Dynamics of Resistant *Plasmodium falciparum* Parasites

NIZAR ENWEJI
Persistence of drug resistant *Plasmodium falciparum* is a major problem to management and control malaria in endemic areas. The focus of this thesis was to study the dynamics of resistant *P. falciparum* parasites. The study was performed in two African countries: 1) Sudan: Asar village in eastern Sudan, here we examined the persistence of drug sensitive and resistant *P. falciparum* genotypes among individuals with single-clone and multiple clones infection during the dry season. We genotyped microsatellite loci in the vicinity of the dihydrofolate reductase gene (*dhfr*) and the dihydropteroate synthase gene (*dhps*). Microsatellite investigation showed that asymptomatic parasitemia persisted in some patients for several months throughout the dry season and into the next transmission season. In some samples mixed infections were detected, and we noted several cases where the microsatellite haplotype varied from month to month, suggesting turnover of different parasite populations in the blood. This demonstrates that even during asymptomatic infections there can be dynamics within the parasite population in an individual. In addition, we calculated the parasite density throughout the dry season to the next transmission season by using allele-specific quantitative PCR. Parasite density during the dry season fluctuated, but was generally lower than in the first transmission season. A significant difference (P<0.05) between dry and first transmission season was found in regard to the parasite density, whereas no significant difference was observed when dry and second transmission season were compared (P>0.05). 2) Ethiopia: West Arsi zone, one of the malaria endemic zones of the Oromia region. In the first study we determined the prevalence of asymptomatic malaria carriages from November-December 2012. According to PCR the prevalence of sub-microscopic *P. falciparum* carriage was 19.2%, microscopy-based prevalence was 3.7% while the prevalence was 6.9% using RDT. Based on this, PCR was considered a better tool for measuring *Plasmodium* prevalence than microscopy and RDT. A second study addressed the genetic diversity of chloroquine resistance (CQR) in *P. falciparum* by analysing four microsatellite markers in and around the *pfcrt* gene. Although CQ was withdrawn for more than a decade, 100% of the parasites still carried the *Pfcrt* K76T mutation. Only the CVIET haplotype was identified. Based on combinations of MS markers, seven different Ethiopian CQR variants (E1-E7) were identified. Both intronic and MS flanking the *pfcrt* gene showed low levels of diversity.

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To the memory of my father
To my mother
To my wife and my daughter
To my wonderful sister and devoted brothers
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I  Enweji N*, Kheir A*, Kerje S, Abdel-Muhsin A, Babiker H, Swedberg G. Dynamics of asymptomatic malaria infections as revealed by microsatellite typing. Manuscript


*Shared authorship

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### Abbreviations

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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin based combination therapy</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
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<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<tr>
<td>CQR</td>
<td>Chloroquine resistance</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EIR</td>
<td>Entomological inoculation rate</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>IPTp</td>
<td>Intermittent preventive treatment for pregnant women</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor residual spraying</td>
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<tr>
<td>ITNs</td>
<td>Insecticide treated bed nets</td>
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<tr>
<td>Kb</td>
<td>Kilo base pair</td>
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<td>MS</td>
<td>Microsatellites</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Pfcrt</td>
<td><em>P. falciparum</em> chloroquine resistance transporter gene</td>
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<tr>
<td>Pfdeltafr</td>
<td><em>P. falciparum</em> dihydrofolate reductase</td>
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<td>PfDhps</td>
<td><em>P. falciparum</em> dihydropteroate synthase</td>
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<td>RDTs</td>
<td>Rapid Diagnostic Tests</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
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<tr>
<td>SP</td>
<td>Sulfadoxine-pyrimethamin</td>
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<td>TB</td>
<td>Tuberculosis</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

Global burden of malaria

Even though it has been more than 100 years since the discovery of the causative parasites of malaria, the disease still remains a major global health problem. Reports from the World Health Organisation (WHO) have shown that half of the world’s population, in 97 countries, live at risk of malaria (WHO 2013a). About 207 million cases of malaria worldwide (uncertainty interval, 135-287 million), associated with almost 627 000 malaria deaths (uncertainty interval, 473 000-789 000), were estimated in 2012. The risk of suffering or even dying from contracting malaria varies considerably between populations. For instance, children less than five years of age, pregnant women, and patients with HIV/AIDS are at higher risk than any other population group. In addition, 90% of malaria-related deaths occur in Africa, where one child dies of malaria every 60 seconds.

It has been shown that malaria is closely associated with economic and social development (Malaney, Spielman et al. 2004). Malaria is known to be both a disease of poverty and a cause of poverty. While poverty can cause malaria by decreasing the capacity for disease prevention (at both individual and government levels), malaria can also cause poverty, for example through indirect economic costs, medical cost, loss of income, or a negative impact on trade. Although the work towards elimination of malaria began several decades ago, the focus has now shifted from eradication to control, due to the dramatic acceleration of disease and mortality rates (Gregson and Plowe 2005). During the last 10 years this trend has been reversed.

The malaria parasite and vector

Malaria is caused by protozoan parasites of the phylum Apicomplexa and genus Plasmodium. There are about 120 Plasmodium species that can infect mammals, birds and reptiles. Five Plasmodium species can infect humans: Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale and Plasmodium knowlesi. It has been reported that P. knowlesi, whose natural host is macaque monkeys, can infect humans as well (Singh, Kim Sung et al. 2004, Cox-Singh and Singh 2008). The most
common human-infecting species are *P. vivax* and *P. falciparum*, which cause the most severe cases of disease and the highest number of deaths. According to WHO 2000, different malaria parasite species vary in geographical distribution, microscopic morphology, clinical presentation, and susceptibility to anti-malarial drugs.

Human malaria is transmitted from one human to another by the bite of an infected female mosquitoes of the *Anopheles* genus. The main vectors are the *Anopheles gambiae* complex in sub-Saharan Africa (Coetzee, Craig et al. 2000), and the *Anopheles minimus* complex in South East Asia (Van Bortel, Trung et al. 2003).

**Life cycle of the malaria parasite**

The life cycle of the malaria parasite is extremely complex and requires both the human host and an insect vector. The life cycle of the malaria parasite is illustrated in Figure 1. When an infected female *Anopheles* mosquito penetrates human skin to obtain a blood meal it injects saliva mixed with an anticoagulant, and also injects sporozoites into the blood stream. The sporozoites travel to the liver and enter the liver cells (hepatocytes). At this stage they can either remain dormant as a hypnozoite form (*P. vivax* or *P. ovale*) (Schlitzer 2008, Winzeler 2008) or, in the case of *P. falciparum*, the sporozoites remain in the liver for 7-14 days and undergo asexual replication to form tissue-stage schizonts (exo-erythrocytic schizonts), in which each sporozoite can give rise to tens of thousands of merozoites. When the hepatocyte ruptures, merozoites are released into the blood stream and invade erythrocytes. Once inside the erythrocyte, the parasite matures from merozoite to early trophozoite (ring stage). The trophozoite develops to schizont.

The erythrocyte ruptures and releases merozoites into the blood stream again. *Plasmodium* enters a sexual phase when some merozoites in the erythrocytes develop into gametocytes, cells capable of producing both male and female gametes. Gametocytes can be ingested by *Anopheles* mosquitoes biting an infected individual, and form micro (male)-and macrogametes (female) in the mid-gut of the mosquito. The gametes undergo fertilization and form a zygote, which rapidly develops into an ookinete that penetrates the intestinal walls of the mid-gut and develops into an oocyst. Asexual replication occurs within the oocyst and produces sporozoites. These sporozoites migrate to the salivary glands of the mosquito, and from there are injected into the blood stream of a human, thus starting the parasite life cycle again.
Malaria symptoms

The symptoms of malaria infection in humans are caused by the asexual blood stage of the parasite life cycle. Uncomplicated or mild malaria symptoms are fever, headache, diarrhoea, vomiting, coughing and abdominal pain. Severe malaria symptoms including respiratory distress, coma, cerebral malaria, anemia, renal failure, splenomegaly and hypoglycemia (Weatherall, Miller et al. 2002). If the uncomplicated malaria infection is not treated it can develop into severe malaria and lead to death.

Transmission and epidemiology

There are about 400 species of *Anopheles* mosquitoes, of which about 40 can transmit malaria. Malaria is transmitted by different *Anopheles* species, depending on the region and the environment. Anophelines that can transmit malaria are found not only in malaria-endemic areas, but also in areas where malaria has been eliminated. The latter areas are thus constantly at risk of re-introduction of the disease (CDC 2012a).

Malaria transmission can be stable or unstable. It can be rather constant from year to year in stable areas, whereas in unstable areas the transmission varies. Although both are endemic, there is a greater risk of epidemics in the unstable areas, i.e. the number of malaria cases considerably exceeds what is
normally expected. The stable transmission is more common in areas where the vector favours feeding on humans and has a high survival rate. On the other hand, in areas where the vector bites animals and human and/or has a low survival rate, the unstable transmission is more common (Reiter 2008). The percentage of people live in areas at risk of malaria is illustrated in Figure 2.

![World map demonstrate percentage of people live in areas at risk of malaria transmission. Adopted from (WHO, 2014).](image)

**Figure 2.** World map demonstrate percentage of people live in areas at risk of malaria transmission. Adopted from (WHO, 2014).

**Malaria control**

Malaria is both preventable and a curable disease. Increased malaria prevention and control measures dramatically has decreased the malaria burden in many places (WHO 2014). The world's first attempt to eradicate malaria came after World War II. The WHO started malaria eradication programs in 1955 in many African countries (WHO 2008).

Malaria diagnosis was historically based only on symptoms, with fever being the main symptom. Fever, however, is not exclusively a symptom of malaria. Therefore, many patients have been given the wrong treatment where antimalarials have been overused. Thus, the WHO recommended a parasitological confirmation of the diagnosis of malaria before starting treatment. The two main methods for malaria diagnosis are:

1). **Microscopic diagnosis:** Microscopy is the gold standard and preferred technique for diagnosing malaria. Examination of thick and thin blood films reveals malaria parasites. Further, thick films are more sensitive than thin films for detecting low density malaria parasitaemia.
2). Rapid Diagnostic Tests (RDTs): These are also known as dipsticks. If microscopy is not available or not feasible, a rapid diagnostic test (RDT) should be used as an alternative way of diagnosing malaria in patients. The test provides results in 2-15 minutes (CDC 2012c, WHO 2013c). HRP2 antigen is targeted by RDT and is specific to the \textit{P. falciparum} parasite; pLDH and parasite aldolase are target antigens for RDT and are specific to species other than \textit{P. falciparum} (Moody 2002, Hawkes, Conroy et al. 2014).

The tools currently used for malaria control are:

\textbf{Insecticide treated bed nets (ITNs):} This is simply a mosquito net treated with insecticides. ITNs are much more protective than untreated nets. In Africa, ITNs have been shown to reduce the death of children younger than 5 years old, from all causes, by about 20% (CDC 2014).

\textbf{Indoor residual spraying (IRS):} The objective of this method is to kill mosquitoes by spraying the inside walls and other surfaces of a house with a residual insecticide. For several months, the insecticide will kill mosquitoes that come in contact with these surfaces. IRS usually kills mosquitoes after they have fed if they come to rest on the sprayed surface. Therefore, IRS prevents transmission of infection to other people (CDC 2012b).

\textbf{Intermittent preventive treatment (IPTp):} This prophylactic treatment is recommended for pregnant women. IPTp reduces maternal malaria episodes, maternal and fetal anaemia, low birth weight, and neonatal mortality (WHO 2013b). IPTp with sulfadoxine-pyrimethamine (IPTp-SP) has been shown to successfully reduce the burden of pregnancy-associated malaria (PAM), and is currently part of the national malaria prevention programme in most African countries (Bertin, Briand et al. 2011).

\textbf{Larvicides:} This is an insecticide used to kill immature mosquitoes before they become adults (mosquito larva stage) (CDC 2013).

A limited number of antimalarial drugs are under current clinical use; these include: Chloroquine, antifolate (Sulfadoxine-pyrimethamine), lumefantrine, artemisinin and its derivatives (artesunate, artemether), as well as antibiotics. The purpose of antimalarial treatment in severe malaria is to prevent death. For uncomplicated malaria, the purpose is to cure, i.e. eradicate the infection to prevent development to severe disease.

\textbf{The \textit{P. falciparum} parasite}

The \textit{P. falciparum} clone 3D7 nuclear genome covers 22.8 megabases (Mb) of DNA, consists of 14 chromosomes, and encodes about 5300 genes. It also contains apicoplast and mitochondrial genomes, which are extrachromosomal DNA of sizes 35kb and 6kb, respectively. The malaria
parasite is found to be rich in Adenine/Thiamine (A+T), which comprises about 80% of the *P. falciparum* genome (Gardner, Hall et al. 2002).

**Microsatellite loci**

Microsatellites (MS), known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are repeating sequences of 2-6 base pairs of DNA. MS are considered to be molecular markers that can provide valuable information on population structure in the malaria parasite and are common in the *P. falciparum* genome (Su and Wellems 1996, Su, Ferdig et al. 1999). MS are widely distributed in eukaryotic genomes, and are highly variable. In addition, microsatellites can be amplified for identification by the PCR process, using the unique sequences of flanking regions as primers, and they have been used in many different research areas, such as forensics, diagnosis and identification of human diseases, population studies, and conservation biology.

MS-based studies have the limitation that they only reflect relatively recent evolution of *P. falciparum*. Therefore, markers with lower mutation rates are more powerful in studying long-term evolutionary history (Ferdig and Su 2000, Su and Wootton 2004).

Mutations associated with drug resistance in *P. falciparum* have occurred within the past 50 years, and therefore provide a unique chance to identify allelic associations.

These drug resistance genotypes are characterised by reduced diversity around the major resistance alleles (Su and Wootton 2004). In Asia and Africa, microsatellite markers with multiple allele flanking genes associated with drug resistance, such as *dhfr* and *pfcrt*, were investigated. This allowed for typing of specific haplotypes flanking these regions and enabled identification of drug resistance origin, as well as tracing the spread of these mutations (Nair, Williams et al. 2003, Roper, Pearce et al. 2003).

**Antimalarial drug resistance**

In 1973 the WHO defined drug resistance as the ability of the parasite to survive and/or multiply despite the administration and absorption of a drug given in dose equal to or higher than recommended, but within the limits of tolerance of the subject (WHO 1986).

Drug resistance may cause treatment failure; however, treatment failure does not always have to be caused by drug resistance. Other factors may lead to treatment failure: inadequate dose or duration of treatment; fake drug with no or little active ingredient; or poor absorption. In malaria treatment, resistance to all known anti-malarial drugs, including the newly introduced
artemisinin based combination therapy (Carrara, Zwang et al. 2009), has developed to various degrees in several countries.

Chloroquine
Chloroquine (CQ) was discovered in 1934 by Hans Andersag and co-workers at the Bayer laboratories in Eberfeld, Germany and given the name Resochin (Dondorp, Yeung et al. 2010). CQ has been used for decades as an anti malarial medication, is cheap to produce and relatively well tolerated compared to Quinine (QN). The standard dose of CQ recommended by WHO for treatment of uncomplicated malaria is 10mg/kg on days 0 and 1 and 5mg/kg on day 2, giving a total dose of 25mg/kg. CQ may cause side effects, such as: diarrhoea, nausea temporary hair loss, dizziness and worse psoriasis (Taylor and White 2004, AlKadi 2007). CQ is also used for treatment of other diseases, including viral infection, cancer and arthritis (Savarino, Boelaert et al. 2003).

Sulfadoxine-pyrimethamine
Sulfadoxine-pyrimethamin (SP) or Fansidar is currently the main option to treat malaria in pregnancy. Use of SP began after the widespread resistance to chloroquine. The drug is easy to use because treatment involves only a single oral dose and it is cheap. SP acts by interfering with two key enzymes in the folate biosynthesis pathway. Sulfadoxine is a competitive inhibitor of dihydropteroate synthase (DHPS). Pyrimethamine is a competitive inhibitor of dihydrofolate reductase (DHFR). The inhibition of these two enzymes affects the tetrahydrofolate synthesis that is required for DNA synthesis (Sibley, Hyde et al. 2001). The WHO recommends intermittent preventive treatment for pregnant women (IPTp) in all areas with moderate to high malaria transmission in Africa (WHO 2013b).

Artemisinin based combination therapy (ACT)
The World Health Organisation (WHO) recommended a switch of first-line treatment against uncomplicated malaria to artemisinin-based combination therapies (ACTs) for countries where conventional antimalarial treatments such as chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) have become ineffective (WHO 2006). Combination therapy for treatment of infectious diseases is common in diseases such as TB, HIV infection, and in cancer. This strategy is also applicable to malaria treatment. By using ACT for malaria infection the chance that the parasites develop resistance as a result of genetic mutations to two drugs with different modes of action becomes much lower than that of parasites developing resistance to a single treatment (White 1999, Nosten...
and White 2007). Therefore, there are a number of ACTs being used or tested in different *P. falciparum*-endemic regions (Kremsner and Krishna 2004). The most common antimalarials used in combination therapy are: artemether-lumefantrine (AL), artesunate-mefloquine (ASMQ), artesunate-amodiaquine (ASAQ), artesunate-sulfadoxine-pyrimethamine (AS-SP) and dihydroartemisinin-piperaquine (DHA-PQ). Artemether–lumefantrine (Coartem) is a fixed-dose oral combination for treating uncomplicated *falciparum* malaria in adults and children (Kokwaro, Mwai et al. 2007). Coartem’s efficacy against *falciparum* malaria has been validated in several clinical trials. Ethiopia adopted artemether-lumefantrine as first line therapy in 2004 (FMOH (Federal Republic Ethiopia 2004, Jima, Tesfaye et al. 2005, Assefa, Kassa et al. 2010). In Sudan, artesunate–sulphadoxine–pyrimethamine (AS–SP) and artemether–lumefantrine (AL) are the recommended first- and second-line treatments for uncomplicated *P. falciparum* malaria, respectively (Abdallah, Ali et al. 2012). In addition, Artemisinin and its derivatives are generally safe and well-tolerated (Cui and Su 2009).

**Epidemiology of drug resistance**

Chloroquine resistance (CQR) was first reported in 1957 in the Cambodia–Thailand border. In 1959, chloroquine resistance emerged independently in South America, and was reported in Africa 17 years after the first cases in Asia. However, once chloroquine-resistant *P. falciparum* strains had gained a toehold in Africa, they spread rapidly from country to country (Payne 1987).

The increase in CQR in East Africa has led to a rise in malaria mortality. Correspondingly, a significant rise in malaria mortality in children under 5 years of age has been observed in Senegal in West Africa, simultaneously with the emergence of CQR in the area. However, antimalarial drug resistance has also been implicated in the increasing frequency and severity of epidemics. The conditions for the development and spread of drug resistance differ between the Asian and African continents. Migration of individuals carrying resistant gametocytes has probably been of major importance for the spread of CQR between different endemic areas in Asia and Oceania and for the initial introduction of CQR to East Africa (WHO 2001). Widespread chloroquine resistance has forced those countries to switch treatment from chloroquine to sulfadoxine/pyrimethamine (SP, trade name Fansidar) (Gatton, Martin et al. 2004). Since the 1980s, SP failures have increased significantly, due to the presence of resistant parasites (Maiga, Djimde et al. 2007). First reports on SP resistance were reported on the Thai-Cambodian border, then increasing resistance was documented in other parts of Southeast Asia, southern China and the Amazon Basin,
rendering them established multi-drug resistant (MDR) areas (Wernsdorfer and Payne 1991, Wongsrichanalai, Pickard et al. 2002).

**Mechanisms of drug resistance**

Many factors affect the emergence and spread of resistance to antimalarial drugs, such as: characteristics of the drug itself, the human host, the vector, the parasite and the environment (see Figure 3).

**Figure 3.** Factors affecting the emergence and spread of resistance to antimalarial drugs.

**Origins and evolution of antimalarial drug resistance**

Resistance in nature occurs through spontaneous mutations, which are rare events. For some drugs a single mutation is enough to cause resistance, while for others multiple mutations may be necessary. The development of resistance is the result of both genetic change and its selection. The intensity and the timing of resistance emergence depend on several factors. The single most important reason is, however, the drug pressure. There are two processes which are necessary for evolution of drug resistance: (a) A resistant genotype is generated by mutation; (b) the spread of this mutation within and between parasite populations (Anderson and Roper 2005).
Mutation rates in *Plasmodium* genes have been found to be in the order of \(10^{-9}\), when measured (Paget-McNicol and Saul 2001).

### Antimalarial resistance genes

Three genes relevant to the study are discussed: chloroquine resistance transporter gene (*pfcrt*), associated with chloroquine resistance; and the genes encoding dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), which are associated with Sulfadoxine/Pyrimetamine resistance.

#### Pfcrt

A major breakthrough in the search for the genetic basis of CQR in *P. falciparum* was the identification of *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) on chromosome 7. PfCRT is a 48kDa protein containing 424 amino acid, 10 predicted transmembrane-spanning domains, and is localised to the digestive vacuole (DV) membrane in erythrocytic stage parasites (Fidock, Nomura et al. 2000).

The main point of mutation, K76T, appears to be necessary for the resistance phenotype, and is the most reliable molecular marker of resistance among the various *pfcrt* mutations (Djimde, Doumbo et al. 2001). The whole section 72-76 of *pfcrt* seems to be important. *P. falciparum* CQR strains carrying haplotype CVIET at position 72-76 of the *pfcrt* locus are common in South-East Asia and Africa, and SVMNT haplotype has been reported in South America (Fidock, Nomura et al. 2000) but rarely in Africa (Alifrangis, Dalgaard et al. 2006). The *P. falciparum* chloroquine-sensitive strains from malarious regions around the world carry CVMNK haplotype at codons 72-76 (Mehlotra, Fujioka et al. 2001).

#### Pfdhfr and Pfdhps

Mutations in the genes that encode the target enzymes of the antifolate drugs have been known for several years to be associated with in vitro resistance to these drugs. The major enzyme targets of malaria chemotherapeutic in the folate biosynthetic pathway are dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS); the inhibition of these enzymes leads to decreased production of tetrahydrofolate (Ferone 1977). Tetrahydrofolate is an essential cofactor for the production of a number of folate precursors, including methionine and dTMP. Moreover, decreased production of these precursors would severely inhibit the *Plasmodium* parasite life cycle (Sibley, Hyde et al. 2001). Mutations in the *dhfr* gene are associated with pyrimethamine resistance and mutations in the *dhps* gene are associated with sulfadoxine resistance (Brooks, Wang et al. 1994, Salgueiro, Vicente et al. 2001).
2010). The mutations found to be associated with pyrimethamine resistance are: S108N, N51I, C59R, and I164L. A change from wild type Ser108 to Asn 108 (S108N) in \textit{dhfr} is enough to cause a low level of pyrimethamine resistance. The other additional mutations (N51I, C59R and I164L) in the \textit{dhfr} gene can yield higher levels of SP resistance than does the mutation S108N alone (Cowman, Morry et al. 1988, Peterson, Walliker et al. 1988). In sulfadoxine resistance, five points of mutation have been involved (S436A/F, A437G, K540E, A581G and A613S/T) in the \textit{dhps} gene. The double \textit{dhps} mutation A437 and K540E is highly associated with SP treatment failure (Happi, Gbotosho et al. 2005, Alker, Kazadi et al. 2008). In East Africa, a triple mutation of \textit{dhfr} (S108N, N51I, C59R) combined with a \textit{dhps} double mutation (A437G and K540E), which is known as a “quintuple mutant”, is predictive for SP treatment failure (Kublin, Dzinjalamala et al. 2002, Sridaran, Mc Clintock et al. 2010, Zeile, Gahutu et al. 2012). \textit{Pfdhfr} (I164L) is linked with high levels of pyrimethamine resistance (Sirawaraporn, Sathitkul et al. 1997).

**Malaria in Sudan (eastern part)**

Eastern Sudan has a dry savanna climate. The epidemiology of malaria is considered to be hypoendemic, with a short transmission period during the rainy season between July and November, followed by a hot dry season between November and June.

The transmission season is thus confined to the short period from November to December. The main mosquito vector in the region is \textit{Anopheles arabiensis}. The predominant parasite species is \textit{P. falciparum}, which accounts for more than 90% of malaria cases, while \textit{P.vivax} causes about 5%. The entomological inoculation rate (EIR) has been estimated to be less than one infective bite per person per year (Babiker, Abdel-Muhsin et al. 1998, Hamad, Nugud Ael et al. 2002). \textit{P. falciparum} parasites that cause clinical malaria during transmission seasons are characterised by great diversity, and are found as multiple clones in infected patients. Thus, it is suggested that these parasites belong to larger parasite reservoirs existing prior to the start of the next transmission season (Babiker, Abdel-Muhsin et al. 1998, Abdel-Wahab, Abdel-Muhsin et al. 2002).

**Malaria in Ethiopia**

Of Ethiopia’s 68 million inhabitants, about 45 million are estimated to be at risk of malaria infection. The most important determinants of malaria transmission in Ethiopia are altitude and climate (rainfall and temperature). In addition, transmission is seasonal and mostly unstable in character. The
major transmission of malaria follows the June – September rains and occurs between September and December. The minor transmission season occurs between April and May following the February – March rains. The major transmission season occurs in almost every part of the country, whereas the bimodal pattern of transmission is limited and restricted to a few regions that receive the small(belg) rains.

There are four major eco-epidemiological strata of malaria in the country:

I. Malaria-free highland areas above 2,500 metre altitude.
II. Highland fringe areas between 1,500 - 2,500 metre (affected by frequent epidemics).
III. Lowland areas below 1,500 metre (seasonal pattern of transmission).
IV. Stable malaria areas (all year round transmission).

The most dominant malaria parasites in Ethiopia are *P. falciparum* and *P. vivax*, which are distributed all over the country and account for 60% and 40% of malaria cases respectively. The parasite is mainly transmitted by the major mosquito vector *Anopheles arabiensis*. However, in some areas *Anopheles pharoensis*, *Anopheles funestus* and *Anopheles nili* also transmit the disease (WHO 2012)
Aims of the thesis

The overall aim of this thesis was to provide a better understanding of dynamics of *P. falciparum* parasites in symptomatic and asymptomatic infections.

The specific aims were:

I. To determine the dynamics of parasite populations during asymptomatic infections by using microsatellite analysis linked to *dhfr* and *dhps* genes.

II. To estimate the parasite density during the first transmission season, the dry season and the second transmission season by using Real-time quantitative PCR.

III. To investigate the presence of sub-microscopic *Plasmodium falciparum* infections using polymerase chain reaction (PCR) and to compare the PCR-based parasite prevalence against microscopy and rapid diagnostic test (RDT).

IV. To address the genetic diversity of chloroquine resistance in *P. falciparum* by analysing four microsatellite markers in and around the *Pfcr* gene.
Material and methods

Study sites

Studies that are included in this thesis have been performed in two African countries, Sudan (Eastern region) and Ethiopia (West Arsi zone, Shalla district).

Eastern Sudan

The study was conducted in Asar village, Gedaref state in Eastern Sudan. The transmission season is short (8-12 weeks) and distinctly seasonal, following the annual rains from July to November and reaching a peak in October. The number of malaria cases decreased substantially by January. A longitudinal survey (papers I&II) was conducted from October 1993 to December 1994; a cohort of 81 patients was followed for 15 months and here we reported 25 patients who showed persistent parasitaemia during the whole period, even during the dry season.

Ethiopia

The study was performed in Shalla district West Arsi zone, which is one of the malaria endemic zones of the Oromia region, with a population of around 177,000 living in malarious areas. The study district is located at a distance of 251 km from Ethiopia’s capital, Addis Ababa, and is 1500-2300 m above sea level (Golassa, Enweji et al. 2014).

West Arsi zone has ten districts, of which Shalla district has the highest number of malaria cases; here, \( P. falciparum \) and \( P. vivax \) are the two common causes of malaria. Two cross sectional studies (papers III and IV) were performed in the area from November to December 2012. A total of 1,453 blood samples were collected from clinical patients and sub-clinical subjects. The clinical patients were diagnosed by microscopy and RDT (SD BIOLINE Malaria Ag P.f/P.v POCT test kits (Standard Diagnostic, Inc, Germany, LOT No: 145021)) were used according to the manufacturer’s instructions.
Genotyping

Blood sampling and storage
All blood samples were collected on Whatman 3MM filter paper for parasite genotyping. After the blood had dried, the filter papers were stored in individual sealable plastic bags at room temperature.

DNA extraction
DNA was extracted from filter paper using the Chelex method (Plowe, Djimde et al. 1995). This protocol is designed for the isolation of genomic DNA from dried blood spots. The blood spots were excised from filter papers using a sterilised hole punch (1/8 inch) diameter. The samples were stored at -20°C for long term storage or at 4°C for shorter storage times.

PCR-RFLP
PCR-Restriction Fragment Length Polymorphism (RFLP) is, equipment wise, a simple way to analyse SNPs and therefore a suitable method to use in the field.
The nested-PCR products were digested with one unit of ApoI restriction enzyme at 55°C for three hours incubation time to identify the K76T mutation. After incubation, restriction fragments were analysed on 2% agarose gels with ethidium bromide and were visualised by UV Transilluminator in a VWR Gel Documentation System.

Sequencing
DNA sequencing samples were purified before sequencing using GeneJET PCR purification kit (Thermo Scientific) and sequenced using an automatic sequencer ABI3730 DNA analyzer at Uppsala Genome Centre.

Microsatellite analysis (STR)
Three MS loci, located 0.3, 4.4 and 5.3kb from position 108 of the dhfr gene and 0.8, 4.3 and 7.7kb from the 3’end of the dhps gene were analysed to determine variation of microsatellites flanking the drug resistance genes (paper I).
Four highly polymorphic MS loci (msint 2, msint 3, mscrt -2 and mscrt -29) were used for determining variation among pfcrt gene; each MS marker was amplified by semi-nested PCR in a LifePro thermal cycler (Bioer). Fluorescent PCR products were analysed in ABI 3730 DNA Analyzer
GeneMapper v4.0 software was used for analysis of the fragment data (paper IV).

Real-time PCR
Real-time quantitative PCR was used for determining the parasite density among asymptomatically infected patients (paper II). A LNA®-dual-labelled fluorogenic probe (Sigma-Aldrich) was designed to detect the dhfr108 wild-type sequence. The probe was dual labelled with a reporter dye at the 5' end and a quencher moiety at the 3' end. The MiniOpticon real-time PCR detection system (Bio-Rad) was used for all reactions.

Statistics
In paper II, GraphPad Prism software v5.0 was used for the statistical analyses. The data are expressed as the mean (95% confidence interval (CI)), if not otherwise indicated. A paired-sample t-test was performed to determine the differences between groups. Differences were regarded as significant when the P value < 0.05.
In paper III, statistical analysis was performed using Stata version 11. The $X^2$ test was used to compare the plasmodium carriage among different age groups. The test was considered statistically significant if the P value was < 0.05.
In paper IV, the heterozygosity (He) for each MS locus was calculated using the following formula: $He = \left[ \frac{n}{(n-1)} \right] \left[ 1 - \sum p_i^2 \right]$ where n is the number of alleles in the sample and $p_i$ is the frequency of the $i^{\text{th}}$ allele.

Ethical considerations
Ethical approval for Paper I and Paper II was obtained from the ethical committee of the Ministry of Health in Sudan. For Paper III and IV ethical approval was obtained from institutional review boards of Aklilu Lemma Institute of Pathobiology, Addis Ababa University and of the Armauer Hansen Research Institute, as well as the National Research Ethics Review Committee in Ethiopia.
Results and discussion

Paper I

As shown in Table 1, a remarkable variation in the microsatellite markers neighbouring both the \textit{dhps} and \textit{dhfr} genes during the dry season was identified. Most samples contained unique MS haplotypes, as expected. In addition, very few haplotypes appeared in more than one patient.

Out of the 25 individuals that were selected for this study, only five patients are presented in the table below. From the first transmission season to the middle of the dry season only one haplotype persisted in patient 1. However, as shown by marked changes in both the \textit{dhfr} and \textit{dhps} MS patterns, the patient was obviously infected with a new variant after the 8th month of the dry season.

In patient 2, MS genotyping showed a fluctuation between single and double parasite clones. In the second transmission season, another parasite clone appeared to have infected the patient, though parasitemia was still quite low, and the patient was asymptomatic with no fever.

When \textit{dhfr} MS were analysed in patient 3, mixed infections in the first transmission season were indicated. In addition, 3 different parasite clones persisted in the dry season, as shown by both the \textit{dhfr} and \textit{dhps} MS patterns. In contrast, patient 4 was infected with the same parasite clone (\textit{dhps} MS loci) from the first transmission season to the 4th month of the dry season. MS genotyping revealed a shift in the parasite population around the 5th month that remained stable until the second transmission season, when a new shift appeared. On the other hand, the \textit{dhfr} MS analysis for the same patient showed that the same clone persisted during the first transmission season, but that a shift in parasite population occurred around the 4th month. In the dry season, a mixed population of parasites (\textit{dhfr} MS) was detected around the 6th month and then remained stable from the 7th month until the second transmission season, when a new shift appeared.

There was no variation at all in either \textit{dhps} or \textit{dhfr} MS patterns throughout the dry season in patient 5. The patient however was infected by a new variant in the following transmission season, as shown by marked changes in both the \textit{dhps} and \textit{dhfr} microsatellite patterns.

The results show one of the few instances in which different patients harboured parasites with the same haplotype. This is seen in patients 4 and
5, who carried parasites with nearly the same *dhps* MS patterns during the dry season.

Table 1. Neighbouring microsatellite loci located 0.8, 4.3 and 7.7 kb downstream of the *dhps* gene, and neighbouring microsatellite loci located 0.3, 4.4 and 5.3 kb downstream of the *dhfr* gene among 25 *P. falciparum* isolates from Asar village in Eastern Sudan. Patient number (1, 2, 3, 4, and 5).

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Our study has exploited the unique malaria epidemiology settings of Asar village in eastern Sudan, where a long dry period followed by brief annual rains and the appearance of Anopheles mosquitoes causes malaria transmission to be short and seasonal (Babiker, Lines et al. 1997, Hamad, Nugud Ael et al. 2002). During the dry season, a high degree of fluctuation between different parasite clones was observed. The absence of mosquito transmission during the dry season in Asar suggests that this fluctuation is possibly driven by competitive interaction between different parasite clones within the human host (de Roode, Culleton et al. 2004, Wargo, Huijben et al. 2007). Such competition could eliminate genotypes with survival disadvantages, including mutations causing drug resistance in the absence of drug pressure. Data from our study show that the parasites found in patients during the dry season are not simply resting. In addition, the parasites are shown to turnover from month to month. Therefore, several people carry mixed infections

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● 1st transmission season, ● Dry season, ● 2nd transmission season
of at least two variants of the parasite. These variants grow during the dry season without causing any symptoms.
Paper II

In this study, 25 patients were recruited and followed throughout three different seasons: the first transmission season, the dry season and the second transmission season. The parasite density was calculated during a period of 15 months in each patient.

When examining the data as a whole, the mean parasite density in the first transmission season was 616 (95% confidence interval (CI): 302-1288) parasite/μL, which was considered to be a high level (Figure 4). During the dry season the parasite density fluctuated with a mean of 219 (95 % CI: 98-490) parasites/μL, which was significantly lower than in the first transmission season (P<0.05).

The mean parasite density in the second transmission season was 209 (95 % CI: 110-400) parasites/μL. In addition, when compared with the dry season, there was no significant difference between the means of parasite density between the two seasons (P>0.05).

Figure 4. Diagram illustrating the difference in parasite density between (A) the dry season and the 1st transmission season (P<0.05) and (B) the dry season and the 2nd transmission season (P>0.05). Error bars indicate mean with SEM.

The decrease in parasite densities when entering the dry season is explained by the fact that all patients were treated for malaria infection during the first transmission season. It is possible that some limited mosquito transmission of P. falciparum continued at a local level during this time. In the absence of mosquito transmission, the newly appearing clones could only have originated from parasites already present in the patient but otherwise undetectable at the earlier times of sampling. Furthermore, such parasites could have been either sequestered outside the peripheral circulation at this time or have been present at levels too low to have been detectable (Babiker, Abdel-Muhsin et al. 1998).
The large difference in parasite density between individuals in the first transmission season may be explained by differences in immunity when infection was acquired, meaning that those who displayed high parasitaemia had low immunity due to the absence of previous infections. The relatively low parasitaemia observed in the second transmission season can be explained by the remaining immune response sustained by the persistent parasitaemia during the dry season.
Paper III

Out of the total number of collected blood samples, the prevalence of *P. falciparum* infection diagnosed by microscopy was 3.7% (54/1,453) while that diagnosed by RDT was 6.9% (100/1,453). From the remaining 1, 299 negative samples, 400 were randomly selected and tested by PCR. The prevalence of sub-microscopic *P. falciparum* carriages with infections detected by PCR was 19.2% (77/400). Although sub-microscopic infections were lower in females than males, the difference was not statistically significant between the two groups.

As shown in Table 2, when the 154 samples that were shown to be positive by microscopy and RDT were tested by PCR to evaluate the two diagnostic tests against PCR, the latter detected 90.7% (49/54) and 80% (80/100) of the microscopy and RDT positive samples, respectively. The prevalence of *P. falciparum* by age group as diagnosed by the 3 different tests is shown in Figure 5. The highest prevalence of microscopic as well as RDT *P. falciparum* was among the youngest age group (2-5 years) while the older group (>35 years) had the lowest prevalence. PCR showed an unequivocal superior diagnostic performance compared to both RDT and microscopy.

Table 2. Evaluation of rapid diagnostic test- and microscopy-positive results against polymerase chain reaction

<table>
<thead>
<tr>
<th>PCR</th>
<th>Microscopy (n=54)*</th>
<th>RDT (n=100)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. falciparum</em></td>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>7</td>
</tr>
</tbody>
</table>

*Only microscopy and RDT positive samples were subjected to PCR (the overall results of microscopy and RDT not indicated in the table).*
It is noticeable that the PCR analyses in our study helped to establish two relationships, i.e. the rate of false positives and false negatives given by microscopy and RDT, respectively. Both have implications for malaria control measures.

Regarding the false positive rate and its implications, anti-malarial drugs were prescribed for 9.3 and 20.0% microscopy- and RDT-based parasite-negative patients, respectively, as observed by PCR. Similar percentages of parasite-negative patients receiving antimalarial drugs have been also shown in countries such as Tanzania (Masanja, McMorrow et al. 2010), Uganda (Sserwanga, Harris et al. 2011) and Zanzibar (Msellem, Martensson et al. 2009), where high malaria transmission settings are observed.

For false negatives, the discussion revolves around 19.2% prevalence of sub-microscopic P. falciparum. Our study results indicate that considerable numbers of P. falciparum infections were missed by both RDT and microscopy. Therefore the need for a more sensitive assay for the detection of sub-microscopic parasitaemia can be suggested from the study findings. The presence of sub-microscopic asymptomatic P. falciparum infections may represent a significant challenge to malaria control programmes since such parasitaemic individuals may serve as a reservoir of infection and contribute to mosquito infection (Ochola, Vounatsou et al. 2006, Okell, Ghani et al. 2009). The agreement between RDT and microscopy was 89.4% (42/47) and 71.4% (five out of seven) in detecting P. falciparum and mixed infections, respectively. Both RDT and microscopy underestimated the true parasite prevalence in the study area. In this study, microscopy and RDT detected 38.9 and 60%, respectively, of the infection identified by PCR. In addition, the false positivity rate of RDT was 2.1% higher than microscopy.

The detection of a high prevalence of PCR-based submicroscopic P. falciparum in the study area would provide significant implications for malaria control measures.
control measures in Ethiopia since such infections are important contributors to the infectious reservoir (Schneider, Bousema et al. 2007, Diallo, Ndam et al. 2012), and could, even at low densities, be a potential source of transmission for vectors and for malaria attack within the population. The increased sub-microscopic carriage in older age groups, which was observed in our study corroborates similar finding in Tanzania (Manjurano, Okell et al. 2011). However, asymptomatic carriage is probably a common occurrence in the study area.

Further studies to reveal the magnitude of sub-microscopic asymptomatic carriage will be necessary for guiding and monitoring future malaria elimination efforts in Ethiopia.
Paper IV

PCR results from pfcrt analysis showed that 100% (n = 99) of the patients harboured the pure mutant allele of pfcrt 76 T. Of the fragments that were resistant to ApoI digestion (76 T genotype), 20 were randomly selected for complete sequence for amino acids positions 72-76. All the isolates harboured the pfcrt-CVIET genotype. No single wild-type, CVMNK pfcrt genotype, was detected in the study area.

Four MS loci were analysed for all the isolates, two intronic (msint 2, msint 3) and two flanking (mscr -2, mscr -29). Only 79 samples were successfully analysed for MS haplotypes, leaving 20 of the isolates to be excluded from the analysis due to unsuccessful amplification or because they had either multiple alleles (>one peak in the sequencing electropherogram) or missing data at one or more codons. As presented in Table 3, the 79 isolates grouped into seven different CQR haplotypes (E1-E7). The most prevalent haplotype, E1 (82.3%), had allelic combination of (149-179-198-204). When looking at the isolate sources, it is noticeable that two allelic combinations in E3 and E5 were found in isolates collected from asymptomatic subjects; while another two allelic combinations in E6 and E7 were specific to isolates collected from symptomatic subjects.

Table 3. Pfcrt allelic types defined by mutations in positions amino acids 72-76 and polymorphisms in MS markers (intronic and flanking) in 79 P. falciparum isolates in Shalla district, south-central Oromia, Ethiopia.

<table>
<thead>
<tr>
<th>Haplotype ID</th>
<th>Ms loci</th>
<th>Genotype of Pfcrt</th>
<th>Isolate sources</th>
<th>Isolates n=79</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>149</td>
<td>179</td>
<td>198</td>
<td>204</td>
<td>CVIET</td>
</tr>
<tr>
<td>E2</td>
<td>149</td>
<td>181</td>
<td>198</td>
<td>204</td>
<td>CVIET</td>
</tr>
<tr>
<td>E3</td>
<td>149</td>
<td>179</td>
<td>198</td>
<td>209</td>
<td>CVIET</td>
</tr>
<tr>
<td>E4</td>
<td>149</td>
<td>179</td>
<td>198</td>
<td>215</td>
<td>CVIET</td>
</tr>
<tr>
<td>E5</td>
<td>149</td>
<td>179</td>
<td>198</td>
<td>197</td>
<td>CVIET</td>
</tr>
<tr>
<td>E6</td>
<td>149</td>
<td>181</td>
<td>198</td>
<td>215</td>
<td>CVIET</td>
</tr>
<tr>
<td>E7</td>
<td>149</td>
<td>177</td>
<td>198</td>
<td>204</td>
<td>CVIET</td>
</tr>
</tbody>
</table>

Haplotypes ID (E1-E7) were classified as different if they contained ≥ one different alleles across all loci. Allelic size at each MS locus is shown.

The widespread resistance to chloroquine in Ethiopia has triggered and urged the distribution of ACT for falciparum malaria. Therefore, artemether-lumefantrine has been the choice for P. falciparum therapy in Ethiopia since 2004.

The high level of pfcrt K76T mutation observed in this study is in accordance with a study conducted in a rural hospital in southern Ethiopia (Mula,
Fernandez-Martinez et al. 2011), although CQ has, since 1999, been withdrawn in Ethiopia. This is the first report in Ethiopia to reveal the presence of a single CQR mutant pfcrt genotype, CVIET the most common haplotype in Africa.

The CQR isolates from our study findings showed reduced diversity with respect to all MS markers (two introns and two flanking). The isolates were 89.9, 100, 88.6 and 100% identical with respect to msint 2 (0 kb), msint 3 (0 kb), mscrt -2 (-2.814 kb) and mscrt -29 (-29.268 kb), respectively. Of the seven haplotypes identified in the study area, haplotype E1 is the most prevalent allelic type. It can be inferred from this study that parasites identified from asymptomatic subjects are more diverse than those present in symptomatic infections (clinical) sample sets. Even though malaria transmission setting of the study area was low, asymptomatic parasite carriage is still associated with a higher multiplicity of infection. The number of MS alleles varied among the pfcrt introns and flanking loci that were successfully analysed, with msint 2 displaying the greatest number of alleles (n = 4). On the other hand, msint 3 and mscrt -29, showed no polymorphisms. The low heterozygosity of intronic and flanking MS alleles shown in this study may indicate that the parasite population in the study area is genetically homogeneous, which could be the result of the CQ-selective sweep.
Conclusions

- The parasite population can be dynamic in an individual even during asymptomatic infections. Microsatellite analysis showed clear signs of variation in infected individuals during a long period with no transmission of malaria.

- Real-time PCR was demonstrated to be a reliable method for measuring varying amounts of *P. falciparum* parasites collected from old samples, and the parasite levels observed matched those obtained earlier by microscopy studies.

- PCR was found to have good sensitivity to detect a higher number of infected subjects with low and sub-microscopic parasite densities than RDTs and microscopy.

- Decreased diversity in markers surrounding *pfcrt* in chloroquine resistant isolates suggests that chloroquine selection has led to homogenization among the parasite population in the study area in Ethiopia.
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أبي الحبيب أعلم أن هذه الكلمات التي تعبّر عمّا في داخلي من شكر وحب وتقدير لجهودك وتضحيتلك ليكي أصل إلي ما أنا عليه الآن لتصلك، ولكنني أعلم أشدي العلم بأن ذهاني لك يوصله. أبي الغالي يصعّب عمّا في تصديق أنني لن أراك مرة أخرى وأنك قد رحلت عني بدون أن أودعك. وكم كنت أمتنى أن تكون بجانبي يوم حصولي على شهادة الدكتوراة لأنك أنت من يستحق التكريم. لطالما كنت حريصا جدا على دراستنا ونجاحنا وأنا وأخوتي والحمد لله الذي وفقي في تحقيق أحد أمانيعك.

أسأل الله العلي القدير أن يرحمك ويغفر لك وينير قبرك ويجعله روضة من رياض الجنة (أمينٍ بارب العالمين).

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من الصعب أن أجد الكلمات التي تعبر عمّا في داخلي من حب وتقدير وعرفان على كل ما قدمته لي ولازلي تقديميه وبارك الله فيك ورزقني برك وأطل الله في عمرك. أسأل الله العلي القدير أن يوفقني على أرضاك. أحبك يا أمي!

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