>7</td>
</tr>
<tr>
<td>MF (n=22)</td>
<td>7 (31.8%)</td>
<td>15 (68.2%)</td>
<td>0</td>
</tr>
<tr>
<td>Total (231)</td>
<td>89</td>
<td>54</td>
<td>13</td>
</tr>
</tbody>
</table>

The proportion of homogenic infections was significantly lower than that of heterogenic infection (P=0.009, Fisher Exact Test) whether or not SM and UM were considered together. The maximum number of clones in an individual infection was four in SM and five in UM.

### 3.1.1.2.3 Multiplicity of infection (MOI) in different clinical grades of malaria infection:

The total number of clones of parasites isolated from the 231 *P. falciparum* infected individuals at D0 was 339, giving an overall MOI of 1.5 (339/231). The MOI in parasite carriers with ASUM was 1.0, which was significantly lower than in patients with clinical malaria infection (UM and SM) who had MOI of 1.52 (P=0.001, Mann-Whitney Rank Sum Test). However, when we compared the MOI in UM (1.53) and SM (1.5) patients, the difference was not significant. Furthermore, when the individual complications of SM were considered separately, there was no significant difference in MOI between patients with HTN (1.56), SMA (1.52), CAM (1.52) and CM (1.47) (Table 3.4). But the MOI was significantly higher in patients with SMA and non-fatal CM (1.7) when compared with patients with
ASUM infection. Patients died of SM (all had CM, fatal-CM, [n=7]), had less complex infections, with MOI of 1.14.

**Table 3.4:** Mean clone number and the frequency of infection with FC27 allele family alone in; MF donors (asymptomatic-sub-microscopic infection) and individual complications of SM, including fatal and non-fatal CM.

<table>
<thead>
<tr>
<th>Molecular Parameters</th>
<th>ASUM Malaria (n=22)</th>
<th>CM</th>
<th>SMA (n=48)</th>
<th>CAM (n=23)</th>
<th>HTN (n=9)</th>
<th>UM (n=106)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOI (range)</td>
<td>I</td>
<td>1.14</td>
<td>1.70*</td>
<td>1.52*</td>
<td>1.56</td>
<td>1.53*</td>
</tr>
<tr>
<td></td>
<td>(1 – 1)</td>
<td>(1 – 2)</td>
<td>(1 – 4)</td>
<td>(1 – 3)</td>
<td>(1 – 4)</td>
<td>(1 – 5)</td>
</tr>
<tr>
<td>FC27 only (frequency)</td>
<td>68.2%</td>
<td>00%</td>
<td>20%</td>
<td>20.1%</td>
<td>8.7%</td>
<td>22.2%</td>
</tr>
</tbody>
</table>

**3.1.1.2.4 The frequency of IC1 and FC27 genotype families and allele size in different types of malaria infection:**

Based on MSP2 locus analysis, the parasites were either belonging to IC1 or FC27 allele family. The overall frequency of IC1 and FC27 alleles in all malaria infections was; 57.2% (194/339) and 42.8% (145/339), respectively.

However, within each allelic family there were considerable degree of polymorphism based on allele size, the later was measured by the number of base-pairs (bp) in each allele. Accordingly, we found that the total number of alleles in the IC1 and FC27 families were 30 and 23 allele types, respectively.
Figure (3.4) The MSP2 allele-size polymorphism in uncomplicated (black circles) and severe (open circles) malaria, each circle points to the number of parasites with specific allele size

Not only the number of IC1 alleles in UM (24 type) and SM (26 type) were similar but also the frequency of the different allele sizes were comparable, (CC=0.594, p<0.001, Pearson Product Moment Correlation), (Figure 3.4A). Although, the number and types of FC27 alleles were less common in SM (n=57, types=13) compared with UM (n=73, types=23), their distribution was comparable between SM and UM (CC=0.718, p<0.001, Pearson Product Moment Correlation, see Figure (3.4B). The most commonly recognized alleles, based on size typing, were IC1-420-bp and FC27-420-bp in both SM (18 & 14 times, respectively) and
UM (14 & 9 times, respectively), See Figure (3.4). The prevalence of the genotype FC27 alone as a cause of infection was similar in patients with UM (23.6%, 25/106) and SM (16.5%, 17/103), and between the individual complications of SM; CAM (8.7%), CM (11.8%) SMA (20.1%) and HTN (22.2%), see Table (3.4). However, in patients with ASUM infection the prevalence of FC27 infection was 68.2% (15/22), while no patient died of CM (fatal-CM) carried the FC27 genotype as a single clone (0/7), the prevalence of FC27 in the two groups was significantly different (P=0.012, Fisher Exact Test). Only one fatal-CM infection was found to be IC1/FC27 mixed infection.

3.1.1.2.5 Dynamic of parasite clone numbers and types in persistent SM infections after quinine treatment:

After quinine treatment, twelve patients with SM had persistent parasitemia, the later was revealed by PCR after analysis of blood samples collected during the days of follow up (D0, D3, D7 and D28). All forty eight parasite DNA samples were genotyped. Seven patients were found to have had an initial single clone infection, 3 patients had 2 clones and 2 patients had 3 clones. As seen in Fig. (3.5), there was marked vertical heterogeneity indicating the genetic diversity of SM parasites that were resistant to quinine treatment in this sub-group of patients, only 2 out of 12 (16.7%) patients had similar parasite genotypes (IC1-330) at diagnosis (D0). However, there was a clear horizontal homogeneity, 3 patients had novel genotype at D28, while only 1 patient had carried new genotype in D7, but no other patient had novel genotype in the other follow up days. None of the patients infected with more than a single clone had maintained all the infecting clones during the follow up, they all had only one clone at D28. Generally, only 6 out of 39 (15.4%) clones identified during the follow up of this subgroup of SM patients were found to be novel (were not detected at diagnosis, D0).
<table>
<thead>
<tr>
<th>Did</th>
<th>Clone No</th>
<th>Allele type at diagnosis</th>
<th>D3-allele</th>
<th>D7-allele</th>
<th>D28-allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG038</td>
<td>1</td>
<td>FC27-350</td>
<td>FC27-350</td>
<td>FC27-350</td>
<td>FC27-700</td>
</tr>
<tr>
<td>SG042</td>
<td>1</td>
<td>IC1-390</td>
<td>IC1-390</td>
<td>IC1-390</td>
<td>IC1-390</td>
</tr>
<tr>
<td>SG101</td>
<td>1</td>
<td>IC1-330</td>
<td>IC1-330</td>
<td>IC1-330</td>
<td>IC1-330</td>
</tr>
<tr>
<td>SG096</td>
<td>1</td>
<td>IC1-330</td>
<td>IC1-330</td>
<td>IC1-330</td>
<td>IC1-330</td>
</tr>
<tr>
<td>SG098</td>
<td>1</td>
<td>FC27-480</td>
<td>FC27-480</td>
<td>FC27-480</td>
<td>FC27-480</td>
</tr>
<tr>
<td>SG104</td>
<td>1</td>
<td>FC27-420</td>
<td>FC27-420</td>
<td>FC27-420</td>
<td>FC27-420</td>
</tr>
<tr>
<td>SG047</td>
<td>1</td>
<td>FC27-517</td>
<td>FC27-517</td>
<td>FC27-580</td>
<td>FC27-517</td>
</tr>
<tr>
<td>SG037</td>
<td>2</td>
<td>IC1-340</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FC27-517</td>
<td>FC27-517</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG066</td>
<td>2</td>
<td>IC1-420</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FC27-650</td>
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<td>FC27-580</td>
<td>FC27-580</td>
</tr>
<tr>
<td>SG95</td>
<td>2</td>
<td>IC1-420</td>
<td>IC1-420</td>
<td>IC1-420</td>
<td>IC1-420</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FC27-450</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG010</td>
<td>3</td>
<td>IC1-350</td>
<td>IC1-350</td>
<td>IC1-350</td>
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<tr>
<td></td>
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<td></td>
<td>IC1-550</td>
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<td>FC27-517</td>
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</tr>
<tr>
<td>SG065</td>
<td>3</td>
<td>IC1-430</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>IC1-520</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FC27-420</td>
<td>FC27-420</td>
<td>FC27-420</td>
<td>FC27-420</td>
</tr>
</tbody>
</table>

**Figure (3.5):** The genetic diversity of infection in different severe malaria patients at the time of malaria diagnosis

**3.1.1.2.6 Differential clearance of IC1 genotype in multi-clone SM infections after treatment:**

The total number of SM patients who had heterogenic multi-clonal infection (mixed IC1/FC27) at the time of malaria diagnosis was 34 patients (33%). After initiation of quinine treatment, significantly more parasites of IC1 (28/34) genotype were cleared compared to the FC27 (15/34) parasites at D3, P=0.002, Fisher Exact Test (fig. 3.6). The same trend was also recognized in the other follow up days, where the prevalence of FC27 exceed that of IC1 at D7 (12/34 and 4/34, respectively) and D28 (8/34 and 4/34, respectively), P= 0.026 and P=0.34, respectively.
Figure (3.6): The differential and faster clearance of IC1 genotypes in multi-clone infections with IC1 and FC27 parasite clones, in SM patients.

3.1.1.3 Genetic fingerprints of parasites causing severe malaria in a setting of low transmission “one strain one patient”:

3.1.1.3.1 Clinical data:

Among the 943 microscopically confirmed clinical *P. falciparum* malaria infections, only 53 (5.6%) patients were found to have severe malaria, these included 11 (1.2%) patients with cerebral malaria, and among the later 5 (0.5%) patients died. Other severe malaria patients had; convulsions (n=14), severe malaria anemia (n=14), hypotension (n=9) and multiple complications (n=5), the details were presented before. From the patients with SM, parasite DNA was obtained from 47 patients.

3.1.1.3.2 Diversity of infection (DOI):

The diversity of infection (DOI), is the parameter for quantitative estimation of the parasite genetic variations, it is based on our hypothesis that; each infection is caused by a parasite of a unique genetic make up; however; the DOI value depends on the molecular marker used. The maximal number of parasite genotypes which can be detected by using any of the four molecular markers; the *pfcrt*K76T, *pfmdr1*N86Y, *GLURP* allele size, *MSP2* family types and allele sizes were; 3, 3, OPEN (depends on number of test samples), 3 and OPEN, respectively. Accordingly, the maximal DOI which can be obtained if we used any of the
above molecular markers would be; 0.064 (3/47) for $pfcrt$, $pfmdr1$ or MSP2 allele families, while if we used GLURP or MSP2 allele size typing, the maximal DOI should be 1.00 (47/47), see Table (3.5).

**Table (3.5):** Estimation of the diversity of infection in severe and uncomplicated malaria, by using different molecular markers ($pfcrt$, $pfmdr1$, msp2 and glurp) and by compiling all markers together

<table>
<thead>
<tr>
<th>Molecular markers (MM)</th>
<th>Diversity of infection (DOI) in clinical malaria</th>
<th>Max. No of Allele-type for each MM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diversity of infection (DOI) in clinical malaria</td>
<td>Max. No of Allele-type for each MM</td>
</tr>
<tr>
<td></td>
<td>SM</td>
<td>UM</td>
</tr>
<tr>
<td>$pfcrt$ 76</td>
<td>0.043</td>
<td>0.057</td>
</tr>
<tr>
<td>(T, K, T/K)</td>
<td>(2/47)</td>
<td>(3/53)</td>
</tr>
<tr>
<td>$pfmdr1$ 86</td>
<td>0.064</td>
<td>0.058</td>
</tr>
<tr>
<td>(Y, N, Y/N)</td>
<td>(3/47)</td>
<td>(3/52)</td>
</tr>
<tr>
<td>MSP2 families</td>
<td>0.064</td>
<td>0.057</td>
</tr>
<tr>
<td>(IC1, FC27, Mixed)</td>
<td>(3/47)</td>
<td>(3/51)</td>
</tr>
<tr>
<td>MSP2</td>
<td>0.553</td>
<td>0.804</td>
</tr>
<tr>
<td>Allele size</td>
<td>(26/47)</td>
<td>(41/51)</td>
</tr>
<tr>
<td>glurp</td>
<td>0.435</td>
<td>ND</td>
</tr>
<tr>
<td>Allele size</td>
<td>(20/46)</td>
<td>ND</td>
</tr>
<tr>
<td>Overall</td>
<td>0.936</td>
<td>0.868</td>
</tr>
<tr>
<td></td>
<td>(44/47)</td>
<td>(46/53)</td>
</tr>
</tbody>
</table>

3.1.1.3.3 Genetic diversity of parasite isolates in patients with severe malaria, based on use of a single molecular marker:

In the 47 SM parasite isolates, only two $pfcrt$76 (T and K), 3 $pfmdr1$ 86 (N, Y and N/Y), and 3 MSP2 families (IC1, FC27 and IC1/FC27) allele-types were detected. However, the glurp locus analysis revealed 20 allele-types; accounting for (20/46) 43.5% of all SM infections, indicating that at least 43.6% of all infections were precipitated by parasites of unique allele-type. While number of parasites with unique allele-type based on MSP2-allele size typing was 26, giving a DOI of (26/47) 55%, as seen in table (3.5).

3.1.1.3.4 Genetic diversity of parasite isolates obtained from patients with uncomplicated malaria, based on use of a single molecular marker:
For parasite isolates obtained from patients with UM (n=53), 3 allele types were revealed by each of the three markers; \textit{pfcr76} (T, K and T/K), \textit{pfmdr1} 86 (N, Y and N/Y), and \textit{MSP2} families (IC1, FC27 and IC1/FC27), and that was the maximum allele-type could be detected. Interestingly, the allele-size-based \textit{MSP2} typing revealed 41 allele-types in UM, giving a DOI of (41/51) 0.804, which was higher than the DOI in SM when \textit{MSP2} allele size typing was used. The \textit{GLURP} locus analysis was not done for UM samples, and not all samples were analyzed for the other three molecular markers, as seen in Table (3.5).

\textbf{3.1.1.3.5 Genetic diversity of parasite isolates obtained from malaria patients, based on use of multiple molecular markers:}

In severe malaria, the genetic profile of the individual parasite isolates in all tested loci; \textit{pfcr7}, \textit{pfmdr1}, \textit{glurp} and \textit{msp2} (allele family and size), is shown in the figure (3.7). This multi-locus genetic profile of parasites had revealed an extreme diversity between the isolates, DOI of 0.936 (44/47), which was statistically significantly higher compared with the DOI when any of the individual molecular markers was used alone (range 3/47 to 26/47), P value < 0.001, Fisher’s exact test, see Table (3.5) and the Figure (3.7). Although, \textit{glurp} analysis was not carried out for parasite isolates obtained from patients with uncomplicated malaria, the multi-locus genetic profile revealed a large degree of diversity, DOI 0.902, which was also significantly higher than the DOI for individual markers (range 3/53 to 41/51)(P=<0.001 to 0.269, Fisher’s exact test), see Table (3.5) and figure (3.7).
Figure (3.7): The multi-locus genetic profiles of parasites isolates from patients with severe malaria.

<table>
<thead>
<tr>
<th>MSP2 families</th>
<th>FC27</th>
<th>IC1/FC27</th>
<th>IC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfcrt allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pfcrt76K (W)</td>
<td>0.19</td>
<td>[9]</td>
<td></td>
</tr>
<tr>
<td>Pfcrt176T (M)</td>
<td>0.81</td>
<td>[38]</td>
<td></td>
</tr>
<tr>
<td>Pfmdr1 alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pfmdr186N (V)</td>
<td>0.255</td>
<td>[12]</td>
<td></td>
</tr>
<tr>
<td>Pfmdr186Y (A)</td>
<td>0.723</td>
<td>[54]</td>
<td></td>
</tr>
</tbody>
</table>

W/M 0.213 [1]
3.1.1.3.6 Homogeneity of parasite isolates in patients with severe malaria:

In figure (3.7), three pairs of patients were found to carry parasites of identical multi-locus genetic profile within each pair which differ between the pairs; (SG19 & SG20), (SG25 & SG42) and (SG49 & SG51). The history, clinical description and laboratory data of these patients shown in table (3.6). Depending on this data, the probability that the parasites of each pair will remain identical if genetic profile testing was extended to involve more genes is high for one pair (SG19 & SG20), intermediate for another pair (SG25 & SG42) and unlikely for the third pair (SG49 & SG51).

Table (3.6): Characteristics of three pairs of patients infected with parasites of the same multi-locus genetic profile in each pair, but differ between pairs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pair 1</th>
<th>Pair 2</th>
<th>Pair 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complication</td>
<td>SMA plus</td>
<td>SMA</td>
<td>SMA</td>
</tr>
<tr>
<td>Gender</td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Age</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Ethnic group</td>
<td>The same</td>
<td>The same</td>
<td>Different</td>
</tr>
<tr>
<td>Village</td>
<td>The same</td>
<td>The same</td>
<td>The same</td>
</tr>
<tr>
<td>House</td>
<td>The same</td>
<td>The same</td>
<td>Different</td>
</tr>
<tr>
<td>Hemoglobin (%)</td>
<td>30%</td>
<td>35%</td>
<td>55%</td>
</tr>
<tr>
<td>TWBC</td>
<td>5100</td>
<td>4000</td>
<td>3600</td>
</tr>
<tr>
<td>Parasite count</td>
<td>144600</td>
<td>108600</td>
<td>8040</td>
</tr>
<tr>
<td>Ab response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP1&lt;sub&gt;19&lt;/sub&gt;</td>
<td>0.610</td>
<td>0.721</td>
<td>0.65</td>
</tr>
<tr>
<td>MSP2&lt;sub&gt;A&lt;/sub&gt;</td>
<td>0.547</td>
<td>0.717</td>
<td>0.33</td>
</tr>
<tr>
<td>MSP2&lt;sub&gt;B&lt;/sub&gt;</td>
<td>0.849</td>
<td>0.074 (-ve)</td>
<td>ND</td>
</tr>
</tbody>
</table>

In the first pair, the patients are brothers, close in age and both brought to the hospital at the same time, both had SMA, admitted and discharged from the hospital on the same dates. For
the second pair of patients, they were of the same ethnic group, living in the same quarter (may be neighbors) and most probably they were relatives and may be living in the same house. In the third pair the patients different in most of the available data, but they were living in the same town and may be the same quarter.

3.1.2 Severe malaria in New-Halfa Hospital (November 2000 to February 2001):

3.1.2.1 Clinical and parasitological categorization of patients:
From the patients seen in the malaria clinic, 120 individuals were recruited to the study. Three study groups were identified based on clinical and parasitological data; 52 febrile patients were clinically suspected to have malaria but were found to have negative blood smears, 40 febrile patients with microscopically confirmed malaria and the 28 apparently malaria free (MF) donors (Table 3.7). In the former two groups of patients there were three clinically diverse subgroups of patients; patients with or suspected to have uncomplicated malaria (UM), severe malarial anemia (SMA) or cerebral malaria (CM).

Table 3.7: Number of individuals, number of patients with coma and mortality rate of comatose patients in the three study groups

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Confirmed Malaria (+ve blood smear)</th>
<th>CS Malaria (-ve blood smear)</th>
<th>Apparently healthy (MF donors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of individuals</td>
<td>40 (27/13)</td>
<td>52 (24/28)</td>
<td>28 (17/11)</td>
</tr>
<tr>
<td>(male/female ratio)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of comatose Patients</td>
<td>10</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Mortality among comatose patients</td>
<td>20% (2/10)</td>
<td>20.8% (5/24)</td>
<td>0% (0/28)</td>
</tr>
</tbody>
</table>

The cerebral malaria patients: Among the clinically suspected group of malaria patients, 24 patients were brought to hospital in coma, suspected to have cerebral malaria (CS CM), the microscopic examinations failed to confirm the clinical diagnosis. The age of CS CM patients (age data is available for 21 out of 24 patients) fall between 1 and 70 years included one child, 3 adolescents and the others were adults, with an overall mean ± (SD) age of 31.2 ±
21.8 years. Half of the patients were males. All the comatose patients were treated initially with quinine infusion; in addition injectable antibiotics were given to two patients. Nineteen patients (79.2%) fully recovered, and five (20.8%) patients died (mean age was 46.2 ± 22.4 years).

The other clinically diagnosed malaria patients with negative blood smear included 14 patients suspected to have severe malarial anemia (CSMA; hemoglobin - mean ± SD of 24.3 ± 4.5%). They had a mean age of 18.6 ± 19.3 (range 1-58) years (age data is available for 9 out of 14 patients). Fourteen patients with a mean age of 4.1 ± 3.4 (range 1-12) years, had symptoms suggestive of uncomplicated malaria (CSUM).

The control groups were patients with microscopically confirmed malaria and malaria-free donors; the former group included 40 patients, 17 had uncomplicated malaria (UM) and 23 patients with severe malaria (10 patients had CM and 13 had SMA). The mean age ± SD (range) of the patients with UM, CM and SMA were; 8.2 ± 9.4 (1-30), 8.4 ± 9.1 (2-33) and 1.6±1.1 (0.3-4). In addition, a group of 28 apparently healthy donors (MF) was included in the study; they had a mean age of 6.8 ± 4.8 (1-21) years [± SD (range)].

The clinically suspected severe malaria group (CSCM and CS SMA) had a mean age much greater than that of the corresponding representative group of the confirmed severe malaria, CM and SMA. However, the mean age for all selected groups, represented the overall age pattern of malaria prevalence in the area16.

3.1.2.2 Parasite prevalence by PCR:

*P. falciparum* parasites were detected by PCR in 17 out of 24 (70.8%) blood samples obtained from the CSUM patients (Table 3.8). All the 5 comatose patients who died had parasitemia revealed by PCR (Fig. 3.7A). For the 14 CSMA, the parasite rate as detected by PCR was 42.8% (6/14). The detection rate of parasites by PCR was 100% for the 14 patients who had negative blood smears and clinical features suggestive of uncomplicated malaria (CSUM). Parasitemia was also detected by PCR in patients with microscopically confirmed SMA, CM and UM, except for one patient with UM (PH08), for whom repeated PCR did not reveal any visible band. On the other hand parasitemia was detected by PCR in only 5 of the 28 asymptomatic (MF) donors (17.8%) and two of them had parasitemia detected also by microscopy (7%). As shown in table 3.6, the differences were statistically significant between the prevalence of parasitemia as detected by PCR between malaria-symptom-free donors (MF) and the other malaria patient categories; UM, CSUM, CM, CSCM and SMA, CSMA
(17.8 % for MF versus 94.1, 100, 100, 100, 70.8 or 42.8, respectively), p < 0.05 for all comparisons.

### 3.1.2.3 Molecular characterization of the parasite isolates:

The predominant sub-patent malaria infections of the comatose patients (CS\textsuperscript{CM}) were single clone infections (82.4%, 14/17), with a mean clonal number (MCN) of 1.23 ± 0.56 while only 50% of the CM infections with patent parasitemia, were single clone infections, with a MCN of 1.70 ± 0.82 (Table 3.8, Fig. 3.8).

**Table 3.8** Parasitemia detected by microscopy and by polymerase chain reaction and the mean number of clones, in different study groups or subgroups in New Halfa area

<table>
<thead>
<tr>
<th>Parasitemia</th>
<th>Patients with +ve BF</th>
<th>Patients with -ve BF</th>
<th>Apparently Healthy, MF Donors (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UM (n=17)</td>
<td>CM (n=10)</td>
<td>SMA (n=13)</td>
</tr>
<tr>
<td>Parasite rate by microscopy</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Parasite rate by PCR</td>
<td>94.1%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Mean clone number</td>
<td>1.69± 0.70</td>
<td>1.70± 0.82</td>
<td>1.62± 0.77</td>
</tr>
</tbody>
</table>

The uncomplicated malaria infections were relatively more commonly multiclonal, both patent UM (56.3%, 9/16; with a MCN of 1.69 ± 0.70), and sub-patent CS\textsuperscript{UM} (71.4%, 10/14; with a MCN of 1.86 ± 0.66). Although there were great variations in clone number between the groups, the mean clonal number was not statistically significantly different between all the groups taken together (p=0.060, Kruskal-Wallis one way analysis of variance on rank). However, when comparing CS\textsuperscript{CM} with CS\textsuperscript{UM} the difference was statistically significant (1.23 ± 0.56 versus 1.86 ± 0.66, p=0.014, Mann Whitney Rank Sum Test). The genotype IC1 was detected in all PCR-detectable infections except in seven patients (7/81). The genotype FC27, was recognized in only 3 infections in CS\textsuperscript{CM} (17.6%, 3/17), while it was recognized in 7/10, 9/14, and 8/17 parasite isolates obtained from CM, CS\textsuperscript{UM} and UM patients, respectively. The
differences were statistically significant when comparing \(^{CS}\)CM to CM or \(^{CS}\)UM, (p=0.013 & 0.012, respectively, Fisher Exact Test).

### 3.1.2.4 Prevalence of antibodies against MSP Antigens:

The prevalence of antibodies against any or all the three MSP fragments (MSP\(_{19}\), MSP\(_{2\text{GF}}\), MSP\(_{2\text{T9}}\)), in plasma collected at diagnosis and before treatment, was calculated for the 3 major groups of donors. Plasma from the \(^{CS}\)SMA subgroup was not obtained.

As seen in figure 3.8, the positivity rate of anti-MSP antibodies was; 84.2% (16/19), 68.4% (26/38), 36.4% (8/22), and 14.3% (2/14) in the groups of the patients; the \(^{CS}\)CM, microscopically confirmed malaria, MF and \(^{CS}\)UM groups, respectively. The difference in the proportion of responders between the \(^{CS}\)CM and microscopically confirmed malaria group (UM & SM) was not significant (p=0.339, Fisher Exact Test). But the difference between \(^{CS}\)CM and MF or \(^{CS}\)UM donors was statistically significant (p=0.004 and 0.001, respectively), as was the difference between the confirmed malaria group and MF or \(^{CS}\)UM (p=0.029 and 0.001, respectively). When considering the clinical subgroups; the Ab prevalence rate in comatose patients, with negative blood smear and negative PCR, was 85.7% (6/7), while it was 100% (5/5) in comatose patients who died thereafter. While the prevalence of anti-MSP1 Abs was low in the MF donors, most of the responders (80%) had the highest level of Abs compared with the other groups as seen in the semi-quantitative estimation of antibody levels (Fig. 3.9A & B).
**Figure 3.9** Comparison between; $^{35}$SM, UM and MF donors. Test parameters are; a. parasite genotype (CN; light shade =1 clone, dark shade=2 clones) b. level of MSP Ab (blank, light, medium or dark shaded boxes for; no response, low, intermediate and high level of Abs, respectively). Panel (A), patients with malaria symptoms ($^{35}$SM and UM); Panel (B), apparently MF donors. ID **bolded** given quinine and benzyl penicillin, ID **italic** given in addition gentamycin

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age year</th>
<th>Genotype</th>
<th>Anti-MSP Ab level</th>
<th>Clinical category</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH01</td>
<td>60</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH02</td>
<td>NK</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH04</td>
<td>35</td>
<td>IC1 FC27</td>
<td>0</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH05</td>
<td>32</td>
<td>IC1 FC27</td>
<td>&amp; CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH06</td>
<td>NK</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH07</td>
<td>NK</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH08</td>
<td>65</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH09</td>
<td>4</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH10</td>
<td>50</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH13</td>
<td>25</td>
<td>IC1 FC27</td>
<td>0</td>
<td>CS CM</td>
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<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
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<td>CH18</td>
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<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>SH13</td>
<td>5.5</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>SH19</td>
<td>26</td>
<td>IC1 FC27</td>
<td>CS CM</td>
<td>CS CM</td>
</tr>
<tr>
<td>CH12</td>
<td>30</td>
<td>IC1 FC27</td>
<td>CS CM died</td>
<td>CS CM</td>
</tr>
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</table>

Blood film & PCR positive +  
" Only PCR positive  
CN = clone number  
Ab = antibodies  
ND = not done  
CS = clinically suspected  
CM= cerebral malaria  
UM= uncomplicated malaria  
NK= not known

*(3.9A)* *(3.9B)*
3.2 Estimation of antimalarial drugs efficacy in Eastern Sudan:
3.2.1 Gedarif area (October-December 2003):
3.2.1.1 The efficacy of sulfadoxine-pyrimethamine alone and in combination with chloroquine:

3.2.1.1.1 Characteristics of the treatment groups:

The two treatment groups, the sulfadoxine/pyrimethamine (SP) and SP plus chloroquine (CQ) groups were similar with regard to age (22.9±15.5 and 22.5±16.4, respectively), weight (42.4 ± 15.4 and 40.9 ± 16.3 Kg, respectively), residence (Daraweesh / Kajara; 16/45 and 65/134) and time of study. However, the proportion of females was higher in SP treatment group (M/F, 26/35 [57.4%] and 97/102 [51.3%]), but the difference was not significant (p=0.490, Chi-square), Table (3.9).

The table 3.9 Characteristics of the two treatment groups

<table>
<thead>
<tr>
<th>Treatment Arms</th>
<th>Total patients</th>
<th>Gender (M/F)</th>
<th>Age Mean ±SD</th>
<th>Median</th>
<th>Weight (Kg) Mean ±SD</th>
<th>Village</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP alone</td>
<td>61</td>
<td>26/35</td>
<td>22.9 ± 15.5</td>
<td>15</td>
<td>42.4 ± 15.4</td>
<td>Daraweesh 16 Kajara 45</td>
</tr>
<tr>
<td>CQ &amp; SP</td>
<td>199</td>
<td>97/102</td>
<td>22.5 ± 16.4</td>
<td>18</td>
<td>40.9 ± 16.3</td>
<td>65 134</td>
</tr>
</tbody>
</table>

3.2.1.1.2 Clinical and parasitological response

3.2.1.1.2(a) Efficacy of SP and SP plus CQ:

The effectiveness of the two arms of treatment, SP and SP plus CQ was not statistically significantly different. Before PCR correction the proportion of patients achieved adequate and clinical response (ACR) following the treatment with SP alone or with SP plus CQ was; 68.3% (41/60) and 63.4% (123/194), respectively. Typing the polymorphic regions of *msp2* revealed that 3.6% (7/194) were re-infections rather than recrudescence in the SP+CQ group only, resulting in a PCR adjusted ACR of SP plus CQ of 67.01% (130/194) the ACR in SP alone is remaining un changed 68.3% (41/60). The treatment failure (TF) inclusive was 31.7% (19/60) for SP alone and 32.99% (64/194) for SP+CQ group. The overall treatment failure was estimated to be (83/254=32.68%). Both early and late TF (ETF & LTF) were recognized in the two arms of treatment see Fig (3.10).
In early treatment failure, all patients with parasitemia detectable at D3 of follow-up, were not given alternative treatment, but were observed up to D7; none of them developed severe symptoms or marked hyperparasitemia. Based on clinical and parasitological findings (between D3 and D7 or after), patients can be categorized into two groups, a. patient without symptoms and undetectable parasitemia at D7 (delayed parasitological response, DPR). b. Patients maintained parasitemia and still had or had no symptoms at D7, that continued for variable periods after D7 (actual early treatment failure, AETF). None of those patients developed severe symptoms, and most of them were given alternative treatment either at D7 or later. The frequency of DPR and AETF in the two treatment groups, SP and SP plus CQ; was 3.3% & 11.7% and 3.6% & 7.7%, respectively. The LTF was higher in SP plus CQ treatment group (25.3%, 49/194) than in SP treatment group (16.7%, 10/60), but the difference was not significant (P = 0.350, Chi-square).

3.2.1.1.2(b) Prevalence of asexual parasitemia during follow-up:
As shown in Fig. (3.11), the parasite clearance by D3 after treatment with SP and SP plus CQ treatment was 83.6% and 86.4%, respectively. Thereafter, the highest proportion of parasitemic patients was observed at D7 & D28 (13.1%), and at D21 (15.6%), in SP and SP
plus CQ treatment groups, respectively. The proportion of patients with parasitemia at D7 were significantly higher in the SP treatment group, 13.1% versus 4%, (p=0.037, Chi-square). There was no correlation between initial parasitemia and treatment outcome over the 28-days of follow-up. Patients had ACR and TF had a median parasite count at D0 of 9130 and 10065 parasites/µL of blood, respectively, the difference was not significant P=0.129 (Mann Whitney Rank Sum Test).

Figure 3.11 The proportion of patients who had microscopically detectable asexual parasitemia during the days of follow up

3.2.1.1.3 Drug resistance and age:

All patients (n=260), who were treated by SP (age, mean ± SD, 22.9±15.5 years) or SP plus CQ (22.5±16.4 years), had a median age of 17 years. The patients in both treatment groups, who had microscopically detectable parasitemia at D3, had a median age of 13 years, which was not significantly different from the age of all malaria patients at D0. However, malaria-treated patients who had parasitemia after D3 (had drug resistant malaria) were statistically significantly younger in age, than all patients at the time of diagnosis (P = <0.001, Kruskal-
Wallis One Way Analysis of Variance on Ranks, Dunn's Method). The median age of patients with ACR (21 Years) was statistically significantly greater than the median age of patients who had parasitemia at D7, D14, D21, and D28, which were; 9, 10, 10 and 10 years, respectively (Fig. 3.12A). Even, when we related treatment failure to age of patients in each treatment arm separately, a similar result was obtained, with few exceptions. For the SP treatment failure group, the patients with parasitemia at D28 had small median age of 9.5 years (but the mean age was relatively high). While, for the SP plus CQ treatment failure group, the age of patients with parasitemia at D7 was not significantly different from the age of patients at D0 of the same treatment group. Since the two treatment regimes were comparable, we compared the age of all patients (both treatment groups) who achieved ACR (median & inter-quartile range; 21 year & [13% – 35%]) and others who had TF (12 years & [9% – 16%]), the difference was strongly significant (P=<0.001), Mann Whitney Rank Sum Test).

### 3.2.1.1.4 Age and gametocyte carriage:

The median age of the patients who didn’t had detectable gametocyte during the study period (n=117) was 22 Years (inter-quartile range, 13–35 Y). While patients had gametocytemia in the follow-up days; D0 (n=7), D3 (n=30), D7 (n=104), D14 (n=81), D21 (n=41), and D28 (n=25), their median age were, 12 [9.25 - 16], 13 [9 - 17], 13 [9.5 - 22], 13 [10 - 21], 13 [9 - 19] and 11[8.75 – 16.5] years, respectively, see (Fig. 3.12B). The age of patients without gametocytemia and with gametocytemia in the different days of follow up were significantly different P = < 0.001, Mann Whitney Rank Sum Test. But, even if the age of all patients with malaria at D0 was compared with age of patients who had gametocytemia in any of the follow up days the differences were all statistically significant (P = 0.01 - <0.001, t-test). Seven patients had gametocytes at the time of diagnosis (D0) in the two treatment groups, see Fig. (3.12).
Figure 3.12A

Figure 3.12B

Figure (3.12): (A) The age of patients who had asexual parasitemia during the days of follow up, after treatment with either SP alone or in combination with CQ. (B) The age of patients who never had microscopically detectable gametocytemia during the days of follow-up.

From the figure (D0₁) stands for D0 without detectable gametocytemia, and the others who had gametocytemia in the 6 days of the follow-up; D0₂, D3, D7, D14, D21, and D28. D0₂ stands for patients with gametocytemia in D0. The data was pooled from the two treatment groups since there was no significant difference between the two groups. The box span
between 25% and 75% percentile and crossed by the median value (heavy line within the box), the mean is represented by the light interrupted lines and the circles are outliers.

However, during the follow-up, the gametocyte carriage in the two treatment groups was similar except for D3, while only 7% of the patients treated with SP plus CQ had microscopically detectable gametocytemia, 26.2% of the patients treated with SP alone had gametocytemia, the difference was significant (P = <0.001, Chi-square). But at D7 the gametocyte carriage rate was equal between the two treatment groups, and reached the highest level of gametocyte carriage (39.3% and 40.2%, for SP and SP plus CQ, respectively) fig. (3.13).

![Graph](image_url)

**Figure (3.13):** The gametocyte carriage rate (%) during the days of the follow up, a comparison between two different treatment regime, SP alone (heavy line and closed circles) and SP plus CQ (interrupted line and open circles).

### 3.2.1.1.5 Gametocytogenesis and drug response:

Gametocyte production was estimated by microscopic detection and count of gametocytes in the blood smears taken during the six days of the follow-up (D 0, 3, 7, 14, 21, and 28). The parasite was considered as a potentially gametocyte producer, if gametocytes of any count were detected in any of the days of the follow up. Based on this definition, the proportion of the gametocytes producing parasites in patient with ACR (44.8%, 73/163) was significantly lower than in patients with TF (72.2%, 70/97), P=<0.001, Chi square test. However, the gametocyte count, in both treatment-outcome groups was comparable in D3 and D7, P=0.603.
and $P=0.414$, respectively, but, it was higher in the TF group in D14, D21 and D28; $P=0.024$, $P=0.002$, and $P=0.061$, respectively, Mann Whitney Rank Sum Test. see Fig. (3.14).

Figure (3.14): The gametocytes count (median) in the different days of follow up, for patients achieved adequate clinical response and in patients with treatment failure

Gametocyte longevity was estimated by the number of times in which gametocytes were detected, over the six times of the follow-up, it varied between zero and six. Accordingly, we found the longevity of gametocytes in patients with TF was significantly higher than in patients with ACR (median & inter-quartile range; 1 & 0 – 2 and zero & 0 – 2, respectively, $P=<0.001$, Mann Whitney Rank Sum Test). However, if we limited the comparison to the gametocyte producing parasites in both treatment outcome groups, the TF and ACR, there was no difference.
3.3 Implication of the dhfr/dhps/pfcrt molecular markers in the evolution of *P. falciparum* malaria parasite beyond the Sulfadoxine/pyrimethamine and chloroquine resistance: more relevance and mutual association

3.3.1 Prevalence of individual *pfdhfr*, *pfdhps* and *pfcrt* mutations;

Of the 168 bloodspots that yielded PCR products, about 4.71% were mixed at *dhfr*, 3.57% were mixed at *dhps* and 1.19% were mixed at *pfcrt*, no majority haplotypes were found. The low number of mixed infections was a reflection of the low level of transmission in the area.

The mutations single nucleotide polymorphism (SNP) of different codons of *dhfr* gene (c51-I, c59-R, c108-N, c164-L) and *dhps* gene (c436-F, c437-G, c540-E, c581-G and c613-S) that associates with SP resistance were estimated in blood samples obtained from patients (n=168) with UM. To attend the most accurate estimation for the prevalence of the mutations in the total population, we assumption that the un-typed ACR samples (n2=82) should mirrored the genotyped ones (n1=86) and we adjusted the mutation frequencies by multiplying by a correction factor of 1.953 (1+ n2/n1) see table (3.10). The prevalence of *dhfr* mutant alleles; c51-I and c108-N, were 0.919 and 0.923, respectively, the mixed wild/mutant isolates, c51N/51I (n=7) were considered as mutant (fig. 3.16).

![Figure 3.15](image-url)

**Figure 3.15**

Mutiplicity of mutations (MOM), and frequency of individual mutations
While only one isolate was found to carry the mutant allele 59-R (prevalence of 0.006), the c164L mutation was not recognized as major infection, but there were 2 patients have it as minor infection. On the other hand, the prevalence of dhps mutant alleles; c437-G and c540-E were 0.902 and 0.793, respectively, and that of c581-G was 0.13. The wild/mutant mixed mutations; c437A/G (n=6), c540K/E (n=6) and c581A/G(n=4) genotypes were considered as mutant (phenotype). While only one isolate (0.006) had c436F mutation as major infection, 6 harboring it as minor infections and 3 harboring 436A also as minor infection, c613S mutation was not recognized as major infection, but there was only one patient harboring it as minor infection.

For chloroquine resistant transporter gene (pfcrt) We found that the wild type (CVMNK) = 11/168= 6.55%, the mutant type (CVIET) = 155/168 = 92.26%, mix infection wild/mutant type = 2/168= 1.19% and the mutant type (SVMNK) was not recognized.

3.3.2 The linkage and the multiplicity of mutations:

The linkage of mutant alleles associated with SP resistance was indicated by the high prevalence of multiple mutations. The prevalence of dhfr c51-I/c108N double mutation was 80%, and that of dhps c437G/540E was 59%.

Regardless of the loci of mutations (codon/gene), parasites were grouped into wild isolates and isolates with single, double, triple, quadruple and quintuple mutations. Some parasite DNA samples (n=23) didn’t show bands for all tested alleles, and they were excluded from the analysis, the included samples were 231. The frequency of isolates with the mutations from one to five were; 0.97, 0.93, 0.87, 0.72 and 0.12 respectively. While only 0.03 (n=4) of the isolates were found to carry the wild alleles of the tested genes.

3.3.3 Association of dhfr/dhps mutations with sulfadoxine/pyrimethamine drug response

In this sub-set of samples, the frequency of the mutant alleles; dhfr 51-I, 108-N and double mutant c51-I/108N (range 0.87–0.95; 0.88–0.92 and 0.68–0.84, respectively) was comparable between the different drug response groups, ACR (n=86), ETF (n= 19) and LTF (n= 63). Similarly was the frequency of dhps mutant alleles; c437-G, c540E, c581G and double mutant 437G/540E (range 0.85–0.94; 0.68–0.89; 0.11–0.26 and 0.37-0.59, respectively) was comparable between the above mentioned drug response groups. However, mostly, the prevalence of mutant alleles was higher in TF groups, but the differences always below the statistical significance.
3.3.4 Multiplicity of mutations, age and the immune factor:

As seen in the Fig. (3.16A & B), patients infected with parasites with different number of mutations or with wild parasites were not different in age. However, the age of patients infected with parasites with the same number of multiple mutations (more than one mutation) was significantly different between those attended full recovery (ACR) and others who had treatment failure (ETF and LTF). Patients achieved ACR infected with parasites with 2, 3, 4, or 5 mutations, their mean ages were; 28.8, 24, 24, and 26.1 years, respectively, which were significantly higher than the ages of patients infected with parasites with a comparable number of mutations; 11.6, 17.5, 17, and 15.6 respectively.

Figure 3.16 (A & B)

The term mutant-clearance immunity factor (MCIF) was introduced to estimate the impact of immunity in clearance of mutant (SP resistant) parasites; age was used as a marker for immunity.
In malaria endemic areas age is the single most reliable indicator for immunity unless otherwise e.g. high prevalence of immune jeopardizing disorder in the area. It was calculated by dividing the prevalence of each individual \(dhfr/dhps\) mutant allele or that of the different scales of MOM, by the actual treatment failure (35.4%) (TF stands for parasite resistance grade I, II and III). The results as shown in table (3.10) varied between 0.34 and 2.74, depending on multiplicity of mutation of the infecting parasites, the higher parasite MOM the higher host immunity, the lower MCIF value.

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<th>LTF n=63</th>
<th>Prevalence n=250</th>
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<td>c50, c164</td>
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<td>0</td>
<td>0.78% (1.95)</td>
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<tr>
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<td>B51</td>
<td>0.872 (75)</td>
<td>0.547 (18)</td>
<td>0.921 (58)</td>
<td>89% (222.5)</td>
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<tr>
<td>N108</td>
<td>0.884 (76)</td>
<td>0.695 (17)</td>
<td>0.921 (58)</td>
<td>89.4% (223.5)</td>
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<tr>
<td>B51/N108</td>
<td>0.756 (65)</td>
<td>0.684 (13)</td>
<td>0.841 (53)</td>
<td>77.2% (196)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular marker</th>
<th>ACR n=86</th>
<th>ETF n=19</th>
<th>LTF n=63</th>
<th>Prevalence n=250</th>
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<td>(dhps)</td>
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</tr>
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<td>G437</td>
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<td>0.937 (59)</td>
<td>87.6% (219)</td>
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<tr>
<td>E540</td>
<td>0.733 (63)</td>
<td>0.684 (13)</td>
<td>0.839 (56)</td>
<td>76.8% (152)</td>
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<tr>
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<td>0.368 (7)</td>
<td>0.683 (43)</td>
<td>56.8% (142)</td>
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<table>
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<tbody>
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<tr>
<td>Single</td>
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<td>1.0 (17)</td>
<td>0.983 (57)</td>
<td>97.0% (224)</td>
</tr>
<tr>
<td>Double</td>
<td>0.91 (71)</td>
<td>0.941 (16)</td>
<td>0.983 (57)</td>
<td>93.1% (215)</td>
</tr>
<tr>
<td>Triple</td>
<td>0.846 (66)</td>
<td>0.882 (15)</td>
<td>0.914 (53)</td>
<td>86.6% (200)</td>
</tr>
<tr>
<td>Quadruple</td>
<td>0.678 (33)</td>
<td>0.647 (11)</td>
<td>0.845 (49)</td>
<td>71.9% (166)</td>
</tr>
<tr>
<td>quintuple</td>
<td>0.103 (8)</td>
<td>0.235 (4)</td>
<td>0.138 (8)</td>
<td>12.1% (28)</td>
</tr>
</tbody>
</table>

### 3.3.5 Multiplicity of mutations and pre-treatment parasitemia:

The parasite density at the time of malaria diagnosis and before treatment in patients infected with wild isolates and with isolates with single mutation, taken together, was significantly
higher than in patients infected with isolates with multiple \(dhfr/dhps\) gene mutations. The difference in parasite density abolished when wild isolates were considered separately, however, the number of wild isolates was too low (n=4). The parasite density was not different between patients infected with isolates had multiple mutations (more than one) irrespective of the number of mutations.

### 3.3.6 Multiplicity of mutations and gametocytogenesis:

We defined gametogenesis as the ability of the asexual parasitemia to produce gametocytes at least once during the 28-days of follow up (D0, 3, 7, 14, 21 and 28), while gametocytes longevity was considered as an index for gametocytogenesis and scaled from 0 (no gametocyte detected during follow up) to 5 (gametocytes detected in all days of follow up). On the other hand, parasites were grouped based on the number of \(dhfr/dhps\) genes mutations per parasite (0[wild], 1, 2, 3, 4 and 5). None of the wild isolates and isolates with single mutation (n=9) produced gametocytes during the follow up. Although, the gametogenesis rate of the parasite groups increased with the number of mutations per parasite, the difference was not significant, it was 60% in the group of isolates with double mutations (n=10) and 70% in isolates with quintuple mutation (n=20). On the other hand, there was no difference in the gametocytogenesis (gametogenesis and gametocyte longevity) among isolates with two or more mutations, that is to say, at least two mutations were needed for influencing the gametocytogenesis, and any further increase in number of mutations have no further effect, see Table (3.11).

#### Table 3.11: The period prevalence of gametocytemia and gametocytogenesis

<table>
<thead>
<tr>
<th>No of mutations</th>
<th>No gametogenesis</th>
<th>Gametocytogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gametogenesis</td>
<td>Gametocyte longevity</td>
</tr>
<tr>
<td></td>
<td>1 (min.)</td>
<td>2</td>
</tr>
<tr>
<td>*Wild (4)</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>*Single (5)</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Double (10)</td>
<td>0.40</td>
<td>0.60</td>
</tr>
<tr>
<td>Triple (21)</td>
<td>0.38</td>
<td>0.62</td>
</tr>
<tr>
<td>Quadruple (93)</td>
<td>0.31</td>
<td>0.69</td>
</tr>
<tr>
<td>Quintuple (20)</td>
<td>0.30</td>
<td>0.70</td>
</tr>
</tbody>
</table>


#### 3.3.1 Submicroscopic \(Plasmodium falciparum\) infections during pregnancy:
Overall, 142 pregnant women were enrolled as they attended antenatal clinic for the first time. Only 17 (11.9%) were found smear-positive for malarial infection (all *P. falciparum*) but 40 (32%) of the 125 smear-negative women were found PCR positive and therefore had submicroscopic *P. falciparum* infections. Among the smear negatives, 14 (38.8%) of the 36 primigravidae, five (22.7%) of the 22 secundigravidae and 21 (31.4%) of the 67 multigravidae (i.e. those who had been pregnant more than once before) were found PCR-positive (P>0.05). Twenty-one (37.5%) of the 56 smear-negative women investigated during the wet season and 19 (27.5%) of the 69 women smear-negative women investigated during the dry season had submicroscopic *P. falciparum* infections (P>0.05).

In terms of age, parity, gestational age, hemoglobin concentration, and the frequencies of anemia (<11 g hemoglobin/dl) and severe anemia (<8 g hemoglobin/dl), the smear-negative pregnant women with submicroscopic *P. falciparum* infection were similar, when enrolled, to those without such infection (Table 3.12).

**Table (3.12) some investigations for the pregnant women**

<table>
<thead>
<tr>
<th>Variable</th>
<th>With submicroscopic infections</th>
<th>Without submicroscopic infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. and (%) of women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Investigated Primigravidae</td>
<td>40 (12.5%)</td>
<td>85</td>
</tr>
<tr>
<td>Secundigravidae</td>
<td>14 (35%)</td>
<td>22 (25.8%)</td>
</tr>
<tr>
<td>Multigravidae</td>
<td>5 (20%)</td>
<td>17 (20%)</td>
</tr>
<tr>
<td>With anemia</td>
<td>21 (52.5%)</td>
<td>46 (54.1%)</td>
</tr>
<tr>
<td>With severe anemia</td>
<td>29 (72.5%)</td>
<td>66 (77.6%)</td>
</tr>
<tr>
<td>Mean value and (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>25.02 (6.0)</td>
<td>26.31 (5.1)</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.01 (2.1)</td>
<td>2.50 (2.4)</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>27.2 (6.8)</td>
<td>25.4 (7.7)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>9.97 (1.3)</td>
<td>9.90 (1.2)</td>
</tr>
</tbody>
</table>

Blood samples from 32 (80%) of those with submicroscopic infections showed only the FC27 allele (of merozoite surface protein-2), six (15%) showed only the ICI allele, and two (5%) showed both of these alleles. Each submicroscopic infection appeared to consist of just one or two clones of *P. falciparum* (mean=1.016; median=1).

**Chapter Four**

**Discussion and conclusions**

4.1 Severe malaria:

4.1.1 In Gedarif area during the two seasons 2000-2002:
4.1.1.1 Clinical pattern of severe falciparum malaria:
In this study, we intended to execute the clinico-epidemiological description of severe malaria from a unique setting in an area of markedly unstable transmission. The malaria transmission in Gedarif area, as in most of central Sudan (∼ 70% of Sudan population), is confined to a short window of time, of two or three months a year, and sometimes is shorter or absent. Under such pattern of transmission, a partially protective immunity is acquired and the inhabitants are best described as semi-immune population16,282.

The overall frequency of severe malaria among the confirmed cases of malaria was 4.4%, and the mortality rate among the severe malaria patients was 6.4% which accounts for 0.3% of all cases of malaria, this finding is coherent with a previously reported rate from other settings291. Only four types of complications were recognized during the study period, the severe malarial anemia (SMA) was the commonest (45.46%), followed by convulsions, cerebral malaria (CM) and hypotension, and only 5.45% of the patients had multiple complications. In general, the difference in the rate of severe malaria morbidity and mortality between the two seasons was not statistically significant, but, the frequency of the individual complications changed considerably. While the SMA represented 26.4% of all complications in the first season it was increased by 2.4 fold (63.2%) in the second season, the high frequency of SMA was not due to multiple visits of individual patients. But, whether or not the unstable transmission of malaria accelerates the change of parasite population and consequently the pattern of clinical complications is not known and worth prolonged observation. Some complications were not recognized during both malaria seasons e.g. respiratory distress syndrome, renal disorders and thrombocytopenia. These complications are commonly recognized in regions like South East Asia292,293.

Previously Giha et al16. reported that uncomplicated malaria in the same area was recognized in all age groups, and individuals aged between 5 and 19 years had the highest risk. In this study, the highest proportion of patients with mild malaria was recognized in the same age group, 5–19 years, while that for severe malaria was in the age group of two to four years. The earlier protection from severe malaria compared with uncomplicated malaria is believed to be due to an earlier and faster acquisition of immunity against parasite strains causing non-cerebral severe malaria55. However, due to the inherent limitations in urban hospital-based studies in general (not all malaria patients seen in hospital) it was difficult to calculate with accuracy the incidence and the age risk for both mild and severe malaria.
There is some evidence that malaria infection in the older age groups results in a different form of complications as compared to that seen in children, in areas of stable transmission. In these areas, distinct age-specific patterns of infection and disease are seen. This study showed that the different clinical groups of severe malaria (anemia, convulsions, cerebral malaria, and hypotension) differ from each other in critically important clinico-epidemiological indices. We compared five parameters; the age, parasite count, hemoglobin level, blood glucose level, and mortality rate, among the groups. Taken together, the four clinical entities of severe malaria were found to be strongly statistically significantly different from each other in all the five tested parameters (p=0.022–0.000, Kruskal-Wallis test). However, when we compared each complication with other complications separately, we found the greatest difference was between CM and SMA, and the least difference was between CM and convulsions. The similarity between CM and convulsions (except for the age), and the dissimilarity between CM and SMA on clinical and epidemiological aspects, looks interesting, and possibly denote resemblance and disparity in pathogenesis of these complications, respectively.

The difference in the age distribution of the different complications in this setting, and between this and other settings, was marked and of special importance. It is well known that, there are differences in the peak age for CM compared to SMA and also in the distribution of CM and SMA depending on the level of \textit{P. falciparum} transmission in the area. The SMA peaks at the age of 6 months to one year and CM at the age of two to three years in hyper endemic areas, in this study, the mean age for SMA was around five years, while that for CM was approximately 14 years. The mean age for both complications, was three times higher in our study area, while the ratio of the peak age for the two complications in each site is constant, (a ratio of approximately 1:3). Hypotension was only recognized during adulthood with a mean age of 35 years; however, the outcome of treatment was good and fast, with the shortest duration of hospital admission. On the contrary, the mean age for malarial convulsions was similar to SMA, five years. In general more than 60% of patients with severe malaria were above five years of age, while all patients who died of severe malaria were above five years of age (with a mean age of 18 years), see table (3.2).

As seen in (figure 3.3), the lowest parasite count was recognized in patients with SMA, the youngest group of patients, a low parasite count was also recognized in individuals with hypotension, the eldest group. The low parasite count in patients with hypotension could be due to the acquired anti-parasitic immunity which increases with age. However, the role of...
age-associated acquisition of immunity can not explain the low parasite count in patients with SMA.

The blood glucose level was highest in patients with CM and lowest in patients with SMA, the difference was significant; also, hyperglycemia was very remarkable in the fatal cases of malaria. Generally, hypoglycemia, is one of the complications of *P. falciparum* malaria with a prevalence of 10% is ascribed to inhibition of gluconeogenesis\(^{300}\), and is considered as independent risk of mortality in severe malaria\(^{301}\). In this study, hyperglycemia in fatal severe malaria was not due to quantitative deficiency of insulin, on the contrary insulin levels were extraordinary higher than in nonfatal severe malaria and above the upper physiological limits. The strong association between hyperglycaemia, hyperinsulinemia and cerebral malaria fatality, need further biochemical explanation. It is of interest to know, how hyperglycemia and hyperinsulinemia coexist, and which preceded the other. The other observation is that the younger patients who died of severe malaria (Table 3.2) had a relatively lower blood glucose level and insulin level, although the sample size is not large enough to make such a judgment. Hyperglycemia, was reported in Vietnamese patients with CM, in whom the blood glucose was found to be 100% derived from gluconeogenesis, as well as, it has been reported that there was a significantly higher blood cortisol in CM patients as compared with a control group\(^{300}\).

In this setting, the CM had an average mortality rate of 39% (45% and 29%in the first and second season, respectively), while there was no mortality from other complications. We compared levels of hemoglobin and blood glucose between severe fatal and severe non-fatal malaria. Surprisingly, patients who died of severe malaria had higher hemoglobin and glucose levels when compared with patients who survived the severe attack of malaria. Interestingly, none of the CM patients who also had SMA died.

**4.1.1.2 Allelic polymorphism of MSP2 gene in severe *P. falciparum* malaria:**

The role of the malaria parasite in development of severe malaria is not a debatable issue, however, how the parasite contributes to development of SM, is not well understood. Certain molecular markers like certain var genes coding for PfEMP1, are known to be involved in parasite rosetting and cytoadherence\(^{302}\), and are believed to be an important pathogenic marker. Otherwise little is known about the markers of virulence or their linkage in the malaria parasite. Enthusiastically, the aim of this study was to identify number or quality (genotype) of clones, both or none are associated with development of SM. This study was conducted in an area of strictly seasonal and markedly unstable and low malaria transmission
in Sudan. Overlap or super-infections are less likely to be frequent, and the inhabitants are semi-immune to malaria. Thus, genotypes, multiplicity and dynamics of infecting parasites are less influenced by non-parasite confounding factors, such as age and immunity.

In this hospital based study, blood samples were obtained and genotyped from 231 individuals, of whom 103 had SM. Although, the individual infections were composed of low number of clones (mean clone number of 1.5), the generally circulating clones were numerous (more than 50 allele types of MSP2). In malaria endemic areas, most of the studies uncovered the huge genetic diversity of infectious parasites, although only a limited number of molecular markers was usually used e.g. MSP1, MSP 2, GLURP and drug resistance associated genes. In fact there are large numbers of potential sources of parasite diversity, thus, it is possible that each parasite has its own unique genetic make up, although it was previously reported that a great homology between isolates obtained from a single household. Whether all data was pooled and analyzed or each clinical group of patients was considered separately, no correlation between multiplicity of infection and age was found, in contradistinction to other studies in other sites of high transmission. In this area people are semi-immune, and both UM and SM affects all age groups although clinical malaria infections are more common in young patients. Further more; there was no correlation between initial parasitemia and the clone number as reported from other sites. However, the influence of both age and parasite densities on multiplicity of infection is likely to be immunity-dependent, so their effect is modest in areas of low transmission, such as in eastern Sudan.

As previously mentioned, the overall mean clone number (MCN) was 1.5. By breakdown of data into groups of malaria infection, ASUM, UM and SM, and sub-groups of SM (CM, SMA, CAM and HTN), a differential pattern of clonality of infection was revealed. The frequency of single clone infections was ranging from 1.0 in individuals with ASUM, to 0.66 in SM and 0.59 in UM. Interestingly, the low complexity of infection was recognized in the two clinical extremes of malaria infection, the asymptomatic infection and the fatal CM infection (1 and 1.1, respectively). So, the general analysis showed that, multiplicity of infection was associated with development of clinical malaria, but didn’t associate with the severity of malaria. This is some how in line with Roper et al. early findings in rural Eastern Sudan, where she reported that the risk of development of UM increases with the increased number of clones in a given infection. However, detailed analysis of this data indicated that fatal CM was not multi-clonal. The controversy of the clinical outcome in relation to the
complexity of infection in this study could be due to; the basically low complexity of infections in the area, or due to the difference in the types, but not the number, of alleles between fatal CM (IC1) and ASUM (FC27) infection. The clinical consequences of multi-clone infections (MCIs) vary considerably between epidemiological settings\textsuperscript{115}, and in different ages and immune status\textsuperscript{71,114}. In Tanzania and Papua New Guinea the MCIs were found to be associated with reduced incidence of clinical malaria\textsuperscript{76,116,118}. But, that protection was not evident in very young; immunologically relatively naïve hosts\textsuperscript{68}. While in other studies conducted in; Tanzanian\textsuperscript{114,117}, western Kenya\textsuperscript{120}, Ghana\textsuperscript{121} and Mozambique\textsuperscript{122} the MCIs were found to be a risk factor for development of clinical malaria. However, most of these studies compared UM with asymptomatic infections, and a very limited number of studies were in fact made the comparison in a broad range of clinical malaria infection.

The overall genotyping data, showed great similarity in the parasite populations that caused UM and SM infections. The prevalence of the 2 allele families of MSP2; the IC1 and FC27 was not significantly different between UM and SM, although more FC27 parasites were recognized in UM. The size polymorphism, in both allele families; IC1 (30) and FC27 (23), was found to be comparable between UM and SM, not only in the total number of alleles, but also in the frequency of individual allele sizes, see Figure (3.5). However, more detailed analysis included clinical sub-groups of infections, revealed some significant variations in parasite genotypes. The frequency of single clone infection of FC27 genotype is relatively low in this setting (<25%) similar to other settings\textsuperscript{305}, however, in other areas, the FC27 genotypes predominates\textsuperscript{75,85}. Interestingly, the frequency of FC27 infections was significantly high (68.2%) in the apparently healthy, malaria free, volunteers who were found to have ASUM infection. In heterogenic multi-clonal infections (mixed IC1/FC27), following treatment with quinine, the clearance of the IC1 genotype was significantly faster than that of FC27 genotype component of the mixed infection (figure 3.5). It was noted that the carriage of parasites during the follow up period was not associated with malaria symptoms (asymptomatic parasite carriage). Two explanations are possible for the later statement; a. infection with IC1 genotypes exceeded that of FC27 in multi-clone infections (super-infection), b. the relative resistance of FC27 genotypes to treatment. In general, these observations indicate that the FC27 genotypes were generally associated with the mildest form of \textit{P. falciparum} infection in this area, and that was consolidated by the reproduction of the results over the two seasons of the study. At least this data prove that the least virulent parasites belong to a subpopulation of derived from a common ancestor which carried the
FC27 alleles. On the contrary, fatal CM was strongly associated with IC1, all patients who died of CM (n=7) were infected with IC1 genotypes, only one of the patients had two clones one of them was FC27 allele type. In French Guiana, MSP-1 allele (B-KI), but not MSP-2 allele (IC1) was associated with SM morbidity\textsuperscript{305}. Thus, in the localities, where certain genotype family is associated with severe disease, it would be easier to zoom-in the search for virulence factors into a sub-group of parasites. Pre-selection of parasites, reduces the efforts and increases the number of candidate molecules to be investigated. Interestingly, it was observed in this study, that the parasites of FC27 genotype had significantly higher prevalence of \textit{pfcr}t and \textit{pfmdr}1 mutant alleles. Furthermore, it was found that mortality was associated with the wild types of \textit{pfcr}t and \textit{pfmdr}1 type\textsuperscript{162}. All these findings were quite consistent and support the association between the FC27 genotypes and the clinical outcome of infection in the study area.

Twelve patients with SM had PCR detectable parasitemia throughout the follow up period (28 days). The D0 parasite genotypes of all 12 patients indicated the large degree of genetic diversity of quinine resistant parasites in this study (vertical heterogeneity) (see Figure 3.6). The genotypes of the parasites in D0, D3, D7 and D28 for each one of the 12 patients, showed very limited genetic diversity (horizontal homogeneity). The later was an indication for parasitological failure of treatment and thus, persistence of the same parasite genotype. This also reflects the importance of the use of size polymorphism, rather than the broad typing as IC1 and FC27 genotype, in the distinction between parasites.

The frequency of multi-clonal infection is comparable between SM and UM and accounts for (35% and 41%, respectively). However, the composition of the multiple infections was either homogeneous (consists of IC1 alone or FC27 alone) or heterogeneous (consists of both IC1 and FC27). Interestingly, most of the complex infections were heterogenic (75%), this and the significantly faster clearance of one genotype (in this study IC1) rather than the simultaneous clearance of all infecting clones, is supporting our previous assumption that multi-clone infections are predominantly due to super-infections. If the multi-clone infections were due to injection of multiple clones in one inoculum, the compositions of multi-clone infections will be random rather than being heterogenic and parasite clearance after treatment would be simultaneous. Also, the predominance of the heterogeneity of multi-clone infections was possibly indicating that infections with certain genotype provide a relatively stronger immunological protection from the same allele family than the other family. In other words
the above might be an indication of a stronger cross-immunity between alleles of the same MSP2 genotype family.

4.1.1.3 Genetic fingerprints of parasites causing severe malaria in a setting of low transmission “one strain one patient”:

The devastating effect of *P. falciparum* is attributed to severe malaria. In sub-Saharan Africa where the heaviest disease toll, cerebral malaria (CM) and severe malarial anemia (SMA), are the main fatal complications\(^{306}\). Usually, only a small fraction of the infections can progress into SM\(^{291}\) as recognized in this area also (1.2\%). Thus, it is conceivable that a small relatively homogenous subpopulation of parasites, described as, “virulent strains” are associated with SM, more specifically CM\(^{261}\). But whether these virulent strains are genotypically identical or are not identical but share a common virulence marker/s, is not known. The present study was carried out to investigate the parasite population genetics in SM, using clinical and molecular data, and here we hypothesized that the parasite subpopulation that cause severe malaria are as diverse as the hosts who develop severe malaria. Since each individual have different genetic make up, each parasite described as virulent strain have a unique genetic fingerprint, and SM occurs by chance as a result of a highly specific host/ parasite interaction, in a key-and-lock manner.

In this study it was found that, the parasites isolated from patients with SM, were extremely genetically diverse from each other, irrespective of the type of complication. It was almost, each single infection (in \(~90\%\) of the infections) was caused by genetically different strain, except for three pairs of infections. Given the limited number of markers used in the study, we speculated that, if more markers were included, there might be only two patients had been infected by identical parasites, as their history and clinical findings suggested. Those are brothers who lived in the same house (hut) and possibly got infected on the same night. However, such degree of diversity would have not been revealed unless multiple genetic loci were examined. Indeed, the more molecular markers used, the better the contrast can be generated, with a more clear distinction between isolates. It is worth noting that we used only 4 markers out of a large number of potential sources of diversity\(^{94,307}\). Using one of the most polymorphic molecular marker for typing, the MSP2\(^{66}\), in the present study it was reaveled that, only half of the expected polymorphism which was statistically significantly lower than the actual polymorphism when all the four markers were used together\(^{305}\), reported an association between specific msp-1 allele (B-K1) with a specific *var* gene (*var-D*), and their
over representation in SM, in French Guiana, a hypoendemic area. However, these findings are not mutual exclusive, as these parasites still could be of different genetic make up.

The diversity of infection (DOI), as explained before, was used to estimate the frequency of the genetically unique isolates in a population or a subpopulation of parasites. On the other hand, the multiplicity of infection (MOI) was generally used for estimation of the average number of clones per person\textsuperscript{17}, however, both parameters depend on the type and number of the molecular markers used. Ideally, a number of markers should be analysed to generate multi-locus genetic profile for each individual parasite. The MOI and distribution of parasite genotypes in SM, was investigated before with a larger sample size (including the samples used in this study), but the \textit{MSP2} gene was the only molecular marker analyzed. In this study it was found that multiplicity of infection (MOI) was comparable between SM (1.45) and UM (1.53), and the detailed analysis showed that; the MOI in asymptomatic sub-microscopic malaria infection (ASUM) was 1.0, which was significantly lower than that of SMA, and non-fatal CM but not fatal-CM. Furthermore, found that the FC27 was over-represented in ASUM, and was not recognized in fatal CM. In addition, it was found that the ratio of IC1 and FC27 allele families was comparable between SM and UM.

Using the multi-locus genetic profile, the molecular data in this study clearly demonstrated a marked diversity between the parasites that caused SM (Figure 3.7). Although virulent strains can have different genetic fingerprints, but still, they could share a common or a limited number of virulence markers (which are not really identified). However, existence of virulent strains with a common or limited virulence markers was unlikely for many reasons; a. the extreme diversity of the clinical presentation and pathogenesis of SM e.g. CM and SMA\textsuperscript{261,306} b. lack of SM clustering per household c. the relatively constant ratio of SM in relation to UM over long time\textsuperscript{291}, compared to the fast expansion of drug resistant in relation to sensitive malaria parasites (the fast propagation in \textit{pfcrf, dhfr} and \textit{dhps} mutations) in different areas\textsuperscript{308}. Thus, a virulent parasite for one individual might not be virulent for another comparable individual. However, the prevalence of SM in non-immune adults is higher than what would be expected from the prevalence of virulent strains\textsuperscript{309}, is indicating a considerable modulation of the host/parasite interaction by acquired immunity.

Clustering of severe malaria is not recognized in this study, although this was a whole season data in an average size town, only 2 sibs were found to have SMA with similar parasite genotype profile. There was another 2 pair of patients infected with strains of similar genetic
profile, but from the history and clinical data, the isolates unlikely to be identical if more markers were used.

Interestingly, the patients with uncomplicated malaria (representing almost 98.8% of malaria patients in this study), had the same DOI, moreover, *MSP2* revealed more diversity than it did in SM. But analysis of *MSP2* data collected over 2 malaria seasons included this season, showed no significant difference in the MOI between the two study groups. In all, this data indicates that the *P. falciparum* isolates from patients with SM are genetically diverse as the genetic diversity of the human host, suggesting a genetic fingerprint for individual parasites.

Many hypotheses were proposed to explain the reasons or mechanisms of diversity in malaria infections in general, although, one view is that natural infections are derived from one or few genotypes, which undergo mating infrequency, implying mainly clonal reproduction. However, recombinations do occur frequently in nature but with higher selfing rate in areas of low transmission. In strain theory, it was suggested that malaria parasites only exist as a limited number of stable antigenic types, called strains, and that cross-immunity between mild and virulent strains is slow. However, many studies affirmed the genetic diversity of parasite populations in different endemic situations, although the magnitude of this diversity was never quantified, it was either described as limited, moderate or large diversity.

**4.1.2 In New Halfa area (November 2000- February 2001):**

**4.1.2.1 Cerebral malaria is frequently associated with latent parasitemia among semi-immune population:**

Severe malaria, including cerebral malaria, has been an important cause of human death since time immemorial and still poses a great challenge. Both the delay or failure of treatment and the delay and failure of diagnosis of malaria are important factors in malaria mortality. The proper diagnosis of malaria begins with clinical suspicion, not only in non-immune travelers but also for the semi-immune inhabitants of malaria endemic regions. This study clearly suggests that a proportion of patients in this area, fairly described on the basis of serology as semi-immune, can develop acute fulminant and often fatal cerebral malaria symptoms with scanty, submicroscopic parasitemia. This phenomenon was predominantly recognized in adults, whose infections were revealed by PCR. Furthermore, the therapeutic response and fatality rates after quinine treatment were comparable with that of cerebral malaria with patent parasitemia in the same region as mentioned before in this study.
In febrile patients with microscopically unproved malaria, presentation with coma narrows the differential diagnosis by exclusion of febrile conditions like pneumonia, typhoid, and other infectious diseases that are not a common cause of coma. Still, other clinical conditions like meningitis, encephalitis and some viral infections could mimic cerebral malaria. Although meningitis can occur sporadically, it occurs in epidemics mainly before the rainy season, and it is mostly (but not absolutely) clinically distinct. However, the other infections occur sporadically but are not clustered during the short window of malaria transmission. Malaria and viral infections can co-exist in patients if either of them can be recognized independent of the other in the same community at the same time. That was not the case for conditions like meningitis, and viral encephalopathy, since no single case of either condition was recorded during the study period. Other non-febrile conditions presented with coma like cerebro-vascular accidents (CVA) can be ruled out since those patients do not present with fever. In this setting, clinicians are confronted in many occasions with such clinical cases making a dilemma in diagnosis and the decision to treat them as malaria or not, is critical and associated with survival or death of patients.

*P. falciparum* infection was detected in a majority of the clinically suspected cases of cerebral malaria (CSCM), as well as in all patients with clinically suspected uncomplicated malaria (CSUM). Obviously, it was the peripheral parasitemia and not the absolute parasitic load, which was estimated by microscopy. In a recent post-mortem study carried out in Thailand and Vietnam, patients who died of cerebral malaria, showed a 26.6 times higher parasitemia in the post mortem brain vessels than in the pre-mortem peripheral blood312. In West Africa, Kurzhals et al.313 reported that between 20 to 25% of the patients with anemia did not have microscopically detected parasitemia. In some non-immune travelers, malaria parasites were detected only when patients were terminally ill or at postmortem310. However, in general, peripheral hyperparasitemia is associated with complications of severe malaria, and it is also considered as an independent risk factor in malaria infection306.

The occurrence of asymptomatic parasitemia is rare in the study region16, as also reflected in this study, with low numbers of positive blood smears or PCR positives among the malaria symptom-free (MF) donors. Although the MF donors were not followed to know whether their asymptomatic infections became overt malaria or not, there was a significant difference in the prevalence of PCR-proved submicroscopic parasitemia between MF donors and patients suspected of having cerebral malaria. This appears different from the situation in Tanzania, where MF donors were shown to have the same prevalence of parasitemia as
patients suspected of having CM. In the latter setting, other neurological diseases were diagnosed and cerebral malaria was excluded.

There was a significant difference between CS CM and uncomplicated malaria (UM and CS UM) infections in the mean clone number, which tends to be smaller in the former group. This is in agreement with other studies, where less complex infections were found to be more associated with CM. However, in this study the mean clone number in CM with latent parasitemia was found to be comparable with that of UM.

Although prevalence and levels of Abs against MSP1 and MSP2, as serological markers for exposure is a rather crude approach at the individual level, it is still informative in epidemiological surveillances. Due to the seasonality of the modest malaria transmission in this study area, the prevalence of Abs against malaria Ags were previously found to be markers for recent exposure. Clinical, molecular and serological data were in-favor of the diagnosis of malaria infection in most if not all of the comatose patients with microscopically undetectable parasitemia. Furthermore, this was supported by the occurrence of CS CM during the peak of the malaria transmission and the fair therapeutic response of the CS CM patients to anti-malarial treatment. In undiagnosed patients with prolonged fever in Pakistan, the thick smears of bone marrow revealed P. falciparum malaria parasite in high proportions of patients with sub-patent parasitemia. A possible explanation for the sub-patent parasitemia in patients with cerebral malaria symptoms is sequestration of infected erythrocytes (IE) in the vascular bed of internal organs, with preference to the brain, which has been found to be associated with severe malaria. Thus, the sub-patent parasitemia was only time-related and coinciding with the cytoadherence of the late stages in synchronized parasite growth. The single-clone infections (dominant in CS CM) logically should tend to synchronize easier than a multi-clonal infection. And by definition, parasites causing cerebral complications are more virulent and are characterized by the ability of cytoadhesion. However, the objective proof for sequestration would be the study of post-mortem brain specimens; the lack of such data is one of the limitations in this study.

### 4.2 Estimation of antimalarial drug efficacy in Eastern Sudan:

#### 4.2.1 Gedarif area (October-December 2003):

#### 4.2.1.1 The efficacy of Sulfadoxine/pyrimethamine alone and in combination with chloroquine:
Although sporadic cases of resistance to SP was observed and reported more than 13 years back in the Sudan, no compiled study was carried out to quantify the resistance to SP in the region before. In this study the prevalence of SP resistance was found to be 32.6% and the efficacy of SP alone and in combination with CQ was comparable. Addition of CQ to SP had no parasitological advantage over SP alone except on halting gametocytismia on D3 of follow up, which rebound later (after D7) to mimic the rate of gametocytismia in SP alone. But whether that difference was due to a short lived gametocidal effect of CQ or not, was not reported before. The findings in this study suggested that, parasites resistant to SP, is less likely to be sensitive to CQ. This statement is supported by the co-existence of SP and CQ resistance associate alleles; the (dhfr 108 & 50/51 and dhps 540 & 581) and the (pfcrt 76 and pfmdr1 86), mutations in parasite isolates obtained from the same study area. It is possibly that, during the parasite evolution the occurrence of mutations in the genes associated with CQ resistance precedes but enhances the mutations associated with SP resistance. However, these findings are in contradiction with a recently published work from West Africa, Nigeria, where the combination of CQ with SP, significantly improved the efficacy of the later in the treatment of UM. While an earlier report from the Gambia showed no additional privilege from the use of CQ in combination with SP, except for symptomatic relief.

The proportions of patients achieved adequate clinical and parasitological response (ACPR) in both arms of treatment were comparable although slightly higher in patients treated with SP alone, but that is more likely due to the smaller number of patients in the SP treatment group. Similarly, the proportion of patients with late treatment failure (LTF) was higher in the SP plus CQ treatment group, but the difference was not statistically significant. The frequency of early treatment failure (ETF) was also similar in the two treatment groups (11.3% and 15%). Patients with detectable parasitemia at D3 were not treated because their parasitemia were not flourishing and because they had only mild to moderate malaria symptoms. However, as the follow up days progressed, we observed that the patients in this group (ETF) were actually belonging to 2 different categories. Patients who achieved complete clinical recovery and cleared their parasitemia by D7, and others who continued to had malaria symptoms and detectable parasitemia, and for whom alternative treatment was given. Thus, the former group was defined as patients who had delayed parasitological response (DPR), and the later group as patients who had actual early treatment failure (AETF), see Fig. (3.9). However, that could be unique to this setting, as microscopically detectable parasitemia rarely dissociates from clinical symptoms and the individuals in this
area are semi-immune to malaria (sluggish parasite clearance). Less than 20% of the patients in the two treatment groups had detectable parasitemia at D3, but at D7, significantly more patients treated with SP alone compared to patients treated with SP plus CQ, were found carrying malaria parasites. This could be one of the few advantages of the added CQ, but that was for only short duration, as the 2 treatment groups were became comparable in the following days of the follow up.

The role of the host immunity in clearance of the drug resistant parasites following treatment, was demonstrated in the field\textsuperscript{176}, as well as in rodent malaria model, \textit{Plasmodium chabaudi}\textsuperscript{322}. The host age is an ideal surrogate marker for protective immunity in individual living in malaria endemic area, as there is no single measurable marker for evaluation of protective anti-malarial immunity. In the present study a statistically significant lower age of patients infected with parasites with any grade of resistance (ETF or LTF, over all the days of the follow up) to any of the two treatment arms, SP mono or combined therapy had been demonstrated. The better parasite clearance in older patients (age; mean $\sim$20, median $\sim$18 years) was most likely attributed to some sort of anti-malarial immunity. Previously in this same study area, the risk of development of malaria episodes reduced to the half after the age of twenty, although all age groups are prone to develop malaria\textsuperscript{16}. On the other hand the studies about malaria infection and immunity carried out in the same region, support the notion that inhabitant of this area are semi-immune to malaria\textsuperscript{31,277}. All studies carried out in the recent years in region showed that the prevalence of SP resistance genes, \textit{dhfr} and \textit{dhps} were more than 70%\textsuperscript{323}, while the prevalence of SP resistance as shown in this in-vivo study was only 32.6%. Taken together, the above findings strongly support the theory about role of immunity in clearance of drug resistant parasites. That means some parasites carried the SP mutant alleles were cleared, most likely through immunity. This study is the first demonstration for the role of partial immunity in clearance of drug resistant \textit{P. falciparum} parasites in human, supporting the rodent malarial model\textsuperscript{322}.

Further more, in the longitudinal surveillance for gametocytes carriage (gametocytogenesis), in this study it was observed that patients carried gametocytes longitudinally were statistically significantly below the median age of the total malaria patients. This finding too, can best be explained by the role of the partially acquired immunity in clearance of the sexual stage of the parasite. The association between the longevity of gametocytemia and the clonal complexity of the infection was exploited before in the same setting, when a more sensitive
tool, the rt-PCR\textsuperscript{324}, was used. It was found that, gametocytemia persist for longer periods in multi-colonal infection compared with monoclonal infections\textsuperscript{325}.

The bursting of gametocytemia following SP treatment compared with other antimalarial drugs like CQ\textsuperscript{326}, quinine\textsuperscript{327}, or artemisinin\textsuperscript{328} is a consistent finding, that worth meticulous exploitation, possibly at molecular level since the parasite genome was already known. It was assumed that the fast propagation in SP resistance, either due to intrinsic parasite/drug properties or cross resistance with drugs like septrin\textsuperscript{329}. However, from this data it was hypothesized that, the fast propagation is due to a combination of two factors mutually related; the first is the strong selective effect of SP i.e. following malaria treatment with SP, only the resistant strains that can exist. Secondly, the robust gametocytogenic effect of SP, which leads to expansion of the selected resistant population at D14 and/or thereafter (Fig. 3.13).

4.2.1.2 Implication of the \textit{dhfr/dhps/pfcrt} molecular markers in the evolution of \textit{Plasmodium falciparum} malaria parasite beyond the Sulfadoxine/pyrimethamine resistance: more relevance and mutual association:

The world is left with only limited feasible options for curative malaria treatment after the resurgence and fast propagation of the anti-malarial drug resistance, namely to CQ and SP as reviewing by White\textsuperscript{172}. A number of studies have investigated the association between mutations (SNPs) of certain genes (\textit{dhfr} and \textit{dhps}) and the parasitologic and/or clinical response to SP treatment at an individual level\textsuperscript{182,234,330,331,332,333}. The relatively recent emergence of mutations and their implication in drug resistance\textsuperscript{64,241}, and the recovery of the wild alleles in the parasite population after the drug usage cease\textsuperscript{334}, indicates an advanced parasite tactic. However, that is also inviting for drawing an evolutionary scenario for this organism, using the molecular markers as a tool. Although, the re-emergence of the wild alleles is explained by outgrowth of sensitive strains in absence of drug pressure\textsuperscript{166}, the assumption of reverse mutation (mutant to wild) is an enticing hypothesis that needs to be disproved.

The present study is the first large scale, comprehensive analysis for SPR molecular markers to be carried out in Sudan in addition to the CQ resistant mutation at \textit{pfcrt} gene; it is coupled with a reliable clinical data and suffice treatment failure that permit the intended analysis. However, the main limitation was the exceedingly high \textit{dhfr/dhps} mutation rate. Approximately, one third of the malaria infections were found to be resistant to SP treatment (\textit{in-vivo}) as seen before in this study, while the frequency of the SP resistance associated
alleles of the \textit{dhfr/dhps} genes were on average 3 times the actual TF. That means two thirds of the mutant infections were surmounted by the host immunity. From these results it had been suspected that in this study area, the mutations in \textit{dhps} but not \textit{dhfr} were associated with fansidar resistance. However Alifrangis\textsuperscript{335} in a study comparing different sites of endemicity in Sudan, Tanzania and Mozambique suggested that \textit{dhfr} genotypes alone may be a suitable marker of the overall SP resistance level while others\textsuperscript{232,234,336} observed that, combination of \textit{dhfr} and \textit{dhps} mutations may still prove to be important mainly in areas of low endemicity such as Sudan.

The role of immunity in clearance of resistant parasites was supported by the higher age of the successfully treated patients, as age stand-in for immunity in the same area\textsuperscript{337} and in other malaria endemic areas\textsuperscript{176}. However, the magnitude of immunity varies considerably in different epidemiological settings; thus, the immunity factor is not fixed but varies with malaria transmission intensity, and possibly between individuals in the same area. However, it would be interesting to know if immunity can operate successfully in clearance of mutant parasites in the absence of treatment, although the later is not functioning? If the answer is yes, then probably two thirds of the malaria infections in this area were missed, which is very unlikely as asymptomatic parasitemia is rare. Otherwise, that means, the use of the useless drugs (to which the parasite is resistant) is still needed for the immune clearance of the mutant (resistant) parasites. In this study, the immune factor varied between 0.37 (for quintuple mutants) and 2.97 (for single mutant), and interpretation here is that the lower the immune factor the more efficient the immunity.

The mutant-clearance immunity factor (MCIF) is comparable to genotype resistance index (GRI), the later was similarly calculated from different sites in Mali over 3 years, the drug was CQ and the GRI values varied between 1.6 and 2.8\textsuperscript{186,187}. The term MCIF was introduced because the variables other than immunity e.g. micronutrients, host genetics and others, were considered to be of minor role, as all the patients were of similar socioeconomic and racial background. The MCIF values were coherent with previous findings that malaria incidence is reduced to the half after the age of twenty due to acquired immunity\textsuperscript{16}, and comparable with GRI in Mali if we consider the differences in malaria intensity between the two areas.

The mean age was not different between patients infected with parasites of different multiplicity of mutations (1 to 5), although, the age of patients who achieved ACR was significantly higher than the age of patients who presented with TF. Interestingly, patients attained ACR and others who had TF were similar in age when infected by wild parasites or
parasites had a single mutation. However, when the infecting parasites had two mutations or more, significantly older patients who attained ACR compared to those who had TF. That indicated, the susceptibility to immune clearance required at least two mutations, then it reached a plateau as the parasites that had more mutations were not more susceptible to immune clearance. It is worth noting that, the inhabitants of the area were semi-immune rather than being fully immune to malaria, which reflect the immensity of the fitness cost of mutations and supporting our previous finding. The association of older age with wild-type parasite infection cannot be worked out from this data, as half of the samples obtained from patients with ACR were not genotyped. However, a larger sample size or a higher prevalence of wild \textit{dhfr/dhps} variants is needed to assess for any.

It is apparent that there are at least three factors directly contributing to parasite clearance (ACPR), a. drug (SP), b. immunity (age), and c. parasite mutations (multiplicity of mutations). While the mutations provide the parasite the resistance to chemotherapy renders it more susceptible to host immunity as an incurred fitness cost. Interestingly, the resistance to SP increases with increasing number of \textit{dhfr/dhps} mutations (which did not reach statistical significance in this study), while the susceptibility to the immune clearance needed only double mutation and did not increase with increasing number of mutations. Generally, from the above data, it is appealing to suggest that for plummeting parasite fitness only 2 \textit{dhfr/dhps} mutations were needed, while the parasite benefit from the mutations, acquisition of resistance to SP, needs and depends on higher MOM. To define the magnitude of the influence of the individual mutant alleles in the double mutants, a larger sample size is needed; however, it is apparent that \textit{dhfr}51/108 and \textit{dhps} 437/540 were the major players in this setting at this time.

On the other hand, the in-vivo parasite growth which is reflected by parasite density, as sequestration is limited to severe malaria infections, is also affected by the \textit{dhfr/dhps} mutations. The in-vivo growth rate of wild and single mutant parasites taken together was significantly higher than that of multi-mutant parasites. However, when wild isolates were taken separately, the growth rate was not different from multi-mutant parasites, but that was mainly due to the very low number of wild isolates. Interestingly, while at least two mutations were needed to retard the parasite growth dramatically, the multiplicity of mutations of more than two did not have further effect on parasite growth.

It was reported before in this study, that the age (median, 22 years) of patients without gametocytemia during the follow up days, was significantly higher than the age of patients...
who had different rates of gametocytemia (11 to 13 years). It was also shown that gametocytogenesis was significantly higher in parasites bearing the mutant rather than the wild alleles of \textit{dhfr/dhps} genes, as recognized elsewhere\textsuperscript{171,338}. However, the new finding is that; at least two (double) mutations were needed to trigger gametocytogenesis, and possibly that was all needed i.e. the further increase in mutations (up to quintuple mutants) did not influence the gametocytogenesis any more. In the in-\textit{vivo} study, it was found that patient who achieved ACR had significantly lower frequency of gametocytemia and shorter gametocyte longevity compared to patients had TF. That is interesting because, there was no difference in the frequency of \textit{dhfr/dhps} mutations between the two treatment-outcome-groups of patients, the ACR and TF group, see Table (3.10). The differences can’t be due to gametocytogenic effect of SP treatment, as both ACR and TF groups were treated with SP. Although, the gametocytogenesis is the corner stone in the spread of the resistant strains, there are only few studies which had addressed the issue in molecular terms\textsuperscript{169,171}.

\textbf{4.3 Submicroscopic \textit{P. falciparum} infections during pregnancy:}

This appears to be the first research on submicroscopic \textit{P. falciparum} infections during pregnancy in an area of unstable, low intensity malaria transmission (eastern Sudan). Approximately one in every three of the pregnant Sudanese women who had been found smear-negative for malaria had PCR-based evidence of submicroscopic \textit{P. falciparum} infection. This prevalence is comparable with those, of 18\%–46\%, previously reported from areas of other African countries with high-intensity transmission\textsuperscript{339,340}. Among both the pregnant (present study) and non-pregnant women of eastern Sudan\textsuperscript{280}, the prevalences of submicroscopic \textit{P. falciparum} infections in the wet season appear similar to those in the dry season. The reasons for this surprising lack of seasonality have yet to be elucidated.

In the present study, the age, gravidity and gestational age of the pregnant women with submicroscopic \textit{P. falciparum} infection were similar to those of the other smear negative pregnant women. Similar observations were recently made in an area of Gabon with intense malaria transmission\textsuperscript{339}. In Ghana, however\textsuperscript{340} reported that the prevalence of submicroscopic \textit{P. falciparum} parasitemia among pregnant women increased with increasing gravidity and gestational age — each pregnancy in a woman’s life enhancing her immune protection and reducing the likelihood that she would develop a parasitemia that was intense enough to be detected by routine microscopy.
The most prevalent MSP-2 allelic family in the parasite populations characterized in the present study was of the FC27 type, as also recently seen in Gabon. The mean number of clones/submicroscopic infection seen in the pregnant women of New Halfa was only 1.016. In areas with intense transmission, the multiplicity of *P. falciparum* infections in pregnant women tends to be higher than this, with means of one to four clones/infection. The extent of allelic diversity in the MSP-2 gene, as estimated by the total number of different alleles found in a given parasite population and the mean multiplicity of infections, appears to be positively correlated with the local level of endemicity.

In the present study, as in Mozambique and Gabon, there was no evidence that submicroscopic *P. falciparum* infections led to increased risk of anemia in pregnant women. In endemic areas of Ghana, however, such infections may be associated with anemia among pregnant women, and 20%–25% of children with anemia do not have parasitemia that are detectable by routine microscopy, although many such children have other evidence of malarial infection.

### 4.4 Conclusions:

In Gedarif area, the pattern of severe malaria under the prevailing epidemiological situation in Eastern Sudan varies considerably from the classic sub-Saharan pattern of severe malaria. Only in such epidemiological setting a clear distinction between the individual complications of severe malaria can be made. This has broad implications, from the molecular understanding of pathogenesis, to case management and control programs at large. It is believed that, this is the first report about severe malaria in the Sudan and it is important for filling the paucity in the knowledge about the epidemiology of severe malaria.

While complexity of infection was associated with clinical malaria compared to asymptomatic malaria infections in this study, the MCN was not significantly different between UM and SM. The low complexity of infection was associated with both asymptomatic malaria and fatal CM, but in the former the predominant single clone infections were FC27 genotype while in the later were only IC1 genotype infections. Otherwise, MSP2 genotype families, IC1 and FC27, and individual allele types (sizes) of both families were similarly distributed in SM and UM. Although the FC27 genotypes were associated with the milder forms of malaria infection, after quinine treatment of multi-clone infections, IC1 clones were cleared significantly earlier than FC27 clones. In general, in this setting the molecular characteristics of malaria infection including SM, varies from that in...
areas of high malaria transmission and inclusion of detailed clinical data in analysis is critical for accuracy of conclusions.

Irrespective of the molecular marker that can be used, addition of any marker should be meaningful to disclose hidden diversity, would have not been revealed even with the most polymorphic markers when used alone. Furthermore, this data indicated an extreme diversity of parasites causing severe malaria including cerebral malaria, and possibly each individual infection was caused by a genetically unique parasite isolate.

In New Halfa area, cerebral malaria may be associated with latent parasitemia in partially immune adults. One of the implications of this finding would be that the diagnosis of CM malaria should not be ruled out if the blood film result is proved to be negative, and there is strong clinical suspicion of CM. That urges development of a robust and friendly to use molecular tools for diagnosis of scanty peripheral parasitemia especially in naive and semi-immune populations. Development of such tools might also result in reduction of malaria infection following use of microscopically screened blood for transfusions, in areas of low malaria endemicity. In conclusion, uniquely in this setting cerebral malaria can be associated with subpatent parasitemia, thus a more sensitive diagnostic tool than microscopy is needed.

For the estimation of drug efficacy in Eastern Sudan, Gedarif area, this is the first and most comprehensive study for estimation of the magnitude of SP resistance in Eastern Sudan and the deployment of SP in combination with CQ for treatment of UM. While more than one third of the clinical malaria infections were resistant to SP, no advantage for the SP/CQ combination therapy over SP monotherapy. The finding about the role of the semi-immunity in clearance of residual parasitemia following therapeutic treatment of malaria is strengthened by the association of resistance with young age. Moreover, the disproportionately high prevalence of CQ and SP drug resistance associated alleles compared with the in-vivo drug resistance rate, is a further evidence for the role of semi-immunity in parasite clearance. We also showed that gametocytogenesis was provoked by SP treatment, and equally it was associated with young age and treatment failure.

Although SP resistance is propagating fast in the area, semi-immunity to malaria contributes in clearance of SP resistant parasites was demonstrated. Furthermore, this data suggested that, the fast propagation in SP resistance is likely to be attributed to the selection of SP resistant parasites and the expansion of the resistant population through the gametocytogenic effect of SP.
The implication of *dhfr/dhps* mutations in parasite growth and gametocytogenesis was more prominent than the implication in resistance to SP. At least 2 mutations in the *dhfr/dhps* genes were needed to retard the parasite growth and to enhance gametocytogenesis considerably, while the drug response to SP treatment was reduced with increasing multiplicity of mutations but is not correlating with the in-vivo result. At least three factors were contributing in parasite clearance, the SP treatment, the parasite mutations and the host immunity.

Fansidar and chloroquine resistance in Sudan was found to be very high; it seems that chloroquine is not working at all, since the percentage failure in the two groups was likely to be the same. Fansidar resistance is propagating rapidly in the area and it associated also with gametocyte carriage. Thus it is suggested that the policy of malaria treatment in the area should be changed by excluding fansidar from the first line of treatment. The fansidar and chloroquine can be reused again once resistance to them has decreased as they are both cheap and affordable drugs.

The findings in the detection of submicroscopic parasitemia in pregnant women in New Halfa area in this study showed that, although the present study failed to demonstrate a significant difference in age, parity, gestational age or hemoglobin concentration between the pregnant women with and without submicroscopic *P. falciparum* infections, the prevalence of malarial infection during pregnancy in Sudan is clearly much higher than indicated by the results of routine microscopy. Many women found smear-negative at the delivery of their babies probably have the placental malarial infections that are associated with low birthweights and other adverse outcomes. Submicroscopic *P. falciparum* infections could represent a significant problem in terms of materno–foetal health.

**REFERENCES**


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Severe Malaria Protocol, Clinical questionnaire
Gedarif Teaching Hospital & New Halfa Teaching Hospital

Sample ID: ………..SC. Dates: admission: …/…/2000; discharge …/…/2000
Name: ................................................................. Gender: M ( ), F ( )
Date of birth: ........................................... age: .................................................................
Residence (detailed): .................................................................
Contact address: .................................................................
Occupation: .................................................................
Tribe: .................................................................
For females: pregnant ( ), lactating ( ). Last menstrual cycle: .........................
C/O:
1. History obtained from: patient ( ), relative ( )
2. Fever:
   A/ Duration: ............... days. B/ Grade: mild ( ), moderate ( ), severe ( )
   C/ continuous ( ), intermittent ( ). D/ regular ( ) irregular ( ). E/ rigors (Yes, No).
   F/ sweating (Yes, No).
General ill health, headache ( ), joint pain ( ), back pain ( ), fatigue ( ), bitter taste ( )
Bleeding: no bleeding ( ), epistaxis ( ), haematemesis ( ), haemoptysis ( ), bleeding
from other site (…………………)

CNS: confusion ( ), convulsions ( ), loss of consciousness ( ), Hallucination ( ), coma (Y, N), duration …… Seizures : (partial, general), (L side, R side), duration……

Abdominal symptoms: pain ( ), nausea ( ), anorexia ( ), vomiting ( ), diarrhoea ( ).

3. Respiratory symptoms: Cough ( ), breathlessness ( ).

4. Urinary system: anuria, polyurea, haematurea

Medical History:

1. Malaria history

   Number of attacks ≥ 1 a year ( ), 1 every two years ( ), 1 ≤ every three years ( ), not at all ( ).

   1. History of severe malaria (Yes, No), what type………………………………………..

   2. Last clinical attack : ………….. Method of diagnosis : laboratory ( ), clinical ( )

   3. Treatment received: ………………………………………………………………………

   3. History of: Diabetes mellitus ( ), hypertension ( ), renal diseases ( ), asthma ( ),

      other diseases : …………………………………………………………………………..

   4. History of prolonged drug use (more than 1 week): ………………………………………

Clinical examination:

General condition: A/ ill ( ) well ( ); B/ Normal ( ), pale ( ), jaundiced ( ), cyanosed ( )

C/ drowsy ( ), confused ( ), comatose ( ), convulsions ( ), stiff neck ( ).

Oral Temperature ……..°C           Weight : ……..kg, Nails : normal ( ) clubbing ( ), spooning ( ).

Blood pressure: ………….. Pulse ………….. RR …………..

Speech: normal ( ), slurred speech ( ), dysphasia ( ).

Tongue: normal ( ), dry ( ), coated ( ), angular stomatitis ( ).

Skin: dry ( ), rash ( ).

Systemic examination

1. Central Nervous System : …………………………………………………………………

   Position: normal ( ), decerebrate ( ), decorticate ( ), opisthotonus ( ).

2. Respiratory system: rhythm (regular, irregular), hyperventilation ( ), deep slow breathing ( ), shallow fast breathing ( ) intercostals recession ( ).

   auscultation…………

3. Gastrointestinal system : Liver size, ………cm, spleen,………cm

   others…………………………………………………………………………………………

4. Genitourinary system: …………………………………………………………………

5. Muscle-skeletal system: …………………………………………………………………

   ………………………………………………………………………………………………………

Investigations:

Blood film for malaria: BF………..

Species: falciparum ( ), vivax ( ), malariae ( ), ovale ( ).

Parasite count: …………/µl

Complete haemogram:

Hb ……..% (………gm/dl), Haematocrit …….. Reticulocytes…………TWBC: …………………,

lymphocytes ……..%, Neutrophils ……..%, ……..Basophils ……..%, Esinophils………………% 

Thrombocyte count ………………………………ESR (1 hour) ………………………………..

Biochemical analysis:

Blood glucose: ……………………………Urine general: ………………………………………
Renal Function Test:
- Blood: pH ........ Urea .........., serum creatinine........., plasma bicarbonate. .......
- Widal test for typhoid: ...................Widal test for brucellosis: ...................
- Chest X-ray (when needed): .................................

Current treatment
1- anti-malarial. ........................................2. Fluid: ................................

Annex (I) For Cerebral malaria (Adults)
- Eye: Ptosis ( ), Nystagmus ( ), squint ( ). Pupils: normal ( ), dilated ( ), constricted ( ), fixed ( ).
- Fundi exam: ...........................................................
- Cranial nerves: ....................................................
- Upper limbs: .........................................................
- Lower limbs: ......................................................
- Plantar reflex (down going, equivocal, up going)

Modified Glasgow coma scale ( )

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>5 - Obeys commands ( )</td>
<td>- Oriented ( )</td>
<td>- ...............</td>
</tr>
<tr>
<td>4 - Localized pain ( )</td>
<td>- Confused ( )</td>
<td>- Spontaneously ( )</td>
</tr>
<tr>
<td>3 - Flexion to pain ( )</td>
<td>- Inappropriate words ( )</td>
<td>- To speech ( )</td>
</tr>
<tr>
<td>2 - Extension to pain ( )</td>
<td>- Incomprehensible sounds ( )</td>
<td>- To pain ( )</td>
</tr>
<tr>
<td>1 - None ( )</td>
<td>- None ( )</td>
<td>- Never ( )</td>
</tr>
</tbody>
</table>

Annex (II) For Children
- Fontanelle; normal ( ), sunken ( ), bulging ( ), absent ( ).
- Can the child sit without support (Y,N), drink/breastfeed (Y,N).
- Nasal flaring (Y,N)

Blantyre coma scale

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>2. - Localized painful stimulus</td>
<td>- Appropriate cry</td>
<td>-</td>
</tr>
<tr>
<td>1. - Withdraw limb from pain</td>
<td>- moan / inappropriate cry</td>
<td>- Directed</td>
</tr>
<tr>
<td>0. - Non-specific or absent response</td>
<td>- None</td>
<td>- Not directed</td>
</tr>
</tbody>
</table>

Annex (III) For pregnant ladies

- Gravidity: ......................................................
- Abortions: No................. causes: fever ( ) others ( ).
- Current pregnancy: ......weeks.
- 1st trimester ( ), 2nd trimester ( ), 3rd trimester ( ).

Follow up
Day 3 ...........................................................................
Day 7 ...........................................................................
Day 14 ..........................................................................
Day 28 .........................................................................
Appendix 3: Schematic representation of MSP-1 of *P. falciparum* and of recombinant Merozoite surface protein-1 (MSP-1). The division into 17 blocks is as outlined by Tanabe et al., (1987); blocks of conserved sequences are denoted by open boxes.

Appendix 4: Schematic representation of Merozoite surface protein-2 (MSP-2) of *P. falciparum*
Appendix 5: Schematic representation of Glutamate rich protein (GLURP) of *P.falciparum*
الموافقة على علم

مركز أبحاث المalaria

**Appendix 6: The consent**