Parasitological and Molecular Evaluation of Artemether Plus Lumefantrine (ACT) For The Treatment of *Falciparum* Malaria (New Halfa, eastern Sudan)

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By

Sahar Mohammed Ahamed

B.Sc of Zoology 1999

Faculty of Science

University of Khartoum

Supervisors

Dr Saadia Ahmed Younis (assoc Prof)

Faculty of Science. U of K

Prof Mustafa Idris Elbashir

Faculty of Medicine. U of K

Faculty of Science

Department of Zoology

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Dedication

To My Family

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ABBREVIATIONS

ACR adequate clinical response

ACT artemisinin-based combination therapy
AL artemether-lumefantrine combination

AQ amodiaquine

ART artemisinin

AS artesunate

CQ chloroquine

DHFR dihydrofolate reductase

DHPS dihydropteroate synthetase

ETF early treatment failure

L lumefantrine

LTF late treatment failure

MQ mefloquine

MQ mefloquine

P. falciparum Plasmodium falciparum

P. malariae Plasmodium malariae

P. ovale Plasmodium ovale

P. vivax Plasmodium vivax

PCR polymerase chain reaction

Q quinine

RDT rapid diagnostic test

SP sulfadoxine–pyrimethamine

WHO World Health Organization

ABSTRACT

The present study aims to provide more information on the therapeutic potential of (AL) for the treatment of uncomplicated malaria in New Halfa Eastern Sudan.

Eighty three uncomplicated falicparum malaria symptomatic patients (male, female, children, and adults) were treated with AL on days 0, 1, 2, and followed for 28 days. Results showed that 95.1% were aparasitemic and afebrile from day 7 until day 28. No significant adverse effects were recorded. Late clinical failure was experienced by 4 patients (4.9%).

Genotyping of Plasmodia parasite from these 4 late clinical failure (LCF) patients showed the following: 1 (25%) recrudescence, 2 (50%) new infections, and 1 patient (25%) with both recrudescence plus new infection. Therefore the true cure rate of (AL) as adjusted by PCR was 97.6%, hence AL appears to be effective and safe for the treatment of uncomplicated falicparum malaria in children and adults of both sexes in New Halfa Eastern Sudan.

خلاصة الدراسة

الدراسة الحالية هدفت إلي الي توفير معلومات أكثر عن الإمكانية العلاجية لعقار AL في علاج الملاريا غير المعقدة في حلفا الجديدة شرق السودان.

83 مصاب بالملاريا غيرالمعقدة (رجال نساء اطفال و بالغين) تم علاجهم بإستخدام عقارAL في اليوم الأبتدائي و الأول و االثاني و تمت متابعتهم حتي اليوم الثامن و العشرون، أظهرت النتائج أن 95.1% كانوا خالين من الطفيل و الحمي من اليوم السابع وحتي اليوم الثامن والعشرين بدون أي اثار جانبية حادة.

الفشل العلاجي أختبر في أربعة مصابين، اجراء إختبار تفاعل التسلسل المتبلرلطفيل الملاريا في المصابين الأربعة أظهر الأتي: 1 (25%) أعادة ظهور الأصابة، 2 (50%) إصابة جديدة، 1 (25%) إعادة ظهور الأصابة + إصابة جديدة.

و بالتالي فأن الأثر العلاجي الحقيقي لل AL قد عدل بعد عمل التفاعل التسلسل المتبلر إلي 97.6% . بالتالي أظهر AL كفاءة علاجية و سلامة في علاج الملاريا غيرالمعقدة في الأطفال و البالغين من الجنسين في حلفا الجديدة في شرق السودان.

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CHAPTER ONE

Introduction Literature Review & Objectives

Malaria is a serious challenge facing world health. Present estimate of clinical burden, varies from 350 to 650 million annually (WHO, 2005). Malaria presents a public health problem for 2.4 billion people, representing over 40% of the world's population in over 90 countries; each year an estimate o.7-2.7 million die from malarias more than 75% of them are children (Breeman, 2001). More people are now dying each year from malaria than 30 years ago, and malaria is returning to areas from which it had been eradicated and entering new areas such as Eastern Europe and Central Asia (Marsh, 1998).

Malaria disaster in Africa is due to drug resistant *Plasmodium falciparum* (Marsh, 1998), A child is estimated to die from (*P. falciparum*) malaria every 12 or so seconds, in sub-Saharan Africa, where 5% of children die from the disease before reaching 5 years(WHO, 2003). Among the newborns of Africa, an estimated 3 million suffer complications from low birth weight, or even death, arising from in utero malaria infection (WHO, 2003). During the last 50 years, many workers (reviewed by Wongsrichanalai 2002) have reported the development and spread of resistance in *P. falciparum*, to most of the drugs used in the first line treatment and also prevention of malaria. The reduction of malaria attributable mortality depends on accurate and early diagnosis followed by prompt treatment with effective drugs.

1.1The causative agent and its life cycle: -

The disease is caused by a protozoan parasite of the genus *Plasmodium*. The vector responsible for malaria transmission in man is the female Anophelene mosquitos which belongs to various genera. In 1897, Ronald Ross discovered that the malaria parasite could infect the female mosquito. Later, he showed that the parasite completed its life cycle in the mosquito and, when that mosquito took another blood meal, it passed on the parasite. Then he wrote that "the discovery was, perhaps, as really important as the discovery of America" (Ross, 1928)

The life cycle of the parasite is quite complex; its begins with <10 sporozoites released from the salivary gland of a feeding female mosquito, they quickly invade the liver cells (about 30 minutes). Within approximately two weeks each sporozoite undergo asexual multiplication resulting in hundreds of merozoites (hepatic schoizont), which cause the hepatocyte to burst, this result in the release of hundreds, of merozoite in the blood stream. Subsequently and within seconds, each merozoite invades an erythrocyte and multiplies producing several merozoites (blood schoizont), this cause the RBC to rupture and the released merozoites invade new RBC and this continues every 48-72 hours according to the plasmodia species. Eventually some merozoites invade RBC but do not divide, instead, each differentiate into a male or a female gametocyte which when taken by a feeding anophlene mosquito initiate the sexual cycle in the gut wall. This

eventually ends up in to hundreds of sporozoites in the salivary gland to infect humans (Appdix-1).

In *P. vivax, P. ovale* some of merozoites released in blood many reinfect other hepatocytes and remain dormant (hypnozoites) may be for years and suddenly become active and cause relapses. However, in case of *P. falciparum* dormant merozoites may be found in RBC only, this is called recrudescence. There are very numerous species in the genus Plasmodium, which infect a wide range of vertebrates including primates and reptiles but mostly birds. These are useful in research. However, man may be infected only by four species of Plasmodia, namely *P. vivax, P. ovale, P. malariae* and *P. falciparum*. The latter is the most virulent and most wide spread geographically.

The distribution of malaria is worldwide, involving tropical areas such as, subSaharan Africa, South Africa, West Africa, South East Asia, Pacific Island, India and central & South America (Ashley *et al*, 2005).

Malaria transmission does not occur at temperatures below 16◦c and altitudes greater than 2000m above sea level because such conditions do not support parasite development in the mosquito vector. The optimum conditions for transmission are high humidity and ambient temperature between 20◦c and 30◦c besides rain fall provides breeding sites for mosquitoes (Whit, 1996a).

Malaria transmission to man as any other parasites depends on several factors. Important among these are the presence of infected humans (gametocytes) specific

anophelene vector and availability of mosquito breeding sites (Schmidt et *al*, 1996).

1. 2 Pathology of Malaria:

- Uncomplicated malaria is caused by one or more of four species of Plasmodia: *P.malariae, P. vivax, P. ovale, and P. falciparum*.

- Most symptomatic malaria infections as caused by *P. falicparum* are uncomplicated and manifest as fever, chills, malaise, often abdominal discomfort, and mild anemia. In falciparum malaria, the mortality associated with this presentation is approximately 0.1%, if effective drugs are readily available. In a small proportion of *P. falciparum* infection, uncontrolled parasite multiplication leads to heavy parasite burdens, which produce vital- organ dysfunction with impairment of consciousness, acidosis, and more severe anemia, Seizures, hypoglycemia, and severe anemia are common manifestations of severe malaria in children, whereas jaundice, pulmonary edema, and renal failure are more common in adults. The mortality despite treatment rise to 15-20% (White, 2004).

- The characteristic chill and fever, associated with malaria correlate with the maturation of blood merozoites and the rupture of infected red cells. Fever is stimulated by the waste products of the parasites released when the erythrocytes lyse. Aneamia arise from the enormous destruction of parasitized and non-parasitized (due to immune mechanisms) red blood cells, inability of the body to recycle the iron bound in the insoluble hemozoin and the cytokines suppressed inadequate erythropoietic response of the bone marrow. Destruction of

erythrocytes lead to an increase in blood bilirubin, which is a breakdown product of hemoglobin; when excretion cannot keep up with formation of bilirubin, jaundice develops (Schmidt *et al*, 1996).

- Almost all of the deaths are caused by *P. falciparum* because the infection could turn to a severe complicated form. This is why all research on drug resistant malaria concentrates on this species. The particular virulence of *P.falcparum* is due to the way it changes the surface of the RBC. Hours after invasion parasite proteins appear on the RBcell surface and promote binding to vascular endothelim leading to severe complications, which will involve the brain. Cerebral malaria is characterized by coma, hypoglycemia, metabolic acidosis, fluid and electrolyte disturbances, acute renal failure, acute pulmonary oedema and respiratory distress this may lead to death (WHO, 2003).

A transient neurological syndrome may follow initial recovery from severe malaria, (post-malaria Neurological syndrome); symptoms include confusion, tremor and seizures. (Ashley *et al*, 2005)

1.3 Diagnosis of Malaria.

Diagnosis of Malaria could be clinical, parasitological, immunological, or genetical.

Clinical diagnosis of malaria have been shown repeatedly to be unreliable, because the first symptoms of malaria are non-specific such as headache, fever, muscular ache and abdominal discomfort. However early clinical diagnosis offers the advantages of speed, and low cost. In areas where malaria is prevalent, clinical

diagnosis usually results in that all patients with fever and no apparent cause be treated for malaria; this approach can misidentify some patients who truly do not need antimalarial treatment (Chandramohan, 2001)

Parasitological diagnosis is based on microscopical examination of Giemsa stained thick and thin blood films. It is the most reliable method for definitive malaria diagnosis. Advantages include differentiation between species, quantification of the parasite density, and ability to distinguish clinically important parasite stages. These advantages can be critical for proper case-management and evaluating parasitological response to treatment (Jonkman, 1995). Intraerythrocytic parasites should be identified, however if identification of parasite species is uncertain, it should be considered as *P. falciparum*) (Field, 1941). Thick blood films are used to detect malaria parasites, thin films are used to detect parasite species, parasite densities and the developmental stages (WHO, 1996).

Immunological diagnosis is based on rapid detection of parasite antigens using different techniques. Such assays are usually based on the detection either of parasite structural elements such as the histidine-rich protein 2 (HRP-2) antigen, or the parasite-specific enzyme lactate dehydrogenase (LDH) found in all plasmodial species (Moody, 2002). Techniques also exist for detecting anti-malaria antibodies in serum specimens. Specific serological markers have been identified for each of the four species of human malaria (WHO, 2001).

Genetic diagnosis is based on the Polymerase Chain Reaction (PCR) to detect parasite genetic material. Any DNA sequence unique to a particular parasite species and which is not found in the genome of the host can be used as target for PCR amplification. Many such sequences are known for the genus Plasmodium. Sequence comparison of Plasmodia species has revealed the presence of DNA stretches unique to each Plasmodium species, this made possible the detection and differentiation of the 4 malaria species that infect man among other nonhuman species. Detection and differentiation are made possible through hybridization to total ribosomal RNA obtained from infected blood. Therefore nested PCR protocol can detect any malaria parasite and identify the species. The technique is becoming more popular in the diagnosis of drug resistance in malaria parasites. Specific primers have been developed for each of the four species of human malaria. One important use of this new technology is in detecting mixed infections or in detection of infection beyond the resolution of the microscope (Beck, 1999), which is necessary in drug efficacy studies.

All non parasitological diagnostic methodes have some limitations such as high cost, high degree of training required, need for special equipment, and absolute requirement for electricity (WHO, 2001).

1.4 Malaria Control:

1.4.1 Vector control:

Malaria control is mainly based on vector control, it is a long established and most used strategy. It includes insecticides, larva control, and personal

protection; such as insecticide-treated bednets (ITNs), insect repellents. Development of resistance to insecticides and insect repellents in most anophelene species reduced the outcome of this strategy. However, effects have been made towards sterilization of male anopheline vectors, or development of a transgenic mosquito incapable of transmitting malaria, which was without success (Ashley *et al*, 2005).

The world's malaria situation is divided into three categories. First, areas where malaria transmission is intense and is unaffected by vector control programs, such as most of tropical Africa. The second category represents the malaria situation in most malarious countries in Asia and the Americas, where large-scale vector control programs are operating—interruption of these programs or a gradual breakdown in the commitment to them leads to outbreaks of the disease. Into the third category are placed areas of rapid economic development and countries seriously affected by social disruption, both of which can lead to environmental disturbances, population movements, and the absence of health care infrastructure (Phillips, 2001).

1.4.2 The chemical control (Chemotherapy):

The chemical control is based on effective elimination of infection by drugs, implementation of such a strategy is rather impossible because malaria prevails among underprivileged people in very vast areas of the tropics and subtropics where people live in poverty, lack of education, and lack of even reasonable health services.

In most of the few privileged affected areas, drug pressure induced resistance to first line and second line drugs in the parasite (Pudney, 1994). Perhaps the answer lies in the development of a vaccine.

1.4.3 Immunological Control:

Immunological control is through the development of a vaccine. This is an area where research continues but without success. Development of an antimalarial vaccine has proven to be a difficult challenge, due to the complexity of the parasite, both in the human and in the mosquito, and also because of its ability to evade the host responses by antigenic variation of the blood stages which are solely responsible for the pathology of the disease. Scientists around the world have been searching for a safe and effective malaria vaccine for several decades. Various antigens have been tried in different combinations but results were always disappointing, because of species specific and stage specific antigenic variation and polymorphism.

The search for vaccine formulation against *P.f* concentrate mostly on identification of major antigens for consideration as vaccine candidates.

However, antigenic variation and polymorphic and immune evasion make this goal difficult to attain.

Various methods of stimulating resistance have been tried with animal plasmodium starting from irradiated sporozoites to isolated antigenic molecules (subunit vaccine). But the knowledge gained cannot be applied on humans with

certainty. Pre-erythrocytic vaccine trials include irradiation attenuated sporozoites (Clyde, 1990) and also a protein on the surface of the sporozoites (CSP) (Zavala, 1983) (Hollingdale, 2005). Of the promising blood stage vaccines candidates are proteins on the surface of the merzoite mainly MSP-1. It is synthesized by the intra-erythrocytic merzoite as a precursor protein, whereas on the released merzoite it is processed into several fragments. However only the carboxyterminal fragment (19 KDa) of (MSP-1) remains on surface of the merzoite and plays an important role in the process of penetrating the RBC. Other antigenic molecules of the merziote which are involved in penetration of the RBC have been isolated from *P.f* then synthesized and combined in a cocktail form named (spf66) and tried on humans in Africa, Latin America, and S.E Asia in 1988 by Patarroyo and co-works but with very limited success. A genetically engineered multistage multivalent vaccine made up of 4 antigens from different stages in the life cycle from sporozoite to gametocyte have been developed but the results were also disappointing (Stanley, 1998)

1.5 *Plasmodium falciparum* genome:

The genome of *P.falicparum* contains 14 chromosomes. It is estimated that the total genome codes for about 6,500 genes (Bowman *et al,* 1999). Various genes that code for antigenic determinants from exo-erythrocytic or endo erythrocytic stages of *P.falicparum* have been isolated and characterized. However, notable among *Plasmodium falciparum* gene intensively investigated as candidates for

vaccine development are those encoding surface proteins of the merozoite , coding for the merozoite surface proteins MSP-1 and MSP-2 (Rich &Ayala, 2000).

Numerous studies have indicated that *MSP-1, MSP-2*, and other antigenic genes are highly polymorphic.

1.6.1 Polymorphism at *MSP1* locus:

The *Msp-1* gene is located on chromosome 9 (Appdx 2). The protein is encoded by two major variant alterative alleles. MSP-1 is a large 185- to 215-kDa protein precursor that is proteolytically cleaved into several membrane protein constituents. The known alleles of *Msp-1* belong to two allelic families (group I and group II). There is considerable nucleotide substitution and length variation between the two families but much less variation within each family occurs (Tanabe *et al,* 1987) (Hughes, 1992). The two families are commonly designated as: K1 (group I) and MAD20 (group II). This protein is synthesized during schizogony and is processed at the time of schizont rupture to generate several fragments on the surface of the free meroziote. The fragments are covalently linked on the surface of the mature merziote. Their molecular weights are:
83 ,42,38,28,30 KDa (Mc Bride, 1987). Subsequent processing of the 42 KD fragment generates the 19 KDaC- terminal fragment. This occurs at the time of RBC invasion where only the 19KD fragment (MSP1 19) is retained on the surface of the invading meroziote the rest of the fragments are shedd off.

Tanabe *et al* (1987) partitioned MSP-1 into 17 (Appdx 2) blocks based on degree of AA polymorphism. They classified 7 blocks (2,4,6,8,10,14,16) as highly variable, 5 blocks (7,9,11,13,15) as semi conserved and 5 blocks as conserved (1,3,5,12,17). Block 2 located with the N-terminus of MSP-1 is highly polymorphic. All sequence variation (about 50) of this block of MSP-1 belong to one or another of 3 families identified as K1- MAD-20 and Ro33. In this block 2 extensive sequence diversity exits. Variation within K1- like and MAD-20 like types of Block 2 differ in tripeptide or hexareplide repeats; whereas block 2 of the Ro33-type is a non repetitive sequence which varies little between isolates (Rich & Ayala, 2000).

1.6.2 Polymorphism at *MSP2* locus.

The *Msp-2* gene is located on chromosome 2 (Appdx 3), it codes for MSP-2 glycoprotein anchored, like MSP-1, in the merozoite membrane, but only 45 kDa in size. MSP-2 is characterized by conserved N and C termini, bracketed within these segments, is the highly variable repeat region. Sequence analysis revealed that the polymorphic block 3 can occur as one of two distinct families namely FC27 and IC1. Different repeated sequence units characterize these two families and the sequence that flank these repeated regions are unique to each family and are shared among all allelic variants (Smythe, 1990, 1991).

This polymorphism at MSP-1and MSP-2 loci are mainly assessed by the number of repeats which can be used to distinguish the different alleles by size after amplification by PCR.

1.7 Drugs available for treatment of malaria:

There are five main classes of antimalarial agents in use: the quinolines and arylaminoalcohols, the antifolates, the artemisinin derivatives, the hydroxynaphthaquinones and antibacterial agents (Ashley *et al*, 2005).

1.7.1Quinolines and Arylaminoahols:

Plasmodia depend heavily on host hemoglobin for nutrition. The hemoglobin is rapidly degraded in the food vacuole within the RBCs. One of the products of hemoglobin digestion is ferri-protoporphyrn in (FP). However, FP inhibits several of the plasmodial proteases, therefore the parasite sequesters FP as an insoluble inert homozoin. But when chloroquine accumulates in the food vacuole, it increases the pH because it is a weak base thus prevent the digestion of hemoglobin. Other quinolines act similalry on infection by affecting the food vacuole (Faley *et al*, 1997). They target the asexual intra- erythrocytic stages of the parasites, which cause the pathology.

1.7.2 The Antifoliates:

Tetrahydrofolate is a cofactor important in various biosynthetic pathways in both host and parasite. An intermediate produc in both pathways is dihydrofolate which

must be reduced by the enzyme hydorfolate reductase into tetrahydrofolate. Anitfolates such as pyrimethamine, proguanil, chlorproguanil, and trimethoprim inhibit the synthesis of dihydrofolate reductase. This enzyme is vital for both host and parasite but fortunately each differ in its affinity for binding the drug i.e it will not affect the biosynthetic pathway of the host (Wellems, 1991).

1.7.3 The Antibacterials:

Although plasmodia have cytoplasmic ribosomes of the eukaryotic type, nevertheless several antibacterials that specifically inhibit prokaryotic protein synthesis (such as tetracycline and its derivatives) have weak antimalarial effect. Tetracycline and some other antibacterials inhibit protein synthesis in *P.falcipraum* (Blum *et al*, 1989), but are not effective enough as monotherapy (Ashley *et al*, 2005).

1.7.4 Artemisinin and its derivatives:

Artemisinin is derived from a Chinese plant named *Artemisia annua*. It was first extracted from the herb *qinghao* which was known to have antipyretic effect and that about 1500 years ago. In 1971 a highly active chemical from *qinghao* known as *ginghaousa* was obtained and is now known as **artemisinin**. Since then many semi-synthetic oil and water derivatives have been developed entering antimalarial clinical trials. Artemisinin is a sesquiterpene lactone structure in which antimalarial activity is limited to an unusual endoperoxide trioxane moiety (Haynes, 2001).

1-8 Development of resistance to antimalarials:

The development and spread of antimlarial drug resistance involves the interaction of drug-use patterns, characteristics of the drug itself, human host factors, parasite characteristics, and vector and environmental factors (Wongsrichanalai *et al*, 200), (Winstanley, 2001) Characteristics of the drug are important determinants of resistance. First, drugs with a long elimination half-life, such as mefloquine (weeks), may exert substantial residual selection on new infections contracted after the treatment of the primary infection when the drug persists at subtherapeutic concentrations in the plasma, (Wernsdorfer, 1994) especially in areas with intense malaria transmission. Second, the maintenance of adequate drug concentrations over a long enough time is important for clearing the entire population of parasites within a given individual. Subtherapeutic drug concentrations eliminate the most susceptible parasites and leave those that may be more fit to recover and reproduce. Third, widespread use of drugs at high intensity serves to increase drug pressure and is a determinant for selection of resistant parasite populations (Wongsrichanalai *et al*, 2002).

A semi-immune patient might be cured by a drug despite the fact that his parasites are partially drug resistant, as a potent immune response increases the efficacy of chemotherapy. Instead, individuals who are naive to malaria generate a non-specific immune response that is not as effective as the specific response elicited by repeated infections. Thus, the introduction of resistant malaria parasites into

non-immune populations such as refugees or migrants increases the opportunity for appearance and spread of resistance (Wongsrichanalai *et al*, 2002).

The level of transmission many also influence the rate of emergence and spread of drug resistant plasmodia but its exact role is complex and not clear. It is most probably multifactorial. Increased risk has been postulated to occur in areas of both low and high malaria transmation**.** The fact that plasmodium drug resistance has initially developed earlier in areas of low transmission (such as Thailand and Brazil) and is still more prevalent in such areas than in those with higher transmission tends to support the low-transmission hypothesis. On the other hand, full chloroquine resistance in children occurred and spread within 2·5 years in an area of high transmission in east Africa (Draper *et al*, 1985). Finally, vector and environmental factors may influence the proliferation of resistant parasites. For example, chloroquine resistant parasites may be more fit for reproduction in certain sub-species of anopheline mosquitoes than non-resistant strains (Wongsrichanalai *et al*, 2002).

1-9 Assessment of Drug resistance:

Drug resistance in malaria has been defined as "the ability of a parasite strain to survive and/or multiply in the host despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject" (WHO, 1973). Later on quantification was added to this definition to specify: "provided that the drug gained access to the parasite or

the infected red cell for the duration of time necessary for its action" (Bruce *et al*, 1986).

According to WHO (1996), drug resistance in malaria can be indicated by a treatment failure and can be graded into different levels depending on the timing of recrudescence following treatment. Traditionally these levels of drug resistance have been defined as (Sensitive /no recrudescence). (RI. delayed recrudescence), (RII. early recrudescence), and (RIII. minimal or no anti-parasite effect). Subsequently a modified protocol based on clinical outcome has been introduced by the WHO in 1996. In this protocol the level of resistance is expressed as adequate clinical response (ACR), early treatment failure (ETF) ,late treatment failure (LTF): -late clinical failure (LCF), or late parasitological failure (LPF) (WHO, 1996).

1-10 Development of drug resistance in *P. falciparum:*

Chloroquine, a potent antimalarial has been used since 1940 as a first line treatment for malaria in all malaria endemic areas worldwide. However *P. falciparum* resistance to the drug suddenly appeared in South East Asia about 50 years ago (Wernsdorfer &Payne, 1991). Subsequent reports came from different parts of Asia where *P. falciparum*, the most virulent species prevails. Chloroquine resistance which spread a cross Africa started in the 50's, and is concentrated in the east part of the continent, and is progressive.

The emergence of Chloroquine resistance lead to the development of alterative antimalarials. Unfortunately resistance to some of the new drugs has already been reported from many malarious areas.

Important among the new alternative antimalarials are (fansidar) sulfadoxine-pyrimethamine (S/P) and also mefloquine. These are the affordable second line antimalarial in Africa, but again sensitivity of *P. falciparum* to (S/P) started to decrease in the late 1980's and is progressing ever since, especially in east Africa, (Deloron, 2000).

No drug resistance has been reported concerning *P. ovale or P.malariae*, but resistance to Chloroquine has been documented in *P.vivax*; only in Indonesia and focally in Asia and South America (Maguire, 2002). *P. falciparum* remains to be the most dangerous and the most virulent. However multidrug resistance has already developed in *P. falciparum*. Multidrug resistance is defined as resistance to more than two antimalarials of different chemical forms. This has been observed in Thailand (S. E. Asia) since 1996 and also in the Amazon. Similar areas are progressively emerging in Africa where chloroqunie, meflquine and (S/P) efficacy is gradually decreasing. Chloroquine has long been the most cheap and potent antimalarial (White, 1999) (Ashely, 2004).

Development of resistance to drugs in *P.falciprum* is genetically based however the exact mechanisms are not very clear yet. The genetic basis for drug resistance has been described for few antimalarials e.g the genetic determinants of resistance to Sulfadoxine is point mutation at 5 sites in the dihydrofolate synthase gene.

Whereas resistance to Pyrimthamine is also due to several point mutations in the dihydrofolate reductase gene. Different combinations of mutations within these genes confer different degrees of resistance to the specific drug and to the combination (S/P). On the whole resistance to S/P is associated with a series of point mutations (substitutions) within the active site of target enzymes of the folate biosynthesis pathway (Peterson, 1988).

Again it has been shown that in chloroquine sensitive strains of *P.falciparum* the drug accumulate in the food vacuole but in case of chloroquine resistant strains, the drug moves out (efflux) rapidly, Chloroquine resistance in *P.falicprum* however is thought to be multigenic and is initially conferred by substitutions in a gene encoding a transporter protein (pfcrt) (Fidock, 2000).

Resistance in *P.falicprum* to mefloquine and other related arylaminoalcohols could result from duplications in *pfmrt* gene which encodes an energy demanding p-glycoprotein pump. The consequences of these various genetic events are reduced intacallular concentrations of the antimalarial quinolines (reduced uptake and increased efflux) (Cowman, 1994). In contrast to *P.falciparum*, the cause of chloroquine resistance in *P.vivax* has not yet been found. Again, a single point mutation in the gene encoding cytochrome (b) confer, Atovaquone resistance in *P.falciparum* (Korsinczky, 2000). As for Proguanil it acts on the mitochondrial membrane but how resistance develops to this mitochondrial action is not known yet (White, 1999).

However all these genetic events may result in moderate changes in drug sensitivity such that the drug still remains effective e.g in case of Pyrimethamine.

P.falciparum resistance to various degrees has already developed to all the antimalarial drug classes except the artmisinin compounds. However artmisinin as monotherapy in *P.f* uncomplicated malaria has been associated with 10% treatment failure (White, 1996b) (Giao, 2001).

1.11 Strategies to over come drug resistance (the role of Combination therapy):

To overcome the threat of resistance of *P.falciparum* to monotherapies, and to improve treatment outcome, combinations of antimalarials are now recommended by WHO (2003) for the treatment of uncomplicated falciparum malaria (White & Olliaro, 1996; White, 1999)

Combination therapy with antimalarial drugs is based on the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite. Such strategy is used to simultaneouly exploit the synergistic and additive potential of individual drugs, the partner drug in the combination must be independently effective. Moreover the strategy protects drugs in a mutual fashion as resistance is based on mutation induced by individual drug pressure (Olliaro & Taylor, 2004).

1.12 Advantage of combining antimalarials:

a- The combination is more effective;

b- If a mutant parasite that is resistant to one of the drugs may arise during the course of the infection, it will then be killed by the other drug. This mutual protection is thought to prevent or delay the emergence of resistance (Krugliak, *et al*. 1991).

c- The combination shorten the duration of treatment (increases compliance).

The possible disadvantages of combination treatments are the potential for increased risk of adverse effects (combination of adverse effects) and the increased cost (WHO, 2006).

1.13 Antimalarial combination Therapy:

All treatment trials are directed towards uncomplicated *P.falciparum* infection as most attacks are uncomplicated. Only about 10% of patients develop complications (WHO, 2003). The idea that drug combination could be used to delay resistance came from Peters (1990), whereby the first trial was in Thailand using mefloquine + SP but the results were negative because of the wide spread resistance to SP that was already there. The following is a review of some of the available antimalarial combination drugs:-

1.13.1 Some of the non-Artmisinin based combinations:-

Sulfadoxine/ pyrimethamine (Fansidar): this is not a single drug but a combination of two drugs where each component inhibits a specific enzyme necessary for the biosynthesis of folic acid in *P.falicprum.* Originally each drug

was used separately in Asia and Africa, however resistance to each drug emerged in the regions (Kremsner, 2004). Subsequently the 2 drugs were combined to ensure inhibition of folic acid in the parasite. Between the 60's and 80's sulfadoxine/ pyrimethamine (S/P) became the most commonly used antimalarial after Chloroquine in all endemic areas (Wernsdorfer, 1994). Eventually resistance to the combination gradually developed, and because of increasing resistance the combination is now hardly used outside Africa. It continues to be used in Africa because it is much cheaper than other alternatives and it is also a single dose treatment (Kremsner, 2004).

S/P + Chloroquine: Chloroquine alone was used as the drug of choice for decades in all malaria endemic areas. Now *P.falicprum* is resistant to Choroquine in all endemic areas except Central America. The triple combination drug proved to have poor effectivness (Bojang, 1998).

S/P + Amodiaquine : Amodiaquine has been shown to cause purities especially in Africans and also rarely cause liver damage in white people. Nevertheless in combination with fansidar it is used in some endemic areas although it has the potential of rare fatal adverse events. The adverse effects appear after repeated exposure during chemotherapy; therefore, it can be used only as a prophylactic by travelers to malarious areas, (Kremsner, and Krishna, 2002).

S/P +Quinine: quinine alone is used against severe complicated malaria. On the other hand to improve efficacy of SP, the triple combination was tried but with unconvincing results in Brazil (Kremsner *et al*, 1989) and Bangladesh (Rahman et

al, 2001), probably because resistance to S/P has already developed in these regions. In South Africa Athan (2001) reported that the triple combination was effective.

Quinine + Tetracycline: The combination has been used for decades in Thailand, but the emergence of parasite resistance will limit its use and also the rare adverse effects (nausea, tinnitus, dysphagia).

Moreover, Tetracycline cannot be used by children or pregnant women (Metzger *et al*, 1995).

Atovacquone + Proguanil:- The combination is successfully used for treatment and prophylaxis of malaria and it shows good tolerability and safety in children and adults with high efficacy against *falciprum malaria* (Lell *et al*, 1998). In western countries it is widely available on the open market, however its use in the tropics is limited by the high price (Olliaro & Taylor, 2004).

1.13.2 Artemsinin Based Combination Therapy: (ACT)

The idea behind using artemisinin-based combination is that artemisinin which is characterized by short half life (≤1 hour) kills rapidly and substantially most of the blood stage parasites, then the higher concentration of the slowly cleared companion drug (long half life) will kill the remaining parasites (Olliaro & Taylor, 2004). In this way the probability that mutant parasites would survive and emerge from these two drugs is low.

Artemisinin and its derivatives (artesunate, artemether, artemotil, dihydroartemisinin) produce rapid clearance of parasitaemia and rapid resolution of symptoms. When artemisinin is given in combination with rapidly eliminated compounds (tetracyclines, clindamycin), a 7-day course of treatment is required; but when given in combination with slowly eliminated antimalarials, shorter courses of treatment (3 days) are effective. The effectiveness of such combinations in comparison to monotherapies has been clearly documented; they reduce parasite numbers by approximately 100,000 in each asexual cycle compared to 10-1000 fold per cycle in case of mono-therapies (Nosten & white, 2007). Again artemisinin has gametocyticidal effect thus it reduces transmission.

Artimisinin monotherapy rapidly reduces parasitaemia and relives symptoms of malaria. They act by inhibition of a *P.falicprum* encoded sarcoplasmic-endoplasmic reticulum calcium At-pase (Eckstein, 2003) However the cure is assiociated with recrudescence (Giao *et al*, 2001). Therefore this suggests that combination with an other antimalarial is necessary for maximum effect and for protection of the arimisinins themselves. Several Artimisinin derivatives have been combined and tried on *P.falicprum* malaria.

i- Artesunate (AS) + Amodiaquine (AQ): this combination was sponsored by the WHO for treatment of *falicprum* malaria in African children. In some African countries it is considered as 1st line treatment for children with uncomplicated malaria (Dorsey, 2007). However disappointing results were reported from a multicenter trail (Adjuik *et al*, 2002)

ii- Artesunate (AS) + Sulphadoxin-pyrimethamine (S/P): this combination was highly effective in Gambia (von Seidlein *et al*, 2000) but showed modest cure rates in East Africa (Adjuik, 2004), probably because resistance to S/P is high in East Africa however, this combination is still effective where SP resistance is low.

iii- Artesunate (AS) + Mefloquine (MQ): This combination has been popular in Thailand for almost a decade (Nosten *et al*,2000), also in South America (Marquino *et al*, 2003), and Africa (Massou *et al*, 2002), It is highly effective and safe. However Mefloquine has a long elimination half life (about 3 weeks) whereas Artesunate elimination half life is about 45 minutes, so wide use in such hyper-endemic area would lead to a long term exposure to low concentrations of Mefloquine, and eventually resistance to Mefloquine will emerge(Palmer *et al*, 1993; Price, 1999).

iv- Artemether (ART)+ Lumefantrine (L): This is the only fixed dose artemisinin containing combination registered after internationally recognized guidelines. The combination was developed and registered in China in 1992, and subsequently in Switzerland in 1999 under dual names of Coartem and Riamet, the former is registered and marketed in malaria endemic countries whereas the latter is available in developed non-endemic countries. A draw back is that there is considerable mismatch in elimination half life of the partner which may end up with emergence of the resistance to lumefantrin which has a half life

of several days compared to Artemether with a half life 45 minutes(White *et al*, 1999).

v- Dihydro-artemisinin (DHA) + Piperaquine: this combination has been developed and registered in China under the brand name of Artekin. It has been evaluated extensively in the Far east. Efficacy was found to be high, tolerablility very good. Dihydro-artemisinin is the main metabolite of artemisinins and has equivalent efficacy. It will be soon registered in Europe but not yet in Africa. (Davis, 2005; Nosten& white, 2007).

1.13.3 Mechanism of action of Arimisinins:

Artemisinins have been found to inhibit endocytosis by the parasite. To investigate the effects of the artemisinins on *P. falciparum* endocytosis of red blood cells, erythrocytes were preloaded with biotinylated dextran and infected with the parasite. Incubation of the infected cells with 110 nM artemisinin for 12 h led to a 76% reduction in the concentration of parasite-associated biotinylated dextran, compared with untreated controls (Hoppe *et al*., 2004). Although no direct link has been reported, changes in cytosolic Ca2+ levels as a result of sarcoplasmic-endoplasmic reticulum calcium At-pase (SERCA) inhibition may have a significant regulatory effect on endocytosis (Hoppe *et al*., 2004). In addition, artemisinins form covalent adducts with four major membrane-associated parasite proteins. None of these adducts has been functionally characterized, leaving the possibility that inhibition of additional proteins may also have a role in artemisinin-mediated parasite killing (Krishna *et al*., 2004; Eckstein-Ludwig *et al*.,

2003). A highly specific, noncovalent interaction between artemisinin drugs and a protein target, as suggested by the PfATPase-dependant model of activity, could result in stereoselectivity, expressed by differences in the antimalarial activity of enantiomeric forms of the drugs. However, enantiomers of trioxanes structurally related to artemisinin showed equivalent levels of activity against chloroquine sensitive, chloroquine-resistant, and multidrug-resistant strains of *P. falciparum* (O'Neill *et al*., 2005). These results imply that activation of artemisinin does not depend on stereospecific interaction with a protein. In addition, introduction of bulky side groups to artemisinin may cause either a decrease in activity or increased activity, depending on which residues are added (Avery *et al*., 1996).

1.13.4 Artemisinins based combination Therapy (ACT) in Africa:

Several artemisinin combination trials have been conducted in Africa with variable results:

Agyepong *et al* (2004) in western Senegal; Zuker, *et al* (2003) in western Kenya, Muheki *et al* (2004) in southern Africa, Zuroyac *et al* (2005) in Zambia.

A big scale study funded by European research organization the WHO, TDR, World bank was carried out in Ghana (Mokenhaupt, 2005), in Rwanda (Rwaga, 2003), Mozambique (Abacassamo, 2004) and in Kampala Uganda (Dorsey, 2002). All the above trials compared Fansider (S/P) plus amodiaquine with (S/P) plus Artusenate and all enrolled children 6-59 month old, and all trials used the some dose and regimen, and all trials ended at 28 days according to the WHO (2003) protocol for evaluation of drug efficacy, and all these studies were conducted in

low transmission areas. This made comparisons of reported outcome possible. They concluded that in all these trials, Fansiar (S/P) plus Amodiqunie (AQ) was better than Fansdiar plus Atresunate at controlling treatment failure at day 28. This could be explained by the fact that (S/P) resistance has spread across Africa, so in combination with a drug which has long elimination half life it is more effective than in combination with artesunate which has half-life of one hour. More over S/P+ Artesunate was more effective in destroying gametocytes.

1.13.5 Artemisinin combination therapy in the Sudan:

The Sudanese policy makers updated their national treatment guidelines in 2004 to ACT (Malik *et al*, 2006).

The combination of **Artesunate (AS)** plus **Fansidar** (**AS + SP**) for the treatment of uncomplicated *P.f* malaria was tried in 10 areas with no reported failure in seven of them and < 1.0% failure rate in two and 8.8 % in one site (Malik *et al*, 2006).

In Malakal Van den Broek *et al* (2005) used the same combination and reported 0.9 % treatment failure, also in the southern region Elamin *et al*, (2005) reported complete cure with no failure using **AS +SP** combination. Muktar *et al* (2007) also tried **AS** plus **SP** compared to AL in eastern Sudan (Gedarif), the results revealed supremacy of the former.

The same combination (**AS +SP**) in western Sudan (Nuba Mountain) resulted in 8% failure rate reported by Hamour *et al*, (2005). And in eastern Sudan Adam

(2005a, b) reported 0.7% treatment failure. Again in central Sudan Mohd *et al*, (2006) stated that there were no cases of treatment failure.

Another artimisinin combination, **Artesunate** plus **Amodiaquin** was tried in western Sudan with a treatment failure of 7.3% (Hamour *et al*, 2005) ad also in southern Sudan where Van Broek (2005) reported 1% treatment failure.

1.14 Assessment of Drug Efficacy:

Evaluation of crude rate of adequate cure rate can be determined by parasite clearance as detected by microscope and also determined by resolution of symptoms and clearance of fever. However, precise evaluation requires molecular assessment.

Molecular genotyping is a simple and practical tool to distinguish between recrudescence and re-infection in antimalarial drug efficacy studies and that by using paired samples taken from a patient before and after treatment (Babiker, 1994; WHO, 1996 b). In this method infecting strains of malaria parasites can be fingerprinted through PCR amplification of polymorphic genes (Viriyakosol *et al*, 1995). The fingerprint pattern of isolates causing successive episodes of malaria can then be compared to distinguish recrudescent from newly infecting parasites (Cattamanchi *et al*, 2003).

Nested PCR with its associated high sensitivity has been used for the amplification of polymorphic repetitive regions, namely block 2 of merozoite surface protein-1 (MSP1), and block 3 of merozoite surface protein-2 (MSP2) in order to determine the presence of allelic variants from the MAD20, K1& R033 families of the

MSP1, and the FC27, IC1 families of the MSP2 repeats. Although MSP2 proved to be the most discriminatory marker, inclusion of other markers is of value (Snounou, 1999).

Molecular genotyping techniques are sensitive and are used to distinguish recrudescence from a new infections and that for all patients failing therapy after day 3. Outcome is defined as recrudescence if all alleles are present at the time of pretreatment and as a new infection if otherwise (Snounou, 1999).

1.15 Malaria Status in Sudan:

In Sudan, an estimated 7.5 million patients suffer from malaria each year and this disease accounts for up to 20% of hospital deaths (RBM, 2003) (WHO/EMRO 2001). This puts malaria on top of the most common diseases in Sudan (RBM, 2003), and that due to increasing levels of *Plasmodium falciparum* resistance against the two most commonly used antimalarials: namly Chloroquine and Sulfadoxine-pyrimethamine (fansidar). Malaria accounted for 37.2% of all maternal deaths in Sudan at hospital level (Dafalla, 2003). Also it was found to be the cause of 18.1% of low birth weight (Taha *et al*, 1995).

According to the annual statistical report of the FMOH, 17.4-44.8% of all outpatient clinic visits are for malaria, and 9.6 - 36.3% of all hospital admissions are a consequence of malaria. Case fatality rate is high ranging between 0.9 to 6.9%. It is increasing every year due to poor case management, low public awareness and the emergence of *P.f* drug resistance (RBM, 2003) It is documented

that, in Sudan those affected with malaria were unable to work for 22% of the time during the course of the year (WHO, 1996 a) (RBM, 2003) .

Malaria is endemic throughout the Sudan. Endemicity varies from hypoendemic in the North to hyper-to-holo-endemic in the South. Considering the prevailing epidemiological factors, it is worth to mention here that 80% of the population is living in epidemic-prone areas (unstable malaria transmission) (RBM, 2003).

In Sudan more than 90% of malaria cases is caused by *Plasmodium falciparum*, whereas 10% is due to *P. ovale, P. vivax, and P. amalarie* Put togther. *Anopheles arabiensis* is the primary vector widely spread allover the Sudan but *A. gambiae* and *A. funestus* are also found in many areas of the South (RBM, 2004).

1.15.1 Drug Resistance of *Plasmodium falciparum* in Sudan:

CQ has been used as first line treatment of uncomplicated *P.f* malaria in Sudan and that for decades. Unfortunately parasite resistance for this drug developed and was detected first in Gezira area, central Sudan (Omer *et al*,1978) then in Khartoum in (Tawil,1983), and then in easteran Sudan in 1986 (Bayomi *et al* 1998) and even in upper Nile in southern Sudan (Van den Breok, 2003) where about 80% of CQ treatment proved ineffective. The situation has been worsening ever since, whereby 32% *P.f* CQ resistance was reported from Khartoum (Tagelsir, 2006) and more than 75% from eastern Sudan (Adam, 2004). Sulphadoxin-pyrimethamine has long been used as 2nd line antimalarial drug in Sudan, however resistance to this is progressively developing. First report came from Senar area (Ibrahim *et al*, 1991) and then from Khartoum (Khalil, 2002) and from the East (A-Elbasit,

2006) and the South (Stivanello *et a*l, 2004). As for the treatment of complicated P.*falciparum* malaria the drug of choice has long been quinine, however resistance to this is also developing (Adam, 2002). Recently, in 2005, Sudan among several African countries has chosen artimisinin combination therapy as first and second line treatment for uncomplicated Chloroquine resistance f*alicparum* malaria despite limited studies of their efficacy in Africa (WHO, 2003). The WHO recommended that Artesunate + SP would be the first line and Artemether + Lumefanterin would be the second line (WHO, 2003).

Objectives:

General objective:

The purpose of this work is to provide the Sudanese policy makers with supportive data for change.

Specific objective:

The present investigation was designed to test the efficacy of Artemether plus lumefantrine as first line combination treatment for uncomplicated Chloroquine resistant *falicparum* malaria in an area of low transmission (New Halfa, Eastern Sudan). Drug efficacy parameters to be used are:

- Parasitological and clinical cure at day 28.
- Mentoring fever and parasite clearance time during 28 days follow up.
- Monitoring adverse side effects (Tolerability and safety).
- Molecular genotyping to distinguish a recrudescent infection from a new infection, in case of treatment failure.

There had been no such previous study in this area (N. Halfa).

CHPTER TWO

Materials and Methods

2.1 Ethical considerations:

The local health and institutional authorities approved the research protocol: namely the Sudanese Ministry of Public Health and the Faculty of medicine University of Khartoum. Verbal and written consent for participation were obtained from all participants or their parents or guardians, after thorough information on the study was provided in very simple language. At all times the safety and welfare of the patient were greatly considered. Patients were given the right to withdraw from the study at any time they wished to do so.

2.2 Study area

The study was an open-label one arm clinical trial. It was a non-comparative assessment of the efficacy and safety of Artemether plus lumefantrine (A/L) combination using the 28-day WHO (2003) therapeutic evaluation of antimalarial drugs in low malaria transmission areas. Therefore it was conducted in New Halfa eastern Sudan, which is located 500 kh from Khartoum city; 450m above sea level, and characterized by average annual rainfall of 238 mm, and average relative humidity of 35. There is a permanent irrigation system (open canals). New Halfa district is made up of iso-villages with an over all population of 400,000. The study was carried out in one village, **Elhara Eloula** which is inhabited by

1841 individuals (Sensus 2001). It was conducted in the peak of the transmission season after the rainfall season of (10 Oct- 15 Dec 2006).

This area was selected because it is characterized by low malaria transmission which is suitable for testing the efficacy of antimalarial drugs against uncomplicated malaria according to the WHO (2003) recommendations, as infection in high transmission areas is usually caused by multiple Plasmodia species and more important is that the majority of the population would be immune, whereby the specific immune response would kill some of the parasites irrespective of drug sensitivity i.e. immunity would influence the outcome of chemotherapy.

Determination of sample size:

Due to limited financial budget the study was meant to cover a minimum sample size accepted by the WHO (2003) protocol for the assessment of antimalarial drug efficacy (Appdix 4). Therefore determination of the sample size was based on the following factors:

Anticipated population proportion of clinical failure (P) = unknown

Desired confidence level usually 95%

Desired precision (d) (usually between 5%-10%)

According to WHO (2003) protocol if P is unknown a value of P+ 0.50 is recommended Therefore according to the (Appdx 4) when P is 0.05 and (d) is 10% i.e. 0.01 then (n) would be 96, or approximate to this.

2.3 The Participants in the Study:

Potentially eligible patients of all ages were those who attended the Out-patient clinic of Elhara Eloula health center with a confirmed fever (axillary temperature ≥37.5°C) and a positive Giemsa stained thin and thick blood films for *P. falciparum,*. 93 Participants of both sexes aged 4 to 55 satisfied the following **Inclusion** criteria according to WHO (2003), and were included in the study:

• weight ≥5 kg

• monoinfection with *P. falciparum*.

• parasitaemia >1000 asexual parasite per µL of blood.

• ability to come for the stipulated follow-up visits and easy access to the health facility.

• able to take oral drugs.

• absence of signs/symptoms of severe malaria.

• no major intercurrent illness or history of cardiac, hepatic or renal disorder.

• no known allergy to the study drugs.

Exclusion criteria on the other hand were:

• Pregnant women.

• Patients with clinically evident malnutrition.

•Patients present with repeated convulsion.

• inability to take anything orally.

• severe anaemia. • difficulty in breathing.

• patients with severe malaria (Appdix 5). Or a clear history of adequate antimalarial treatment in the previous 72 h.

On day 0 each selected participant was given a code number and was subjected to general physical examination and recording of name, age, sex, weight (to calculate dose), parasite count in blood, axillary temperature, etc, (Appdix 6).

Subsequently all participants were kept under close medical supervision, and adverse events if any were recorded any time during the follow up period (28 days). Clinical assessment, management, and drug administration were done by medical staff**.**

2.4 Methods:

2.4.1 Parasitological Diagnosis:

Thick and thin blood smears were prepared from finger prick for each patient on days 0, 1, 2, 3, 7, 14, 21, and 28 (or at any day that a patient feels unwell) to detect treatment failure. Thick & thin Giemsa stained blood films were examined according to the WHO (1983) protocol (WHO, 2003). Parasite density was determined on a thick blood film according to the number of parasites per 200-300 white blood cells (WBC) using a handcounter. Assuming a total WBC count of 8,000/µl. Parasite density was calculated per microliter blood using this formula:

$$\frac{\text{The number of asexual parasites counted} \times 8000}{200\text{WBC}}$$

Thus blood films were screened to detect the disease and the species (*P.f*); on admission day 0 and on days1, 2, 3, 7, 14, 21 and 28. If gametocytes were seen, their occurence was recorded. A thick film was considered negative if no parasites were detected after examining 100 microscopic fields each containing approximately 20 leukocytes. All the positive slides and 20% of negative slides

were reread blindly in the National Reference Malaria Laboratory in Khartoum for quality control according to WHO 1996 protocol.

For molecular diagnosis finger-prick blood samples, from each patient was obtained on filter paper (whatman.3mm), dried and kept into a separate plastic bag, on admission day 0 and on days 7, 14, 21 and 28, and on any day of recurrent illness. All samples on slides or filter paper were carefully labeled with name identification code and date.

2.4.2 Treatment:

All patients were treated orally with (Artemether+ Lumefatrine) (20/120).

A full treatment dose of each combination was adjusted according to the WHO protocols (2003). Six doses (twice daily) of the combination was given for three days as follows: 1.5 mg artemether/kg body weight and 9 mg lumefantrine/kg body weight, at 0, 8, 24, 36, 48 and 60 h (10–14 kg 1 tablet, 15–24 kg 2 tablets, 25–34 kg 3 tablets, >34 kg 4 tablets). The 3 days treatment began on the day of enrolment (day 0) and repeated on day 1 and 2. Patients were observed for an hour after administration of the treatment. Full drug doses were readministered if the patient vomited within 30 minutes and half dose if the patient vomited within one hour. Vomiting of re-administered dose would lead to withdrawal of the patient from the study; and a full course of intravenous Quinine would be administered as rescue treatment and the patient would be referred to the hospital.

The tablets were crushed and dissolved in water for children who were not able to swallow them. Lumefantrine is lipophilie so for maximum absorption the drug was always given with a cup of sugar sweetened milk, or after intake of groundnuts.

4.3 The Follow up Phase:

The follow up period of the study was 28 days according to the WHO (2003) protocol on evaluation of antimalarial drug efficacy. After day 0 patients were encouraged to come back to the clinic on days 1, 2, 3, 7, 14, 21, and 28 or any time between scheduled visits in case of unforeseen symptoms. To complete clinical and laboratory investigations, at each visit, the history, clinical signs and symptoms, body temperature and a blood sample for parasitaemia were collected. A blood spot on filter paper for molecular analysis was also collected at day 0 and at the day of treatment failure. If a patient did not appear in the clinic, we gave him/her a home visit. In case of treatment failures quinine was administrated 10 mg/kg/weight- for 7 days. Patients considered lost to the follow up if they missed any dose after the first one.

2.5 Treatment outcome:

2.5.1 Primary and secondary end points:

Primary efficacy end points were parasitological and clinical cure at day 28.

end points also involve drug safety based on adverse reactions that did not exist before initiation of treatment, or is not a classic symptom of malaria, or signs that worsened upon initiation of treatment or during follow up.

Secondary efficacy end points involve fever clearance time, parasite clearance time. Fever was judged by temperature of 37.5 ^{o}C or higher and history of fever during the preceding 24 hours (sign of malaria).

At the end of a 28 day period the out come of the study was classified according to WHO (2003) protocol.

2.5.2 Clincal and parasitological outcome:

These responses were classified into four groups as follows: • Early treatment failure **(ETF**): if a patient had any of the following during the first3 days of follow up: danger signs of severe malaria or severe malaria (Appdix 5) on Days 1, 2 or 3 in the presence of parasitaemia or parasitaemia on Day 2 greater than on Day 0; or parasitaemia on Day 3 with axillary temperature ≥37.5 ◦C; or parasite density at Day 3 equal to or greater than 25% of that at Day 0 Late clinical failure **(LCF)** was defined as the development of one of the following from day 4 to day 28: danger signs of severe malaria in the presence of parasitaemia or parasitaemia with axillary temperature ≥37.5 ◦C, without having been previously classified as ETF.

• Late parasitological failure **(LPF**): was defined as parasitaemia on any of scheduled days of return (7, 14, 21, 28) without fever and without previously meeting any of the criteria for ETF or LCF.

• The primary outcome was the Adequate Clinical and Parasitological Response **(ACPR)** which was defined as the absence of parasitaemia on Day 28 without previously meeting any of the criteria for ETF, LCF or LPF, after Day 7.

Parasitaemia confirmed by PCR as recrudescence was considered as treatment failure.

2.6 Molecular Diagnosis:

Polymorphism at MSP-1 and MSP-2 gene loci resulting from repetitive sequences has been considered the most suitable genetic marker for rapid genotyping of *P.falicparum*. Allelic variants can be distinguished by size upon amplification of polymorphic loci by the polymerase chain reaction and subsequent electrophoresis in agarose gel.

2.6.1 DNA extraction:

Plasmodium falciparum DNA was extracted from blood samples collected on air-dried Whatman3 filter papers, using the chelex methods (Plowe *et al*, 1995). A small sector was excised from each filter paper sample using new clean razor blade which was cleaned with 5M HCl, followed by 5M NaOH, washed with distilled water and wiped before cutting each and every sample. Paper segment cut from each blood sample was placed in-to labeled 1.5 ml micro centrifuge tube soaked in 1.0 ml .05 %saponin in 1× PBS (0.14M NaCL, 0.1M Na2HPO4) pH 7.2; inverted for 10 min and incubated overnight at 4◦C. The overnight solution was then removed and the sector washed and inverted twice with 1 ml PBS; 50µl hot chelex solution (20%), 100µl sterile water was add to each sector and placed in a heating block at 100◦c for 5 minutes. The flitter paper was then removed carefully from the tube using clean forceps which were washed in (HCL 5M & NaOH 5M). The tube was capped gently; vortexed for 30 seconds, and then returned to the heat

block for 10 minutes; The samples were then centrifuged (10.000xg for 2 minutes). Leaving the chelex in the original tube the supernatant was removed to a new micro centrifuge tube and used in amplification reaction (or stored in -20◦c).

2.7 The PCR for the detection of Polymorphism of *P. falcipraum* at MSP-1 and MSP-2 gene loci:

The amplification strategy for genotyping *P.F* is the nested PCR

A Nested polymerease chain reaction was used to distinguish recrudescent resistant genes from new infection genes for all treatment failure samples from day 4- 28 (Snounou 1999, modified). Block 2 of MSP1 gene and block 3of MSP2 gene were genotyped for allelic variation. In the first amplification reaction the appropriate oligo primers pairs will hybridize to conserved sequences flanking the repeat polymorphic regions of each of those 2 genes .Subsequently outer PCR product will be used as template for separate second (nest 2) amplification reactions, together with the appropriate allelic specific oligonucleotide primer pairs. Thus 5 nested PCR reactions were carried out to amplify the MAD20, K1, RO33, allelic families of MSP-1 and FC27, ICI, allelic families MSP-2. The sets of primers used were as follows:

MSP1gene

Primer	Sequence
M1− OF	5′CTAGAAGCTTTAGAAGATGCAGTATTG 3′↔ MSP-1 outer1
M1−OR	5′CTTAAATAGTATTCTAATTCAAGTGGATCA3′↔MSP-1
M1−KF	5′AAATGAAGAAGAAATTACTACAAAAGGTGC 3′ ↔specific
M1−KR	5′GCTTGCATCAGCTGGAGGGCGTTGCACCAGA3′↔K1
M1−MF	5′AAATGAAGGAACAAGTGGAACAGCTGTTAC 3′↔ specific
M1−MR	5′ ATCTGAAGGATTTGTACGTCTTGAATTACC3′↔MAD20
M1−RF	5′ TAAAGGATGGAAATACTCAAGTTGTTG3′↔ specific for
M1−RR	5′CATCTGAAGGATTTGCAGCACCTGGAGATC3′↔Ro33

MSP2 gene

Primer	Sequence
M2−OF	5′ATGAAGGTAATTAAAACATTGTCTATATA3′↔MSP-2
M2−OR	5′ CTTTGTTACCATCGGTACATTCTT3′↔MSP-2↔ outer 1
M2−FCF	5′AATACTAAGAGTGTAGGTGCARATGCTCCA3′↔ specific
M2−FCR	5′TTTTATTTGGTGCATTGCCAGAACTTGAAC3′↔FC27
M2−ICF	5′AGAAGTATGGCAGAAAGTAAKCCTYCTATACT3′↔specif
M2−ICR	5′GATTGTAATTCGGGGGTTCAGTTTGTCG5′3′↔IC

All outer and nested PCR reactions took place in a total volume of 20 µl: containing a final concentration of 0.125 µM for each primer, 125µM each of 4 deoxynucleoside triphosphate (dNTPs): (dATP, dCTP, dGTP, dTTP), and 0.4 units of hot start Tag polymerase (enzyme).

To initiate all the outer reaction 1ul of the extracted DNA was used, then 1 µl of the outer product was used to initiate each of the 5 separate nested reactions.

Genomic DNA from laboratory clones 3D7, HB3, and RO33 were used as control for MAD20, K1 and RO33 MSP1gene families respectively, and HB3 and 3D7 for FC27 and IC1 MSP2 gene families respectively.

All the outer and nested reactions for MSP1 and MSP2 were preformed with an initial denaturation for 2 minutes at 94C, 30 amplification cycles with annealing at 58C and extensions at 65 for 2 min for the outer reaction and 1min for the nested reaction The last extension was carried out for 5 min. Thirty five cycles were performed for the outer reaction and thirty cycles for all nested reactions.

The amplifications were performed on PCR machine (TECHE, TC.321.model: FTC 105D, serial No 13292-1, CAMBRIGE, UK).

2.8 PCR Analysis:

2.8.1 Agarose Gel electrophoresis:

Preparation of 1.5% Agarose Gel: To every 0.6 gm agarose 4 ml of 10x Tris Boric EDTE (TBE) (0.09 boric acid, 0.09M Tris, 0.002M EDTE) and 36 ml of distilled water were added. The mixture was melted and allowed to cool, then 3µl Ethidium Bromide stain (10mg/ml) were added to the gel and mixed. The mixture was poured into horizontal electrophoresis mini gel tank with suitable size combs and the gel was left for 60 minutes to polymerize. 7 µl of PCR product were mixed with 3µl loading buffer and loaded in to the gel. Running buffer was added. It consisted of 250 ml distilled water and 1.5 ml 10x TBE buffer. The run was preformed at 110 volt and a current range 3-8 mA for 90 minutes. Four DNA molecular weight markers were included.

2.8.2 Visualization of gel:

The DNA was visualized by ultra-violet transillumination and photographed using UV camera with 667 Polaroid film. The size of the PCR fragments was estimated as the distance of migration relative to the marker size.

2.8.3 Evaluation of molecular genotyping (Assessment of treatment failure):

Molecular genotyping can distinguish between treatment failure due to resistant parasites or due to new infection and that by comparing genotyping patterns, on day of treatment failure with genotyping patterns on the day of enrolment. Each band assigned a molecular wt. is considered an individual *P.f* strain. If the picture on day 0 and day of treatment failure is considered the same then the treatment failure would be considered a true recrudescent if other wise the picture is classified as new infections. If the picture contains mixture of bands, it can be considered mixed infection (old and new).

2.9 Statistical analysis:

Statistical analysis of data was preformed using Stata /SE version 15.0 statistical software (Stata Corporation, Texas, USA; http:www.stata.com.) Data obtained was entered into a computer database, and analyzed using SPSS (Statistical Package for Social Science).

CHAPTER THREE

Results

3.1 Baseline data

A total of 93 symptomatic malaria patients, 4 to 55 years of age of both sexes were initially enrolled in a drug efficacy study involving artemether plus lumefantrine combination (AL) in the treatment of uncomplicated falciparum malaria. The study was conducted between October to December 2006 Baseline characteristics of participants are shown in Table 3.1.

Table 3.1: Baseline characteristics of uncomplicated falciparum malaria patients at enrollment (day 0) in artemether plus lumefantrine (AL) efficacy study, in New Halfa, eastern Sudan

Characteristics	
Age (years)	
Median (25^{th}; 75^{th})	16 (11; 27)
Sex (male: female)	47:46
Mean weight ± SE (kg)	39.1 ± 1.8
Range (kg)	12 – 76
Mean axillary temperature ± SE (◦C)	37.9 ± 0.06
Range (◦C)	37.2 – 39.1
Parasite density/µl blood	
Median (25^{th}; 75^{th})	7683 (3000; 21665)
Gametocyte carriage N (%)	0 (0)

On admiton day the age prevelance rate of symptomatic patients was receded, Table 3.2 and Fig 3.1 showed that the follow up malaria was more prevent in the younger age group (4-14) and was higher in age group (10-14) (23.7%)

Table 3.2: Prevalence of uncomplicated falciparum malaria according to age on day (0) (n = 93)

Age group (years)	Prevalence rate (%)
4 – 9	19 (20.4)
10 – 14	22 (23.7)
15 – 19	18 (19.4)
20 – 24	9 (9.7)
25 – 29	7 (7.5)
30 – 34	8 (8.6)
35 – 39	3 (3.2)
40 – 44	2 (2.2)
45 – 49	3 (3.2)
> 49	2 (2.2)
Total	93 (100)

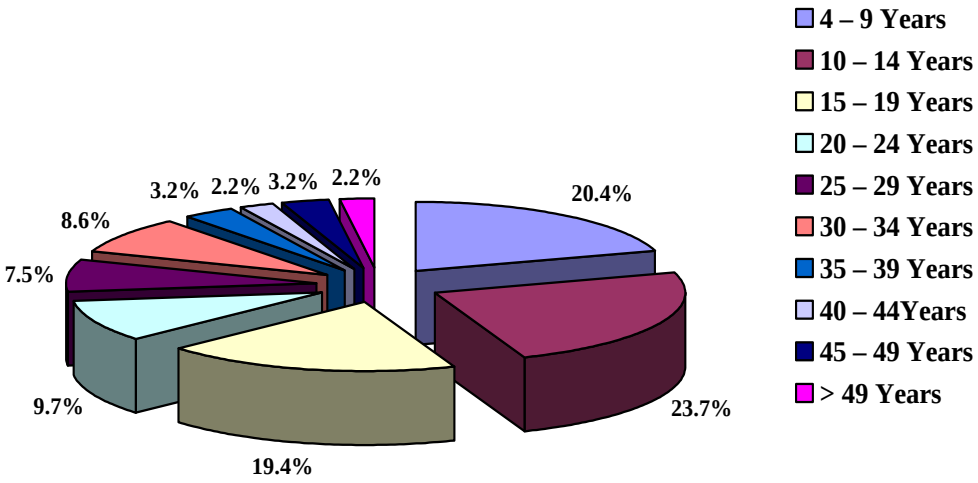

Figure 3.1: Prevalence of uncomplicated *Plasmodium falciparum* malaria according to age at day (0) (n = 93).

When in parasite density load was studied on day 0 (Table 3.3; Fig 3.2), it was observed that the values was quite high with a wide rang of < 4000 to over 20,000. However 35% of those who were in the lowest range of < 4000, which is characteristic of low transmission areas.

Table 3.3: Proportion of initial parasitaemia (Day 0) (n = 93)

Parasite/ µl of blood	Number (%)
< 4000	33 (35.5)
> 4000 – 8000	17 (18.3)
> 8000 – 12000	10 (10.8)
>12000 – 16000	5 (5.4)
>16000 – 20000	4 (4.3)
> 20000	24 (25.8)

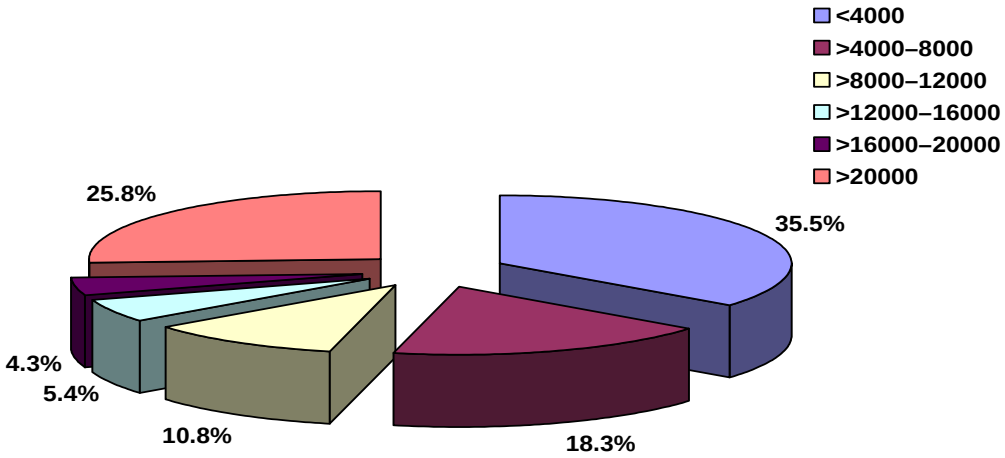

Figure 3.2: Proportions of initial parasitemia (parasite/ µl blood) (Day 0) (n = 93)

However when the age groups were compressed (Table 3.4) it become evident that at day 0 age (4-14) carried the highest load of parasites. And the diffidence between them and older adults was statically significant (P=0.01)

Table 3.4: Initial malariometric indices according to age (n = 93)

Age group (years)	Initial parasitaemia Median (25^{th}; 75^{th})	Parasite clearance time Median (25^{th}; 75^{th})	Fever clearance time Median (25^{th}; 75^{th})
Children (4 – 14)	12840 (5500; 31250)	1 (1; 1)	1 (1; 1)
Adolescents & young adults (15 – 39)	4500 (2409; 10460)	1 (1; 1)	1 (1; 1)
Older adults (> 39)	4000 (1287; 7800)	1 (1; 1)	1 (1; 1)
P- value	0.01*	0.84	0.73

- **= Statiatically significant**

However on day (0) the admission day, two patient had to excluded according to the study protocol: one was excluded due to repeated vomiting of the drug; the other developed convulsion. More over after day 3 the end of the treatment eleven were lost to follow up. Therefore the study was completed for 82 patients up till day 28 post treatment, or till treatment failure.

3.2 Primary effectiveness outcome:

On day 28 and before PCR correction 78 patients experienced ACPR (95.1%). However four patients (4.9%) experienced LTF all of them were LCFs. Three of the LCF were detected on day 21 and one was detected on day 28 (end of follow up). Neither ETF nor LPFs were observed among the study group.

As for parasite clearance time, the proportion of patients with parasitemaeia decreased from day 0 (100%) to (2.4%) on day 2. then all were aparasitemic until day 28 except for four patients between day 21 and day 28 (Table 3.5; Fig 3.3)

Table 3.5: Proportion of parasitemic patients during 28 days follow up after treatment with (AL).

Follow up day	Parasitemic patients: % (N)
Day 0	93 (100)
Day 1	2 (2.4)
Day 2	0 (0)
Day 3	0 (0)
Day 7	0 (0)
Day 14	0 (0)
Day 21	3 (3.7)
Day 28	1 (1.3)

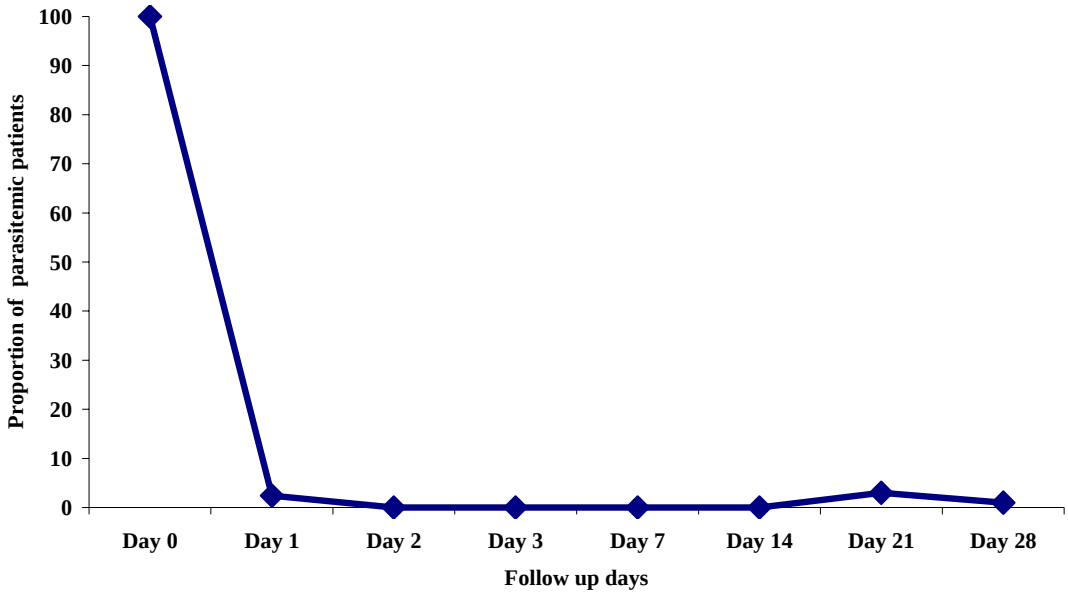

Figure 3.4: Proportion of parasitemic patients during 28 days following treatment with (AL).

3.3 Secondary effectiveness outcome

Fever clearance time; median (25^{th}; 75^{th}) was 1 day (1; 1), ranging between 1 to 7 days. The proportion of patients with fever decreased from Day 0 (100%) to 14.6% at day 1 and 2.4% at day 2, and (2.4%) at day 3. By Day 7 all treated patients were afebrile (Table 3.6; Figure 3.4). However on day 21, 3 were febrile and one also at day 28 these were symptomatic when patterns of response were tabulates against age, it was found that the 4 patients which experienced late

cilincal failure 3 of them in the young group (9. 18. 12) and young adult (29 years) (Table 3.7)

Table 3.6: Proportion of febrile patients following treatment with artemether plus lumefantrine

Follow up days	Febrile patients: N (%)
Day 0	82 (100)
Day 1	12 (14.6)
Day 2	2 (2.4)
Day 3	2 (2.4)
Day 7	0 (0)
Day 14	0 (0)
Day 21	3 (3.7)
Day 28	1 (1.3)

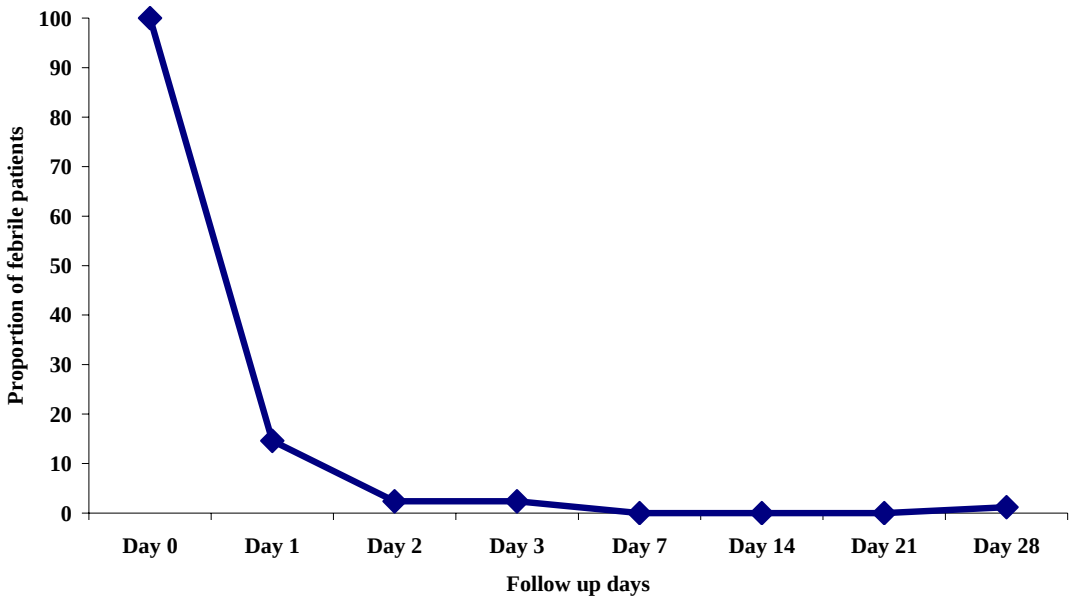

Figure 3.5: Proportion of febrile patients following treatment with (AL)

Parasite clearance time; median (25^{th}; 75^{th}) was 1 (1; 1). There was no statistically significant difference in parasite clearance time between ACPR group and LCF group (P = 0.8). There was a sharp decline in the proportion of patients with parasitemia from day 0 to day 1. By day 2, all the studied patients were aparasitemic and remained aparasitemic till day 14 (Table 3.6; Figure 3.5).

Table 3.7: Patterns of response according to age at the end of follow up period (28 days) after treatment with (AL).

Age group (years)	ACPR Number (%)	LCF Number (%)	Total Number (%)
Children (4 – 14)	37 (97.4)	1 (2.6)	38 (100)
Adolescents and young adults (15 – 39)	36 (97.3)	1 (2.7)	37 (100)
Older adults (>39)	7 (100)	0 (0)	7 (100)
Total Number	80	2	82 (100)
P- Value = 0.91			

- ACPR = Adequate clinical and parasitological response
- LCF = Late clinical failure

No significant adverse event were observed..

Table 3.8: Profile of malariometric indices in the four LCF patients

Patient	Day 0	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28
Patient 1								
Axillary temperature (◦C)	37.4	36.4	36.6	35.8	36.4	36.2	36.7	–
Number of parasites /µl blood	28880	0	0	0	0	0	1020	–
Patient 2								
Axillary temperature (◦C)	38.8	35.9	35.9	35.9	36.1	36.1	37.2	–
Number of parasites /µl blood	8060	0	0	0	0	0	12000	–
Patient 3 Axillary temperature (◦C)								
Number of parasites /µl blood	2366	36.1	36.3	36.8	35.9	35.9	36	39.1
		0	0	0	0	0	0	3000
Patient 4 Axillary temperature (◦C)								
Number of parasites /µl blood	37142	36.7	36.1	36.1	36.4	36.1	36.6	–
		0	0	0	0	0	2000	–

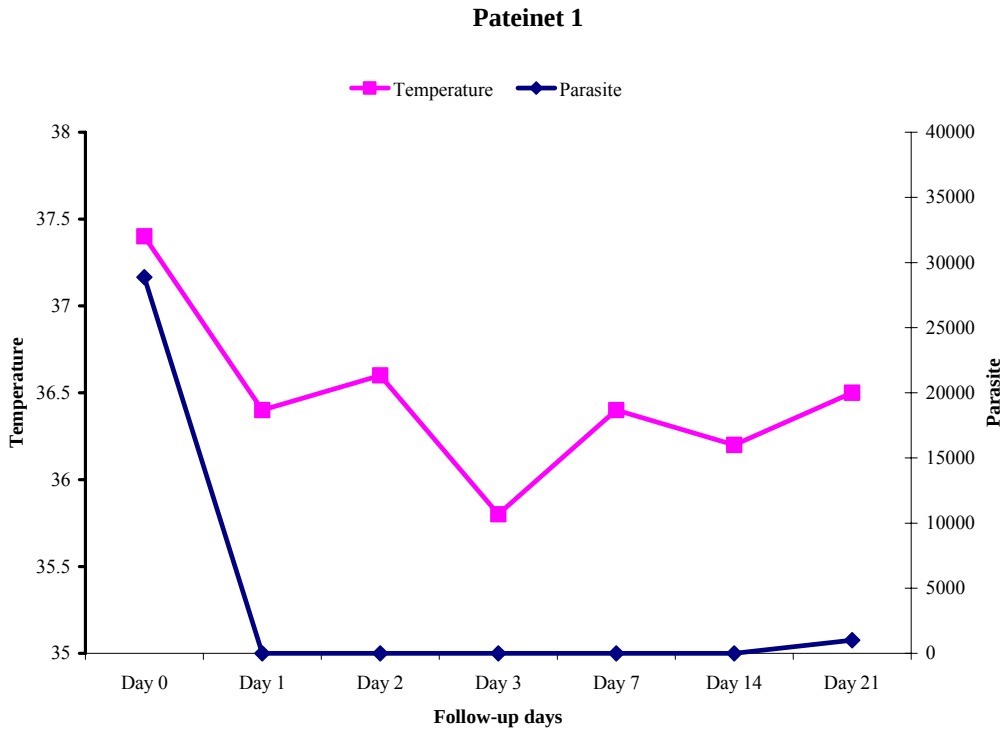

Figure 3.6: Profile of malariometric indices in (LCF) patient No. 1 day 21 post-treatment.

It was clear from Fig 3.6 that this patient improved dramatically from day 1 to day 14. however low level of parasitaemia and slight increase in temperature caused failure to occur on day 21.

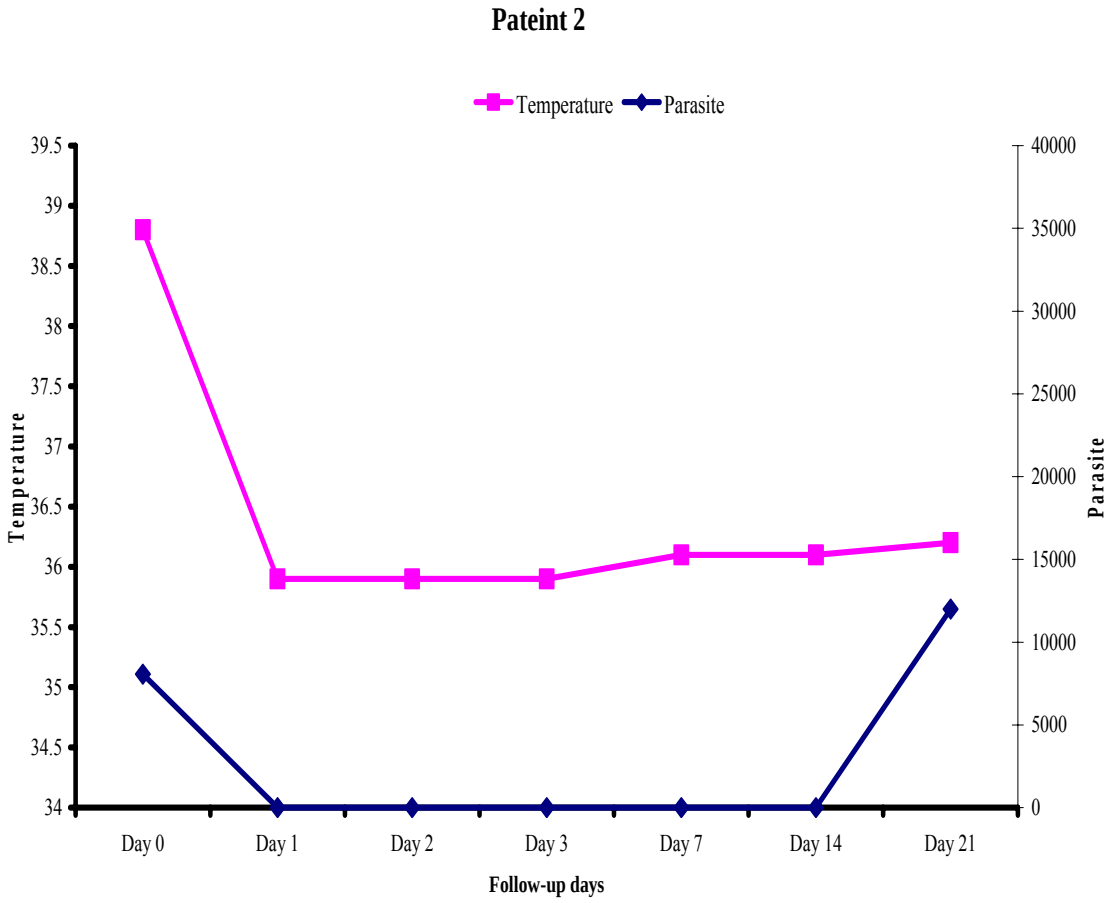

Figure 3.7: Profile of malariometric indices in (LCF) patient No. 2 day 21 post-treatment.

As for LCF patient No. 2 (Fig3.7) although the rise in axially temperature during the 21 days follows up was not considerable parasite density suddenly because elevated between day 14 and bay 21 to a level higher than on enrolment day.

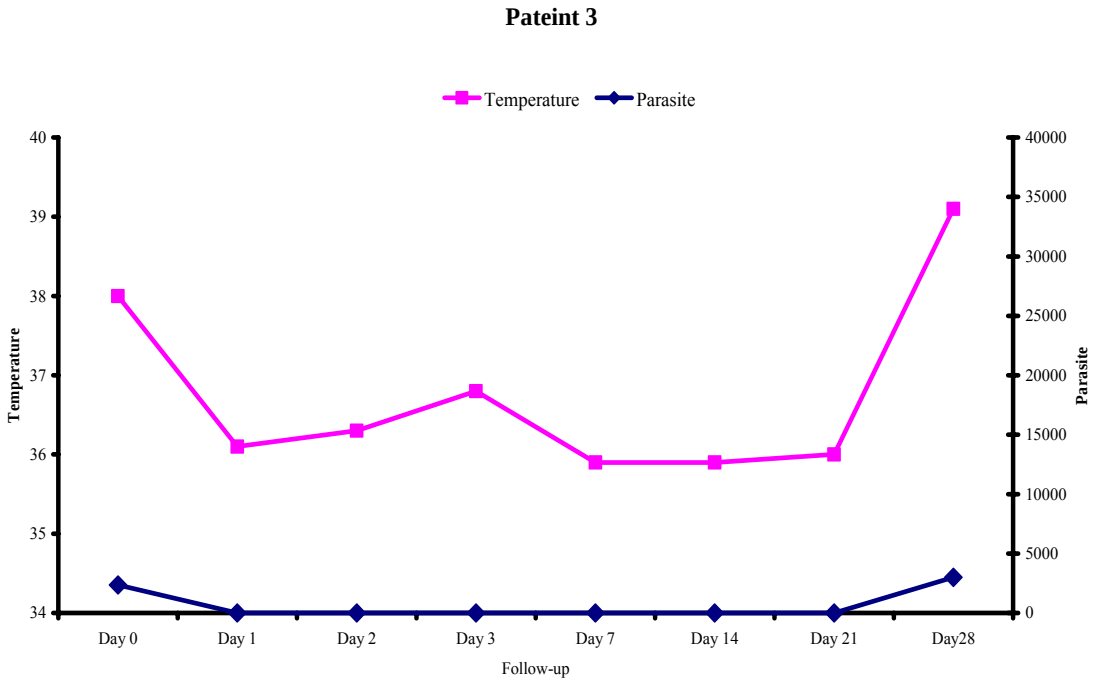

Figure 3.8: Profile of malariometric indices in (LCF) patient No. 3day 28 post-treatment.

LCF patient No. 3 (Fig 3.8) experienced sudden rise in temperature on day 28, however this is was no paralleled by a considerable rise in parasitaemia which repapered at day 28 with lower level comparable to initial density at day 0.

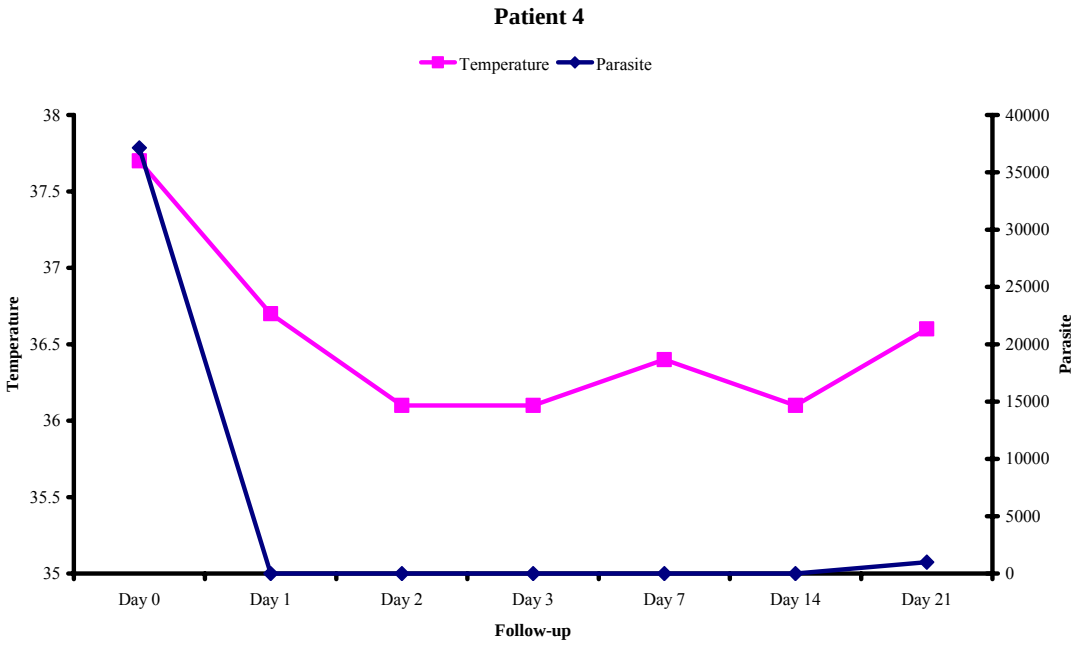

Figure 3.9: Profile of malariometric indices in (LCF) patient No. 1 day 21 post-treatment.

Patient No. 4 (Fig 3.9) although classified as LCF due to detectable parasitaemia ; nevertheless initial density of parasitaemia has been reduced by over 95% at day 21. It can be deduced from the malariomertic profile of the 4 LCF patients that the sharp drop in fever and parasitaemia between day 0 and ady1is defiantly due to drug effect. And also the total absence of parasitaemia there after up to day 21 or 28.

Genotyping of P.f parasites from the 4(LCF) patients using the MSP-1 and MSP-2 marker gene loci pre and post treatment (day of failure), are shown in Fig 3.10 (1-5).

Genotyping of paired samples of day 0 and the day of failure showed that (1 out of 4) had identical pre- and post-treatment patterns, suggesting recrudescent (true failure) in this patient 2 of 4 of the post treatment samples contained alleles different from those observed in pretreatment samples (50%) and were classified as new infections. In addition one pair of samples showed evidence of both new infections and recrudescent parasites, indicating mixed infection. Therefore the 28-day PCR-adjusted cure rate was 97.6% (Table 3.8).

Table 3.9: Crude and PCR-adjusted therapeutic efficacy of (AL) among patients with uncomplicated malaria in New Halfa, eastern Sudan (28 days follow up).

Characteristics	Treatment outcome on day 28: N (%)	
	Crude treatment outcome	PCR-adjusted treatment outcome
ETF	0 (0)	–
LCF	4 (4.9)	2 (2.4)
LPF	0 (0)	–
Total failures	4 (4.9)	2 (2.4)
ACPR	78 (95.1)	80 (97.6)

- ACPR = Adequate clinical and parasitological response
- LCF = Late clinical failure
- LPF = Late parasitological failure
- ETF = Early treatment failure

MAD 20

Figure (3.10.1): Genotypes of paired samples of the late treatment failure parasites using polymerase chain reaction analysis for MAD 20*Plasmodium falciparum* polymorphic loci: merozoite surface protein-1

Lanes: 2,3 patient No 1 D 0& D. 21; Lanes 4,5 patient No 2 at D0 &D 21

Lanes: 6,7 patient No 3 D 0& D. 28; Lanes 8,9 patient No 4 at D0 &D 21

K1

Figure (3.10.2): Genotypes of paired samples of the late treatment failure parasites using polymerase chain reaction analysis for KI *Plasmodium falciparum* polymorphic loci: merozoite surface protein-1

Lanes: 2,3 patient No 1 D 0& D. 21; Lanes 4,5 patient No 2 at D0 &D 21

Lanes: 6,7 patient No 3 D 0& D. 28; Lanes 8,9 patient No 4 at D0 &D 21

RO33

Figure (3.10.3): Genotypes of paired samples of the late treatment failure parasites using polymerase chain reaction analysis for RO33*Plasmodium falciparum* polymorphic loci: merozoite surface protein-1 (MSP-1)

Lanes: 2,3 patient No 1 D 0& D. 21; Lanes 4,5 patient No 2 at D0 &D 21

Lanes: 6,7 patient No 3 D 0& D. 28; Lanes 8,9 patient No 4 at D0 &D 21

IC1

Figure (3.10.4): Genotypes of paired samples of the late treatment failure parasites using polymerase chain reaction analysis for IC1 *Plasmodium falciparum* polymorphic Loci: merozoite surface protein-2.

Lanes: 2,3 patient No 1 D 0& D. 21; Lanes 4,5 patient No 2 at D0 &D 21

Lanes: 6,7 patient No 3 D 0& D. 28; Lanes 8,9 patient No 4 at D0 &D 21

FC27

Figure (3.10.5): Genotypes of paired samples of the late treatment failure parasites using polymerase chain reaction analysis for CF27 *Plasmodium falciparum* polymorphic Loci: merozoite surface protein-2.

Lanes: 2,3 patient No 1 D 0& D. 21; Lanes 4,5 patient No 2 at D0 &D 21

Lanes: 6,7 patient No 3 D 0& D. 28; Lanes 8,9 patient No 4 at D0 &D 21

Table (3.10) Circulating *P. falciparum* population diversity for the *msp-1* and *msp-2* genes

	Msp1			Msp2	
	Mad20	K1	R033	IC1	FC27
Patient1 D0	-ve	-ve	+ve	+ve	-ve
D21	-ve	+ve	-ve	+ve	+ve
Patient2 D0	+ve	-ve	-ve	+ve	-ve
D21	+ve	-ve	-ve	+ve	-ve
Patient3 D0	-ve	+ve	-ve	+ve	-ve
D28	-ve	-ve	-ve	+ve	-ve
Patient4 D0	+ve	-ve	+ve	+ve	+ve
D21	-ve	+ve	-ve	+ve	-ve

Table (3.11) prevalence (%) of MSP I&2 families in the LCF patients (n=4)

MSPI Family	**prevalence (%)**
MAD 2**0**	37.5
KI	37.5
RO33	25
MSP2 Family	
IC1	100
FC27	25

. Result of genotyping showed that MSP-2 is a more sensitive genetic marker than MSP1 **(**Table 3.11**)** as IC1 family recorded 100% prevalence this has also mention by Snounou **(**1999**)**.

CHAPTER FOUR

Discussion

Falciparum malaria is the most dangerous parasitic disease of man, where mortality in spite of treatment rises to 15-20% (White 2004). Malaria is estimated to kill about two million people each year; these are mainly children in the South East Asia and Africa (Breeman, 2001).

In Africa treatment has relied solely on Chloroquine CQ and more recently on Sulfadoxine-Pyrimethamine SP. These two drugs are cheap and have been known to be effective and slowly eliminated from the body. However, misuse of Chloroquine and the long decades of its use have provided a great selection pressure on *P. falicparum* to evolve mechanisms of resistance (Checchi, 2005). Thus in all malaria endemic areas (except Latin America) CQ has became completely ineffective. Moreover, resistance to SP in South East Africa has developed much more rapidly than Chloroquine. Therefore the emergence of resistance in *P. falciparum* towards these two drugs has been a major cause of accelerating mortality rate especially among children in tropical Africa.

The epidemiology of *P. falciparum* in Sudan reveals that by the end of the year 2003 evidence proved that CQ was no longer effective (reviewed by Hameed 2003). At the same time SP was introduced as first line treatment of uncomplicated

falicparum malaria, but subsequent, reports came from different parts of Sudan showing up to 7.5% treatment failure for SP (reviewed by Salah *et al* 2005).

Meanwhile *P. falciparum* resistance to the other alternatives among the quinolines and antifolates is progressively developing where such drugs are widely used as in Thailand, Vietnam, Brazil and other regions in S.E. Asia (Bosman, 2007). Therefore it has become urgent that extensive research be directed towards planning strategies that probably only delay but not prevent the emergence of resistance in *P. faliparum*. The currently leading strategy recommended by the WHO (2003) is the use of antimalaial drug combination (of 2 or more drugs) with different modes of action and therefore different resistance mechanisms. The rationale behind this is that the parasite would have to mutate simultaneously in several sites to become resistant to all partner drugs in the combination. This event is less likely to occur than a single mutation conferring resistance to a single drug. Another advantage is the combined effectiveness. This stimulated a change in Sudan towards employing SP + CQ as first line therapy for malaria especially because SP resistance in Sudan is limited. However treatment failure rates were reported (reviewed by Adam *et al,* 2004). This is due mainly to ineffectiveness of CQ. All drugs in the combination should be at least partially effective.

Inclusion of artemisinin derivatives in any *P.f* antimalarial combination therapy has been strongly advocated by the WHO (2003) strategy. Artemisisnins (derived from a Chinese plant) in combination clear parasitaemia and resolve *P.f* malaria symptoms in a matter of few hours. *Invivo* resistance to this group of antimalarials

has not been reported yet (Ashley & white, 2005). However, to prolong the therapeutic life of Artmisinins, in other words to protect them from mechanism of resistance in *P. falciparum*, these derivetaves should ONLY be used in combination with other antimalarials.

Most research worldwide has focused on the use of Artesunate (derivative of Artemisinin) combined with **standard** antimalarial drugs namely, mefloquiune, amodiquine, CQ and SP. Results showed that cure rates vary widely in different geographical areas and depend mainly on the level to resistance of standard antimalarial drugs in a specific malaria endemic area. For example, Artesunate combination with CQ in Sudan resulted in treatment failure (Adam, 2005b) due to wide spread CQ resistance. Similarly 31% failure rate of CQ + dihydroartemisinin has been reported from Gedaref, eastern Sudan (Osman, 2007).

Registration of artimisinins for use in malaria endemic countries and regions has been pursued but only one fixed dose oral combination is so far available, namely Artemether plus Lumefantarein (AL). Considering the current status of CQ , and SP resistanant malaria in Sudan and predicting the possible emergence of resistance to potentially newly introduced alternatives, the Sudanese Ministry of Health up graded its malaria treatment policy to the use of Artesunate + SP as first line therapy and AL as second line therapy. This stimulated the present study designed to monitor the effectiveness of AL as potential first line therapy in a sample of 93 symptomatic patients infected with *P.falicparum* uncomplicated malaria. The sample included all ages, (males, females, children and adults)

attending the local heath centre in a small village (Alharra Aloula) of New Halfa town characterized by low malaria transmission (non immune patients).

Results of this study revealed high efficacy of this combination whereby 95.1% cure rate was obtained by the end of 28 days follow up period (Table 3.8)

Fig (3.5) and Fig (3.6) reflect the rapid effect of artemether (with half life ≥one hour) which lead to parasite reduction in number with time. Time here is an important factor, as timely inhibition of parasite multiplication (a new generation of merzoite every 48 hours) prevents the progression to complicated malaria and also leads to resolution of fever and other symptoms.

The pyrogenic density Table (3.4) (the density of parasite at which symptoms and fever occur together) reflects the ecology of the area which is characterized by low malaria transmission and thus individuals of all ages are highly infected due to the lack of specific immunity. The artemether will never be presented to this relatively large number of parasites (Table 3.3) at intermediate drug concentrations because with its short half life it is eliminated **within** the 2 days (48 hours) asexual life cycle of the parasite. This means that no parasites are exposed to artemether (during one asexual cycle) **without** lumefantrine being present in plasma.

For the combination to be so highly effective in 3 days ragimen, the elimination half life of the artemisinin partner drug should be more than 24 hours, in other words it should be a slowly eliminated drug. This requirement is satisfied by partnering with lumefantrine which has a half life of 3-4 days, thus lumefantrine will provide complete protection for artmether from selection by prospectively

resistant parasites. However a small portion of **residual** parasites are left to lumefantrine. This is reflected in the Table (3.5) where 95.4% of patients became aparasitemic at day 1and at day 2 after one cycle of parasite multiplication (48 houre) complete clearance was achieved. Thus artemether proved to have a very high killing rate.

In low malaria transmission area, fever, as a clinical symptom is always associated with parasitaemia (parasitaemic is always symptomatic). Thus in this study the great reduction in parasite density was paralleled by reduction in axillary temperature and resolution of symptoms (Fig 3.5, Fig 3.6).

Results also revealed that no significant adverse effects after treatment were observed, except mild headache, or fatigue i.e minor complaints that did not result in loss to follow up.

As the choice of the sample was determined by practical considerations, there was insufficient number of cases among older people. The choice of sample was not random in the true sense as the study consisted of symptomatic malaria patients who attended the outpatient clinic (October to December). However to minimize this effect, for detailed analysis of prevalence rates the cohort was divided into 10 age groups Table (3.2). this showed that maximum prevalence of malaria (67.5) occurred in childhood and adolescence (age 4-19), typical of low transmission areas (WHO, 2003). However when the interval between age groups was compressed as appears in (Table 3.4) there was an inverse relationship between parasite density and age and the difference was statistically significant. However

this can not be considered as due to development of immunity like in high malaria transmission areas, but could be due to insufficient number of adult cases.

Table (3.4) again reflects the killing capacity of this combination where the parasite clearance time was the same for all levels of parasitaemia.

However the 95.1% cure rate left 4.9% (4 patients) infected, 3 at day 21 and 1 at day 28. Is this due to parasite recrudescence or due to the short half life of artemether or due to reduced parasite sensitivity to lumefantarine? or is it due to new infections contracted during the long follow up period (28 days)? To elucidate this point molecular genotyping of *P. falciparum* was conducted on filter paper blood samples taken on day 0 (admission day) and on treatment failure day (21 or 28).

Genotyping of *P. falciparum* by PCR at the allelic variants in the polymorphic regions of MSP-1 and MSP-2 marker gene loci Fig 3.9. (1- 5) revealed the following:

Two patients (50%) aged 9 and 18 showed different allelic patterns on day 0 and 21 genotypes, were compared, these are classified as definite new infections.

Only one patient (25%) aged 29 showed both new allelic forms together with day 0 patterns and that on day 28 genotype. This was classified as mixed infection.

One patient (25%) aged 12 retained the same allelic pattern when genotype of day 0 and day 21 where compared this was classified as recrudescent.

Thus the true cure rate of AL corrected by PCR is 97.6%. (Table 3.8)

The results of genotyping LCF revealed 50% definite new infections, beside one case of mixed infection (old + new); this gives 75% of new infection, which is more likely to occur in hyper endemic high transmission areas not in hypoendemic areas as this study. It is possible that in this case (low transmission area) there may have been a strain at very low density undetectable by PCR at day 0, that may have emerged later on (day 21 or day 28) to cause treatment failure leading to misidentification of recrudescence as a new infection.

Among the 4 LCF patients (3), (75%) of them were ≤18 years, they belonged to an age group with high initial parasitaemia (Table 3.4). Is there an association between age or initial density of parasites and treatment failure? However because of the small number of treatment failure patients no conclusion could be drawn.

The results also showed that even in the 4 cases of treatment failure the combination at least reduced the complexity of the infection. In other words the infection did not develop to complicated malaria. Parasitaemia and axillary temperature in these patients are shown in (Table 3.8) and Figs 3.8.1-4).

The axillary temperature fluctuated throughout the follow up period but below the initial level (day 0).

As for parasitaemia it starteddisappearing from day one and continued so up to day 14 to reappear between day 14 and day 21 or between day 21 and ay 28 though at a much lower level than day 0. All these observations are due to drug effect.

The infection in 3 treatment failure patients was capable of production of few but detectable gametocytes cause in absence of artemether effect. Artemisinin is

known to have gametocyticidal effect; however this could not be verified in this study as no gametocytes were detected on day 0 or subsequent follow up days.

The Sudanese experience with various artemisinin derivatives in combination therapy, is limited to the use of Artesunate + SP which proved to be effective almost all over the country as first line treatment for uncomplicated *P. falciparum* malaria (in literature review). However the combination under study (artemether plus lumefantrine) has been tried twice before, one trial in central Sudan by Mohd (2006) and he reported 100% cure rate. And anther by Mukhtar *et al* (2007) in Gedarif and the results obtained were 91.3% ACPR. Mukhtar *et al*, (2007) attribute the 8.9% failure rate to the reduced absorption of lumefantrine due to low fat diet.

The present investigation is the third of its kind in Sudan testing the efficacy of AL. It can be concluded that the cure rate of the oral antimalarial combination AL corrected to reinfection by PCR was 97.6% . The combination AL proved to be very effective, tolerable, and safe in children and adults of both sexes when used as first line therapy for uncomplicated *falicparum* malaria. Therefore it should be saved as the second line therapy as suggested by the policy makers in Sudan. Its use should be closely monitored to prolong its therapeutic life because today's second line will be tomorrow's first line treatment.

4.2 Recommendations

1. This new effective drug combination of Artemether+ Lumefantrine is too expensive for wide spread use as the Chinese plant extract is very expensive. The full course of treatment costs about 8.4 U.S dollars (WHO, 2003), this is not affordable by the public, therefore the government should subsidize the cost of this lifesaving drug.

2. This effective drug combination should only be obtained by doctor's prescription and that to avoid self treatment. Rational drug must be use enforced by law to limit drug pressure.

3. *In vivo* resistance to artemisinins has not happened yet, but it could happen any time. To delay this happening, knowledge of local resistance patterns of standard antimalarials is required because combination of artemisinins with ineffective drugs will expose them to the possibility of inducing resistance in *P. falciparum.*

4. Today's second line drug may become in the near future a first line drug, so its' use must be strictly limited as second alternative to SP + AS. According to Wongsrichanala (2002) malarious areas with signs of progressive loss of SP efficacy are considered at risk of emergence of multi drug resistance. Progressive loss of SP efficacy has already been reported from some parts in the Sudan.

5. Follow up periods should extend beyond 28 days in order to monitor efficacy after recrudescence, as patients with recrudescence face the risk of progression to severe malaria and may end fatally. However extended follow up periods allow drug levels in the blood to fall below the minimum therapeutic threshold,

reappearance of parasites before this threshold if reached may be due to drug resistance. but reappearance of parasites after this threshold, is reached is not necessarily related to resistance it may be recrudescence, because even sensitive parasites may recrudesce if blood levels are subtherapeutic. Therefore resistance may manifest as recrudescence, differentiation need molecular genotyping.

6. There is considerable mismatch in the pharmacokinetic properties of AL combination, Artemether has half life of less than one hour compared to Lumefantrine with elimination half life of 3 days. Lumefantrine provides complete protection to artemether, but artemether will soon leave the slowly eliminated "tail" of lumefantrine unprotected such lingering sub therapeutic concentration of lumefantrine will induce (in the long run) resistance in *P.falicparum,* to this drug if this happens then artemether will act as a monotherapy which will result in a considerable percentage of treatment failure because of the very short elimination half life. Therefore to further prolong the efficacy of artemether the companion drug should have comparable elimination half life.

7. Blood samples taken at day 7, 14, 21, after treatment should be subjected to PCR analysis to detect the possibility of any *P.falicparum* parasites at any low density below microscopic detection, and also to mark at what time reinfection may have occurred.

Although this has no clinical significance i.e.it and does not a effect therapeutic efficacy of AL but absence of parasitaemia in all post-treatment samples may not be absolute.