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Efficacy and Tolerability of Antimalarials and Molecular Resistance Markers of Falciparum Malaria in Jimma Region, Ethiopia

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List of Publications

This thesis is based on the following publications:

1. Gürkov R, **Eshetu T**, Barreto Miranda I, Berens-Riha N, Mamo Y, Girma T, Krause1 E, Schmidt M, Hempel J, Löscher T: **Ototoxicity of artemether/lumefantrine in the treatment of falciparum malaria: a randomized trial.** *Malaria Journal* 2008, **7**:179.

2. **Eshetu T**, Berens-Riha N, Fekadu S, Tadesse Z, Gürkov R, Hölscher M, Löscher T, Barreto Miranda I: **Different mutation patterns of *Plasmodium falciparum* among patients in Jimma University Hospital, Ethiopia.** *Malaria Journal* 2010, **9**:226.

Summary

Ethiopia as one of the malaria endemic countries has adopted artemisinin-based combination therapy (ACT) in the form of Artemether-Lumefantrine (AL) as first-line treatment for uncomplicated falciparum malaria in 2004. The broad introduction of the drug was achieved in 2006 including our study area Jimma, where we conducted a prospective open-label randomized trial by comparing AL to Quinine (Q) and Atovaquone-Proguanil (AP) to address the controversial reports concerning neuro-ototoxicity and resulting hearing loss due to artemisinin based treatments of uncomplicated falciparum malaria. In addition, the clinical and parasitological efficacy of the study drugs were evaluated and underlying molecular markers correlated with resistance were determined in the parasite isolates.

Patients $\geq$ 5 years of age and eligible for complete audio-vestibular evaluations with uncomplicated falciparum malaria were recruited from April to August 2006 in Jimma and the patients were randomized to receive either AL (n=30) or Q (n=35) or AP (n=32). Tolerability assessments and comprehensive audio-vestibular evaluations were also performed after chemotherapy on follow-up days 7, 28, and 90. Clinical assessments included otoscopy, Rinne and Weber tests, neurological examinations (Romberg, Unterberger's stepping, gait, finger-to-nose, nystagmus, Halmagyi test); neuro-otologic evaluation was performed by transitory evoked otoacoustic emissions (TEOAE), distortion product otoacoustic emissions (DPOAE), pure tone audiometry (PTA), and brainstem evoked response audiometry (BERA).

On the other hand, genotyping of the isolates by molecular analysis of the surface antigens *msp-1* and *msp-2* genes was performed to determine clonality and to distinguish between reinfection and recrudescence (resistance) if parasitaemia occurred after day 7. Molecular markers associated with drug resistance were determined by sequencing of *pfserca* and *pfcytb* genes; and by amplification and enzyme digestion (RFLP) of *pfmdr1* and *pfdhfr* genes.

Tolerability was good, no severe adverse effects were observed related to the drugs upon malaria treatment and as a result patient compliance was high. All malaria symptoms were resolved by day 7 except few patients with headache in all groups. As expected, a significant proportion of patients complained perceived hearing problem in the Q group, but not in the AL or AP group. Tinnitus was experienced with some patients in all groups from day 0 through 7, significantly

increasing in the Q group. There was significant reversible hearing loss elicited on day 7 among patients treated with Q but not treated with AL or AP as depicted by PTA and DPOAE. There was no evidence of drug-induced neurotoxic brainstem lesions from interpeak latencies analysis of BERA measurements in all treatment groups. No early treatment failure occurred in any drug group, but late treatment failures or recrudescences were observed ranging from day 24 to 70, in the Q group (n=4, 11.4%; on day 24, 28, 28, 40) and AP group (n=2, 6.3%; on day 28, 28). In the AL group, one possible recrudescence on day 70 (n=1, 3.3%) was detected. Rate of gametocyte clearance from the peripheral blood was very fast and efficient with AL compared to the other drugs.

The investigation of the molecular markers showed a high proportion of *pfmdr1* N86Y mutation (84.5%) in the parasite isolates of all treatment groups including among the four recrudescent isolates in the Q group. There was a high proportion of *pfdhfr* mutations or single nucleotide polymorphisms (SNPs) in codons N51I (98.8%), C59R (87.6%), and S108N (100.0%), the triple mutation occurred in 83.3% of the isolates. There were twelve SNPs detected in the *pfserca* gene of the AL treatment group, six new and six previously described mutations, but all the strains were treatment sensitive. There was no mutation in codon 268 of the *pfcytb* gene investigated for those recrudescent strains in the AP treatment group.

In conclusion, there was no neuro-ototoxic effect of the AL in the auditory and vestibular system. No transient or irreversible hearing loss was detected in the study except among patients in the Q treatment group with transient cochlear hearing loss due to reversible outer hair cell impairment. Therefore, AL is effective, safe and tolerable for treatment of uncomplicated falciparum malaria. The high degree of mutations observed in the *pfmdr1* and *pfdhfr* are reminiscent of the impact of previously used first-line antimalarials chloroquine and sulphadoxine-pyrimethamine in this area. Mutations in the artemisinin-related gene *Pfserca* were observed in an ACT-naive population and are considered to have spontaneously developed without resistance-conferring effects. However, they may serve as baseline information for further allelic selection that may happen due to long-term use of AL in the area.

Zusammenfassung

Äthiopien gilt als in weiten Teilen als Malaria-endemisches Gebiet. 2004 wurde dort eine Artemisinin-basierte Kombinationstherapie (ACT) in Form von Artemether-Lumefantrin (AL) als Mittel der Wahl bei unkomplizierter Malaria tropica erklärt. Eine flächendeckende Einführung des Medikamentes wurde in unserem Studiengebiet der Region Jimma erst 2006 mit Beginn der Studie erreicht. Aufgrund kontroverser Berichte über Neurotoxizität und in Folge resultierendem Hörverlust unter ACTs bei Malaria tropica führten wir eine prospektive offene randomisierte drei-armige Studie mit AL, Chinin (Q) und Atovaquone-Proguanil (AP) durch. Zusätzlich wurden die klinische und parasitologische Effektivität der Studienmedikamente evaluiert und Resistenz-assoziierte molekulare Marker der Isolate bestimmt.

Patienten mit unkomplizierter Malaria, die älter als fünf Jahre und geeignet für eine audio-vestibuläre Untersuchung waren, wurden von April bis August 2006 in Jimma rekrutiert und für die Studienmedikamente randomisiert (AL: n=30, Q: n=35, AP: n=32). Verträglichkeit und umfangreiche audio-vestibuläre Untersuchungen wurden vor Therapie bei Aufnahme und während des Follow-up an den Tagen 7, 28 und 90 durchgeführt. Klinische Untersuchungen beinhalteten Otoskopie, Rinne und Weber Test sowie neurologische Tests (Romberg, Unterberger Tretversuch, DGI, Finger-Nase-Versuch, Nystagmus-Prüfung, Halmagyi Test); die neuro-otologische Beurteilung wurde mittels TEOAE, DPOAE, PTA and BERA durchgeführt.

Zudem wurde eine Genotypisierung der Isolate mittels molekularer Analyse von Oberflächenantigenen *msp-1* und *msp-2* zur Bestimmung der Klonalität und zur Differenzierung in Neuinfektion und Rekrudeszenz (Resistenz) bei Parasitämie ab Tag 7 durchgeführt. Molekulare Marker, die mit Medikamentenresistenz assoziiert werden, wurden mittels Sequenzierung des pfserca und pfcytb Gens sowie durch Amplifikation und Enzymverdau der Gene pfmdr1 und pfdhfr bestimmt.

Es zeigte sich eine gute Verträglichkeit der Medikamente, keine schweren Nebenwirkungen wurden beobachtet, daher war die Compliance der Patienten sehr hoch. Bis auf Kopfschmerzen in wenigen Fällen gaben die Patienten keine der vor Therapie bestehenden Symptome an Tag 7 an. Wie erwartet gab ein signifikanter Anteil an Patienten in der Q Gruppe Hörprobleme an, jedoch nicht in den anderen beiden Armen. An Tinnitus litten einige Patienten in allen Gruppen

ein signifikanter reversibler Hörverlust mittels PTA und DPOAE gemessen im Vergleich zu den anderen beiden Gruppen. Medikamenten-induzierte pathologische vestibulare Befunde oder Hinweise auf Hirnstammläsionen in der BERA Latenzanalyse wurden in keiner der Gruppen gefunden. Frühe Therapieversager traten nicht auf, späte Therapieversager, so genannte Rekrudeszenzen, wurden ab Tag 24 bis Tag 70 beobachtet. In der Q Gruppe waren es 4 Therapieversager (11,4%), in der AP Gruppe zwei (6,3%) und in der AL Gruppe kam es zu einer möglichen Rekrudeszenz an Tag 70 (n=1, 3,3%). Die Gametozyten wurden mit AL effizienter und wesentlich schneller aus dem peripheren Blut entfernt als mit den anderen Medikamenten.

Bei der Untersuchung der molekularen Marker zeigte sich in 84,5% aller Isolate die Tyrosin-Mutation am Codon 86 des pfmdr Gens, die vier rekrudeszenten Isolate in der Q Gruppe trugen ebenfalls die Mutation. Die Punktmutationen in den Codons N51I, C59R, und S108N des pfdhfr Gens konnten zu 98,8%, 87,6% bzw. 100,0% nachgewiesen werden, die Dreifach-Mutation trat bei 83,3% der Isolate auf. Im pfserca Gen der AL Gruppe wurden sechs neue und sechs kürzlich beschriebene Punktmutationen gefunden, diese fanden sich ausschließlich sensitiven Stämmen. Keine Mutation fand sich am Codon 268 des pfcytb Gens in den rekrudeszenten Stämmen des AP Therapiearmes.

Zusammenfassend lässt sich sagen, dass keine neurotoxischen Wirkungen von AL auf das auditorische oder vestibuläre System festgestellt werden konnten. Bis auf den transienten cochleären Hörverlust unter Chinin durch den reversiblen Schaden an den äußeren Haarzellen kam es zu keinem transienten oder irreversiblen Hörschaden bei den Studienteilnehmern. AL hat sich als effektives, sicheres und gut verträgliches Medikament gegen die unkomplizierte Malaria tropica erwiesen.

Der hohe Grad an beobachteten Mutationen im pfmdr und pfdhfr Gen läßt sich am ehesten auf den noch kürzlich ausgedehnten Gebrauch von Chloroquin und Sulfadoxin-Pyrimethamin in dieser Gegend zurückzuführen. Mutationen im pfserca Gen, das mit der Artemisinin-Wirkung in Zusammenhang gebracht wird, wurden in einer ACT-naiven Population beobachtet; es wird daher angenommen, dass sie sich spontan und wahrscheinlich ohne eine Resistenz-vermittelnde Wirkung entwickelt haben. Sie stellen jedoch eine wichtige Ausgangsposition zur weiteren Beobachtung der Selektion von Mutationen unter Medikamentendruck dar, wie er bei Langzeitgebrauch zu erwarten sein wird.

Introduction

1. Life Cycle and Development of the Parasite

Plasmodium falciparum is a eukaryotic protozoan parasite in the phylum Apicomplexa. The parasite's life cycle comprises several stages, which are morphologically, biochemically, and antigenically distinct developing in mosquito and human hosts (Fujioka and Aikawa, 1999). A schematic representation of this complex life cycle is shown in Fig. 1.

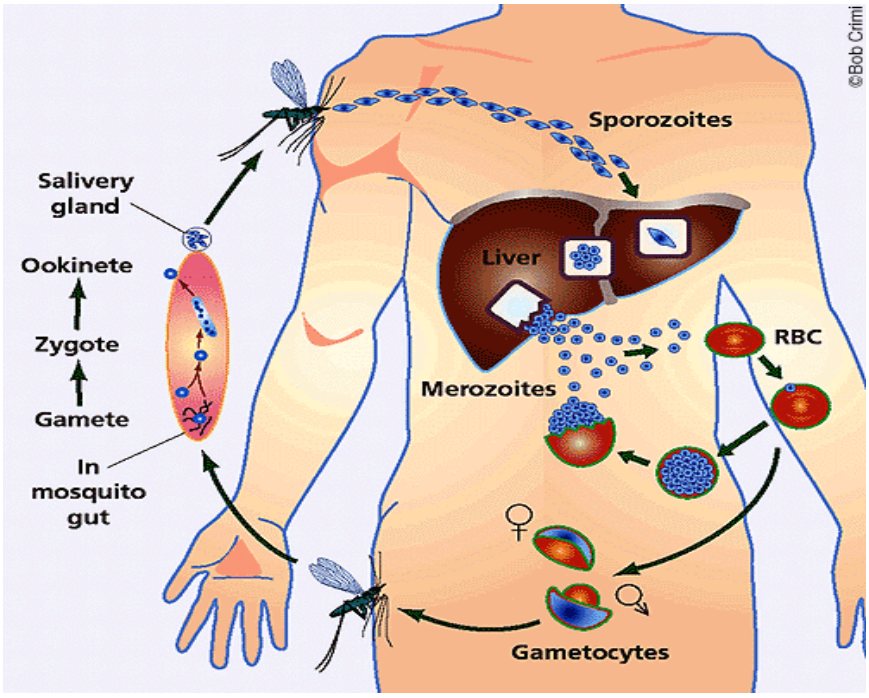


Figure 1. The Life Cycle of *Plasmodium falciparum* (Florens *et al*., 2002)

Infection of the human host starts when an infected female *Anopheles* mosquito takes a blood-meal. This event is accompanied by the injection of the contents of the saliva with anticoagulant, containing usually less than 50 infectious sporozoites (Beier *et al.*, 1991a, 1991b; Ponnudurai *et al.*, 1991). The sporozoites are efficiently cleared within minutes from the blood-stream and reach the liver. Within the hepatocytes sporozoites become round or oval trophozoites and develop into schizonts followed by nuclear multiplication. In an average duration of 6.5 days, tens of thousands of infective merozoites are formed from the schizont and are released into the blood stream and start to invade erythrocytes. No hypnozoites occur in the case of *Plasmodium*

falciparum (Gratzer *et al.*, 1993; Murphy *et al.*, 1989). Then the merozoites adhere to erythrocytes surface, via their apical end, containing rhoptries and micronemes. These organelles then facilitate the entry of merozoites into erythrocytes cytoplasm. Inside the erythrocytes, merozoites being in parasitophorous vacuole membrane develop into ring-forms, trophozoites, and schizonts. When a schizont ruptures, 8 to 32 new merozoites from erythrocyte and cell debris and toxic by-products are released into the blood stream, which is the completion of the first erythrocytic schizogony cycle. This cycle continues to occur resulting the periodic paroxysmal symptoms such as fever (after each 48 h or less for *P. falciparum*) and chills as well as the sequestration of late trophozoites and schizonts in capillaries in almost all organs. After several erythrocytic cycle gametocytogenesis happens where a few percent of the merozoites develop into male and female gametocytes in a ratio of 1:4, respectively (Aikawa, 1988; Read *et al.*, 1992; Ward *et al.*, 1993). They are the sex-cells which can only develop further if they are taken up by a feeding *Anopheles* mosquito.

Once gametocytes are taken into the mosquito midgut the change in temperature, pH and carbon dioxide concentration, together with the presence of a mosquito exflaggelation factor (MEF) (Nijhout *et al.*, 1979), possibly xanthurenic acid (Garcia *et al.*, 1998) triggers them within minutes to escape from the erythrocyte and differentiate into gametes (Carter *et al.*, 1988). A female gametocyte develops into one macrogamete; a male gametocyte develops into 8 exflaggelating microgametes consisting of nucleus associated with a flagellum. The motile microgamete attaches to the female gamete with which they fuse to form a zygote. Fertilization and zygote formation occurs within 10-30 minutes after ingestion of a blood meal by mosquito (Carter *et al.*, 1988). The zygote differentiates into a motile ookinete over the next few hours. After the ookinete has crossed the insect midgut membrane by the help of enzymes, like chitinase, it changes into oocyst in the haemocoel and undergoes multiplication. As a result thousands of sporozoites are formed and migrate to the mosquitoes salivary glands. Then the mosquito will be ready to transmit the sporozoites to human (Shahabuddin *et al.*, 1994; Dinglasan *et al.*, 2007).

2. Aetiology and Pathogenesis of Malaria

Infection by malaria parasites may lead to a variety of clinical syndromes, depending on a combination of different elements including the virulence of the parasite isolate and a variety of

host related factors such as host's immunity or genetic make-up. All types of malaria initially manifest with common mild or uncomplicated symptoms such as fever, chills, malaise, headache, arthralgia, abdominal pain, nausea, vomiting, anorexia, diarrhea or mild anemia and in some patients disease may progress into severe malaria and fatality. Severe malaria and death are more often seen in cases of *P. falciparum* infection. The early paroxysmal manifestations are related to completion of erythrocytic schizogony and release of merozoites along with waste substances. The involvement of red cells makes malaria a potentially multisystem disease, as every organ of the body is reached by the blood (Omer *et al*., 2003; Greenwood *et al*., 2008).

Pathogenesis is a multifactor event from both host and parasite. The waste substances released into the blood, such as red cell membrane products, hemozoin pigment, plasmodial DNA and other toxic factors such as glycosylphosphatidylinositol (GPI), activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators. Tumor necrosis factor, interferon-γ, interleukin-1, interleukin-6, interleukin-8, macrophage colony-stimulating factor, lymphotoxin, superoxide and nitric oxide (NO) are responsible for systemic manifestations of malaria such as aneamia, thrombocytopenia, immunosuppression, coagulopathy, and central nervous system involvement among the others (Mackintosh *et al*., 2004; Clark *et al*., 2006; Parroche *et al*., 2007; Schumann, 2007; Chakravorty *et al*., 2008). Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Awandare *et al*., 2007; Lamikanra *et al*., 2009).

The pathogenesis of severe malaria involves a series of interactions such as rosetting, cytoadherence, and sequestration of infected erythrocytes in the vital organs ultimately blocking blood flow, local oxygen supply, mitochondrial ATP synthesis, and stimulating cytokine production resulting in various organ dysfunctions (Ho and White, 1999; Clark *et al*., 2006; van der Heyde *et al.*, 2006; Maier *et al*., 2008). In addition erythrocytes membrane rigidity; endothelial activation, dysfunction and injury; and altered thrombostasis have been found to be involved in the development of severe malaria (Cooke *et al*., 2004; Chakravorty *et al*., 2008; Park *et al*., 2008).

2.1. Uncomplicated Falciparum Malaria

Uncomplicated falciparum malaria is defined as symptomatic malaria without signs of severity or complications or evidence (clinical or laboratory) of vital organ dysfunction. The signs and symptoms of uncomplicated falciparum malaria are nonspecific. Uncomplicated malaria is the mild form of the disease which presents as a febrile illness with headache, tiredness (fatigue), muscle and joint aches, abdominal pains, rigors (severe shivering), perspiration, nausea, anorexia and vomiting. Parasitaemia for uncomplicated falciparum malaria is defined as 1000 to 100,000 asexual parasite/µl of blood for low to moderate transmission areas and 2000 to 200,000 asexual parasites/µl of blood for high transmission areas (WHO, 2003; WHO, 2006; WHO, 2010).

2.2. Severe Falciparum Malaria

Severe malaria usually manifests with one or more of the following clinical or laboratory features: coma (cerebral malaria), prostration, impaired consciousness, multiple convulsions, metabolic acidosis, severe anaemia, hypoglycaemia, acute renal failure, acute pulmonary oedema, abdominal bleeding, respiratory distress (acidotic breathing), circulatory collapse, jaundice, haemoglobinuria, hyperlactataemia, hyperparasitaemia. By this stage of the disease, the case fatality in people receiving treatment is typically 10–20%. However, if left untreated, severe malaria is fatal in the majority of cases (Chen *et al*., 2000; Miller *et al*., 2002; Mackintosh *et al*., 2004; WHO, 2006; WHO, 2010).

3. Overview of Molecular Biology

Plasmodium falciparum belongs to the subgenus *Laverania* along with *Plasmodium reichenowi*, a chimpanzee malaria parasite, and both are closely related based on phylogenetic studies of small subunit ribosomal RNA (ssrRNA) and Cytochrome b gene sequences. Whereas the other four malaria parasites that infect human, *P. vivax*, *P. ovale*, *P. malariae* and the monkey parasite *P. knowlesi* belong to the subgenus *Plasmodium* and they are not closely related to *P. falciparum* (Qari *et al.,* 1996; Perkins *et al.,* 2002; Tanabe *et al.,* 2004).

The nuclear genome of *Plasmodium falciparum* is comprised of 14 chromosomes, ranging in size from approximately 0.643 to 3.29 Mb, and total size is 22.8 Mb. The genome encodes about 5,300 genes. It is the most (A + T)-rich genome sequenced to date comprising 80.6% (Gardner *et al*., 2002). The *P. falciparum* analogous chromosomes differ considerably in size between isolates; one of the modes of generating chromosomal polymorphism is deletion/duplication (Corcoran *et al*., 1986). Genes that are involved in antigenic variation are concentrated in the subtelomeric regions of the chromosomes. This can be attributed to the feature of subtelomere regions to recombine more readily than other parts of the genome, thus generating variation in gene products that play role in avoidance of host immune response and resistance to chemotherapy (Scherf *et al*., 2001). Apart from the nuclear genome, there are the plastid (apicoplast) DNA (plDNA) genome and the mitochondrion DNA (mtDNA) genome. These also encode factors for vital cellular activities which may serve as target for chemotherapy (Preiser *et al*., 1995, 1996; Wilson *et al*., 1996).

Completion of the entire life cycle offers many opportunities for genetic recombination and mutation events during numerous rounds of DNA replication. Genetic recombination can generate novel beneficial alleles, or combinations of alleles to the parasite, that can spread through the population driven by positive selection. Recombination rate is also influenced by intensity of transmission by mosquitoes, diversity of local parasite populations, and chromosomal locations of specific DNA sequences (Jiang *et al*., 2011). A study by Walliker *et al*. (1987) showed that mixture of cloned *P. falciparum* parasites differing in enzymes, drug sensitivity, antigens and chromosome patterns being transmitted into mosquitoes have resulted extensive rearrangements in their genomes after cross fertilization. Then it was extrapolated that a mosquito infection by mixtures of genetically distinct parasites from patient would provide the mechanisms for generating novel genotypes. These differing genotypes or clones of *Plasmodium falciparum* can be determined using regions of highly polymorphic genes coding for surface proteins; merozoite surface proteins 1 and 2 (MSP-1 and MSP-2), and glutamate-rich protein (GLURP) as markers with PCR-based methods. They are particularly important tools in distinguishing recrudescence from reinfection of the parasite (Färnert *et al*., 2001).

4. Epidemiology

In spite of enhanced control efforts, malaria continues to be a major public health problem in 108 countries mostly in Africa and Southeast Asia and *Plasmodium falciparum* is endemic in most of the countries. WHO estimates that in 2009, a reported 225 million cases of malaria occurred worldwide and 78% of these in Africa followed by 15% in Southeast Asia. About 781,000 fatal cases were registered, 91% in Africa. 85% of the global death toll comprises children under 5 years of age. Almost all deaths are caused by *P. falciparum* (World Malaria Report, 2010).

Malaria in Ethiopia is caused by four human malaria species: *P. falciparum, P. vivax, P. ovale* and *P. malariae.* The prevalence of *P. falciparum and P. vivax* malaria in Ethiopia takes a significant share of about 60% and 40 %, respectively. *P. malariae* is found sporadically in some areas and *P. ovale* is reported rarely (Adhanom *et al*., 2006). The most deadly malaria is caused by *P. falciparum,* but two or more species can overlap in the same area and in a person at the same time (WHO, 2002). Malaria transmission in Ethiopia depends substantially on *Anopheles arabiensis* Patton (principal malaria vector in the country), a member of the *Anopheles gambiae* Giles complex, in the intermediate highlands of Ethiopia. *Anopheles funestus* Giles is the second most important malaria vector. *Anopheles nili* Theobald is an important local malaria vector in the low land region of south-west Ethiopia (Woyessa *et al*., 2002; Federal Ministry of Health Ethiopia, 2007).

Malaria is a leading public health problem in Ethiopia where an estimated 75% of the total area of the country with altitudes below 2000m and about 50 million people (65-68% of the population) live in areas at risk of malaria and the problem is compounded by increasing frequency and magnitude of unstable malaria epidemics (WHO, 2002; Deressa *et al*., 2006; Federal Ministry of Health Ethiopia, 2008). In general malaria in the country is associated with altitude, rainfall, humidity and population movement, where the peak of malaria incidence follows the main rainfall season (July - September) each year. However, many areas in the south and west of the country have a rainfall season beginning earlier in April and May or have no clearly defined rainfall season. Depending on these rainfall patterns, transmission tends to be highly heterogeneous within each year as well as between years. Localized malaria epidemics occur almost every year somewhere in the country with occasional massive outbreaks affecting

most of the country at regular intervals of 5-8 years period (Kiszewski and Teklehaimanot, 2004; Negash *et al.*, 2005, Jima *et al*., 2010). Also frequent malaria epidemics have been observed in highland areas of Ethiopia previously not known to allow transmission (Woyessa *et al*., 2004). In Jimma region, malaria transmission is seasonal with peaks from April to June and from September to December during and after the rainy seasons (Gürkov *et al*., 2008).

In this country, endemicity of malaria was reported for the first time by scientists from Britain and Italy starting from the mid 1930`s. Since then, many malaria epidemics were recorded in the country. For example, in 1958, an estimated 3.5 million people became infected of which about 150,000 died (Fontaine *et al*., 1961). The disease is among the most important public health problems surpassing other communicable diseases such as infections with helminthes, protozoa, bacteria, and HIV (Federal Ministry of Health Ethiopia, 2005). For example, by 2002/2003 alone malaria was reported as the first cause of morbidity and mortality, accounting for 16% outpatient visits, 20% hospital admissions and 27% inpatient deaths. The year 2003 was also a time of large-scale malaria epidemics for the country from April to December resulting in 20 million clinical and confirmed cases and 3000 deaths, affecting 3368 localities in 211 districts (World Malaria Report, 2005). Again in 2007/2008, malaria was the first cause of morbidity and mortality in the country, accounting for 12% of out-patient visits and 9.9% of admissions (Federal Ministry of Health Ethiopia, 2008). Despite the current efforts to control malaria in Ethiopia, the situation has not improved, mainly due to the increasing problems of vector resistance to insecticides, low coverage of malaria preventive services, poor access to health care, rudimentary health service infrastructure, large population movements, limited financial and human resources, and unstable nature of malaria transmission resulting large-scale epidemics (World Malaria Report, 2005; Nigatu *et al*., 2009).

5. Antimalarial Treatment and Resistance

There are different classes of antimalarial drugs with different biological activities, which are being in use or had been used for malaria treatment: (1) 4-Aminoquinolines- (Chloroquine, Amodiaquine, Piperaquine) with blood schizonticide activity (2) Arylaminoalcohols- (Quinine, Quinidine, Mefloquine, Halofantrine, Lumefantrine) with blood schizonticide activity (3) Sulfonamides and Sulfones- (Sulfadoxine, Sulfalene, Dapsone) with blood schizonticide and

tissue schizonticide activity (4) Biguanides- (Proguanil, Chlorproguanil) with blood schizonticide and tissue schizonticide activity (5) Diaminopyrimidine- (Pyrimethamine) with blood schizonticide and tissue schizonticide activity (6) 8-Aminoquinoline- (Primaquine, Etaquine) with very mild blood schizonticide, tissue schizonticide, and gametocytocide activity (7) Sesquiterpene Lactones – (Artemisinin, Arteether, Artemether, Artesunate, Dihydroartemisinin) with blood schizonticide and gametocytocide activity (8) Naphthoquinone- (Atovaquone) with blood schizonticide and tissue schizonticide activity (9) Antibiotics- (Azithromycin, Clindamycin, Doxycycline, Tetracycline) with blood schizonticide and mild tissue schizonticide activity. Despite considerable efforts during the last century to eradicate or control falciparum malaria with chemotherapy being one of the armors, it remains to be the most prevalent and devastating parasitic disease in endemic countries. Emergence of drug resistance has been one of the important factors in hampering the control efforts. This was particularly evident during the Global Malaria Eradication Campaign launched by World Health Organization (WHO) in 1955 that extensive use of antimalarial drugs led to the selection of resistant parasites. The first reports confirming *P. falciparum* resistance to chloroquine came few years after introduction of the campaign (D'Alessandro and Buttiëns, 2001).

Antimalarial drug resistance is due to reduced susceptibility of the parasite. It is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a medicine given in doses equal to/or higher than the usual recommended dosage provided that the drug gets access to the parasite (WHO, 2006). Drug resistance mechanisms that confer reduced susceptibility of the parasite can be due to mutations or changes in (duplications of) the copy number of genes encoding or relating to the drug targets in the parasite or influx/efflux pumps that affect intraparasitic concentrations of the drug. A single genetic event may be all that is required, or multiple unlinked events may be necessary for the resistance. A parasite strain can be resistant to two or more antimalarial drugs of different chemical classes and mode of actions a situation called multidrug resistance. Resistance to one drug may be selected by the parasite for another drug in which the mechanism of resistance is similar, a phenomenon known as cross-resistance (White, 2004). *P. falciparum* resistance to antimalarial drugs can be manifested as early treatment failure (ETF) or late treatment failure (LTF) in patients. ETF means the persistence of clinical symptoms and parasitaemia until day 3 after initiation of chemotherapy

with parasite count ≥ 25% of the day 0 count. In case of LTF, there will be recrudescence with identical clone after being cleared initially following chemotherapy on day 7 or any day after, depending on the type of antimalarial drug. For drugs such as amodiaquine, chloroquine, sulphadoxine-pyrimethamine, and atovaquone-proguanil late treatment failure is expected up to 28 days, for mefloquine up to 63 days, and for artemether-lumefantrine up to 42 days as either clinical symptomatic or parasitological asymptomatic treatment failure (WHO, 2003).

Resistance to antimalarial drugs has been documented in all classes of antimalarials, including the artemisinin derivatives, and it is a major threat to malaria control. Widespread and indiscriminate use of antimalarials exerts a strong selective pressure on falciparum malaria parasites to develop high levels of resistance. This has been observed with chloroquine and sulphadoxine-pyrimethamine having widespread and high level of resistances which made them being ruled out from their service against *P. falciparum* (World Malaria Report, 2009; WHO 2010). Chloroquine resistance in *P. falciparum* may be multigenic and is initially conferred by mutations in a gene encoding a chloroquine resistance transporter (*Pfcrt*) and suggested to be augmented by mutations in a second transporter P-glycoprotein homologue-1(Pgh-1) encoded in multidrug resistance gene (*Pfmdr1*) (Plowe, 2003). Resistance to quinine, lumefantrine, mefloquine and other structurally related Arylaminoalcohols in *P. falciparum* was associated with duplications of copy number of *Pfmdr1* gene and in some cases its mutation (Cowman *et al*., 1994; Price *et al*., 1999; Sidhu *et al*., 2005). Resistance to the sulfonamides and sulfones, results from point mutations in the gene *Pfdhps*, which encodes the target enzyme dihydropteroate synthase (Alifrangis *et al*., 2003). Resistance to atovaquone was associated with point mutations in the parasite's gene *Pfcytb*, coding for cytochrome b, it is usually used in a fixed combination with proguanil. Resistance to pyrimethamine and proguanil's metabolite cycloguanil is conferred by point mutations in the *pfdhfr* gene of the parasite that encodes the target dihydrofolate reductase enzyme (Srivastava and Vaidya, 1999; Alifrangis *et al*., 2003). The target for artemisinin drugs has been shown to be PfATPase6 enzyme (Eckstein-Ludwig *et al.*, 2003), hence some mutations in this gene were suggested to confer resistance in *Plasmodium falciparum in vitro* and *in vivo* (Jambou *et al*., 2005; Jung *et al*., 2005; Uhlemann *et al*., 2005).

5.1. Atovaquone -Proguanil Treatment

Atovaquone-Proguanil is used for treatment and prophylaxis of falciparum malaria (Looareesuwan *et al*., 1999a, Overbosch *et al*., 2001). These partners target different metabolic features of the parasite. Atovaquone acts by inhibiting the mitochondrial electron transport and collapsing membrane potential and exerts its effect by binding to the parasite's Cytochrome bc_1 complex, but proguanil also augments the atovaquone's activity on the mitochondrial membrane. Proguanil in its active metabolite form cycloguanil acts by inhibiting the parasite's enzyme dihydrofolate reductase (DHFR) (Srivastava and Vaidya, 1999). Clinical studies show close to 100% efficacy rate for the combination therapy (Mulenga *et al*., 1999; Looareesuwan *et al*., 1999b; Bustos *et al*., 1999). However, there are few reports of treatment failures among travellers and resistance mechanisms are attributable to mutations in the cytochrome b gene, *Pfcytb* (Codon: Tyr268Asn, Tyr268Ser or Tyr268Cys) and DHFR gene, *Pfdhfr* (Codons: A16V, N51I, C59R, S108N, S108T, I164L) of the parasite against atovaquone and proguanil, respectively (Reeder *et al*., 1997; Fivelman *et al.,* 2002; Färnert *et al*., 2003; Schwartz *et al*., 2003; Wichmann *et al*., 2004). Atovaquone-Proguanil is safe and well tolerated as evidenced from controlled trials either with placebo or other drugs (Nakato *et al.,* 2007).

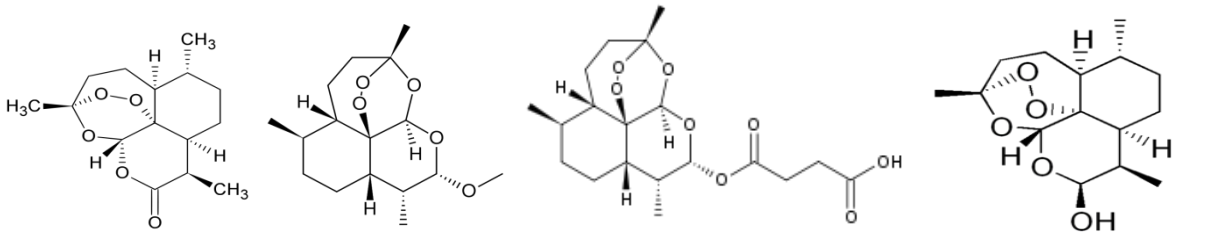
5.2. Quinine Treatment

Quinine is a cinchona alkaloid, Arylaminoalcohol drug with potent activity against asexual forms of all human malaria species, but not hypnozoite of *P. vivax* and *P. ovale*. It is also used for the treatment of sever falciparum malaria. Its potential mechanism of action seems to be the inhibition of heme polymerization in the parasite resulting in toxicity and death of the plasmodium, similar to other quinoline antimalarials (Slater and Cerami, 1992; Foley and Tilley, 1998). Even though it is not completely elucidated, its action is linked to *Pfmdr1*- a multidrug resistance gene that encodes the parasite's P-glycoprotein homologue-1 (Pgh-1). The Pgh-1 is localized in the digestive vacuole membrane of the parasite that may involve in the influx and efflux of drugs (Reed *et al*., 2000; Rohrbach *et al*., 2006). Quinine resistance is associated to *Pfmdr1*, either by increasing copy number of the gene or its polymorphism such as in 86N codon (Price *et al*., 2004; Sidhu *et al*., 2005; Sidhu *et al*., 2006). There are reports of falciparum malaria

quinine resistance mainly from Southeast Asia where it has been used extensively (Verdrager 1986; Bunnag and Harinasuta, 1987; Giboda and Denis, 1988; Karbwang *et al*., 1994) and also in Africa (Jelinek *et al*., 1995). Cinchonism is caused by quinine, which is a complex of symptoms such as tinnitus, reversible hearing loss, vertigo, blurred vision, headache, dysphoria, nausea, and diarrhea at therapeutic concentration of the drug for acute malaria (Bateman and Dyson, 1986; Roch *et al*., 1990; Newton and White, 1999).

5.3. Artemisinin-Based Combination Therapy (ACT) Treatment

Artemisinin is obtained from leaves of the herb *qinghao* or sweet wormwood (*Artemisia annua*). Artemisinin is structurally a sesquiterpen lactone with an endoperoxide bridge. The half-life of artemisinin and its derivatives lasts only for few hours; two to three hours, but can rapidly kill almost all asexual stages of *Plasmodium falciparum* and have effect on early stage gametocytes. The artemisinin derivatives, dihydroartemisinin (DHA), artesunate, artemether, and arteether can be used for treatment of malaria in artemisinin combination therapies with longer half-life partner drugs. The WHO currently recommends the following combination therapies, artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloqiune, artesunate/sulfadoxine-pyrimethamine and dihydroartemisinin/piperaquine for the treatment of uncomplicated falciparum malaria in all areas where malaria is endemic. The ACTs are the most potent and rapidly acting of all antimalarials essentially in response to the global threat of multidrug resistant falciparum malaria (Haynes, 2006; WHO, 2010).



Artemisinin Artemether Artesunate Dihydroartemisinin

Figure 2: Artemisinin and Its Derivatives

Artemether/lumefantrine (AL), a fixed dose in a 1:6 ratio (20mg/120mg) approved as Coartem®, now comprises nearly 75% of the 100 million or so ACT treatments used each year. The six-dose AL regimen based on number of tablets per dose according to pre-defined body weight bands (5–14 kg: 1 tablet; 15–24 kg: 2 tablets; 25–34 kg: 3 tablets; and > 34 kg: 4 tablets, given twice a day for 3 days) is currently the approved treatment regimen for acute, uncomplicated *P. falciparum* malaria. It is used for adults and paediatric patients with a body weight ≥5 kg, irrespective of the immune status of the patients to *P. falciparum* and of the local multidrug resistance situation, in the majority of endemic countries in Africa, Asia, Europe and Latin America where the drug is registered. Until the year 2009, 77 of 81 *P. falciparum* endemic countries including more than 40 sub-Saharan Africa countries had introduced ACT in their national drug policy, as first-line treatment for uncomplicated falciparum malaria (Cousin *et al*, 2008; Makanga and Krudsood, 2009; World Malaria Report, 2009). Ethiopia adopted artemether/lumefantrine (AL) as first-line treatment for the treatment of uncomplicated falciparum malaria in 2004 by replacing sulfadoxine-pyrimethamine, which had a mean treatment failure rate 35.9% (range 21.7 – 53.4%) (Federal Ministry of Health Ethiopia, 2004).

5.3.1. Mechanism of Action

Artemisinin and its derivatives contain a stable endoperoxide bridge, which is suggested to be cleaved by intraparasitic heme. The cleaved endoperoxide becomes a carbon-centered free radical which then functions as an alkylating agent, reacting with parasite proteins (Kamchonwongpaisan and Meshnick, 2006). There have been different assertions about the artemisinins' mechanism of action against *Plasmodium falciparum*, such as interference with parasite transport proteins, disruption of parasite mitochondrial electron transport, and modulation of host immune function (Golenser *et al*., 2006). Either inhibition of heme polymerization or heme dependent activation inside the parasite was also proposed as mechanism of action of artemisinin resulting in parasite toxicity and death (Pandey *et al*., 1999; Meshnick, 2002). Although a number of potential targets have been proposed and investigated, Eckstein-Ludwig *et al.* (2003) have shown with more concrete evidence that artemisinins act by selectively inhibiting PfATPase6, which is the parasite's SERCA-type Ca^{2+}-ATPase and was presumed to be the main target. Whereas the partner drugs along with artemisinins are structurally unrelated and possess different mechanisms of action with longer half-life (Cui and Su, 2009).

The *Plasmodium falciparum* sarco/ endoplasmic reticulum Ca^{2+}-ATPase6 (PfSERCA) is the molecule responsible for refilling of calcium ion into the endoplasmic reticulum (ER) stores, which is critically important for cellular homeostasis and calcium signaling functions. *P. falciparum* has a single copy of SERCA sequence in the genome. PfSERCA protein is evolutionarily well conserved and composed of three cytoplasmic domains (A, actuator; N, nucleotide binding; P, phosphorylation), ten transmembrane (M_1-M_{10}) helices constituting the transmembrane gate and small lumenal loops. In the activated SERCA molecule A, N, and P domains together participate in regulating calcium binding and release into the ER lumen (Dahlström *et al*., 2008; Jambou *et al*., 2010).

5.3.2. Efficacy and Resistance with ACT

The rationale behind ACT is to improve treatment efficacy and to delay emergence of resistance to the potent artemisinin drugs which are the main constituents (WHO 2006). Even though significant clinical drug resistance to artemisinins was not reported at the time when our study was planned and conducted, there had been concern of *in vitro* drug resistance of field isolates from French Guiana and Northern Brazil (Jambou *et al*., 2005) and later high *in vivo* tolerance with evidence of delayed parasite clearance in Cambodia and Thailand to artemisinin derivative combination therapy was observed (Rogers *et al*., 2009). There have been also reports of *in vivo* resistance in Africa and Western Cambodia after a seven-day artemisinin monotherapy (Menard *et al*., 2005; Dondorp *et al.,* 2009). Though the mechanisms of resistance have not been unequivocally described, mutations in the PfSERCA gene are correlated with decreased sensitivity or resistance to artemisinins. In light of this idea, it has been shown that an artificially produced single amino acid mutation L263E in the PfSERCA gene resulted in reduced inhibition of the Ca^{2+}ATPase by artemisinin *in vitro* (Jung *et al*., 2005; Uhlemann *et al*., 2005). Furthermore from field isolate parasites in French Guiana a S769N single nucleotide polymorphism (SNP) in PfSERCA was associated with a significant rise of IC_{50} for artemether, which is a rise in the concentration of the drug that is required to kill half of the parasites (Jambou *et al*., 2005). A combination of two additional SNPs, E431K and A623E, was identified

in one clinical isolate from Senegal, which also demonstrated considerable increase in the IC_{50} to artemether (Jambou *et al*., 2005). Nevertheless, this has not been always the case because from subsequent studies in Africa only one S769N mutation was found in a parasite that has been fully sensitive to dihydroartemisinin *in vitro* (Cojean *et al*., 2006).

More recently, polymorphisms in PfSERCA have been described in 100 *P. falciparum* isolates collected between 2002 and 2006 from South America, Asia and Africa. Full length coding sequence was determined for 56 isolates and partial gene sequence (positions 87 – 2862) was obtained for the rest of the isolates. Overall, 29 codons carried one or more type of mutations (SNPs). A total of 32 distinct SNPs or mutations were observed, of which 19 were non-synonymous and 13 synonymous, resulting in an average of one SNP/115bp. A total of 19 mutations had not been described previously. Some of the SNPs have been shown to be associated with increased IC_{50} of either artemether or artesunate (Jambou *et al.*, 2010). Therefore from these underlying studies the variations or polymorphisms in PfSERCA (PfATPase6) may constitute a starting ground for artemisinins induced selection of resistant isolates with possibility of rise and spread of resistance to artemisinin drugs. Newly emerging polymorphisms or SNPs in the PfSERCA gene might be accountable for resistance to artemisinin derivative drugs.

In addition to the artemisinin derivatives, the partner drugs in the ACT are shown to contribute to efficacy or resistance, for example mefloquine and lumefantrine. This was explained being attributable to *Pfmdr1*, a gene encoding the parasite's transporter protein. There is evidence from studies in Cambodia and Thailand that increased copy numbers of *Pfmdr1* was associated to mefloquine resistance after *in vivo* Artesunate + Mefloquine combination treatment failure; this was at the same time shown by higher *in vitro* mefloquine IC_{50} among the resistant isolates. On the other hand decreased copy numbers of *Pfmdr1* enhances susceptibility of the parasite to mefloquine and lumefantrine (Price *et al*., 2004; Sidhu *et al*., 2006; Rogers *et al*., 2009). Other field studies have also shown selection of *Pfmdr1* 86N codon from recurrent infections after treatment with AL, which suggests that the codon could be a potential marker of lumefantrine tolerance/resistance *in vivo* (Sisowath *et al.*, 2005; Sisowath *et al*., 2007).

5.3.3. Safety/ Tolerability/ Toxicity of ACT

Artemisinin derivatives the main constituents in the ACT antimalarial treatments are considered to be effective and safe/tolerable. However from studies in animal models (mouse, rat, dog, and monkey), they are depicted to cause neurotoxicity targeting mainly the auditory and vestibular pathways. In an experimental study dogs and rats were treated with arteether and artemether at different doses via intramuscular route, the animals with higher dose (20mg/kg/day and above) exhibited ataxia, gait disturbance, loss of brainstem and eye reflexes, loss of pain response and spinal reflexes with histological evidences of neural degeneration and necrosis in brainstem, pons, medulla and spinal cord (Brewer *et al*., 1994). Similar neurotoxic effects were observed in mice with constant administration of oral dihydroartemisinin suspended in water at doses higher than 200mg/kg/day for 28 days (Nontprasert *et al*., 2002). In another study, monkeys treated with high dose of arteether (24mg/kg/day) for 14 days but not 7 days demonstrated neuropathologic findings in brain section as injury to brainstem nuclei, reticular formation, vestibular system, and the auditory system (Petras *et al*., 1997). This phenomenon of toxicity was further shown by *in vitro* tests on cultured brainstem neuronal cells from rat and the cells sustained cytotoxicity and degeneration after treatment with artemisinin (Schmuck *et al*., 2002).

Some case reports and clinical studies carried out in human subjects had conflicting reports whether artemisinin derivatives induce similar toxicity in the treatment of uncomplicated falciparum malaria patients with oral administration. 1,552 uncomplicated falciparum malaria patients, recruited from 2002 to 2004, were treated with a three day dose Artesunate + Sulphadoxine-Pyrimethamine and followed up on day 7 and 14, some of them complained of symptoms as vomiting 55(3.3%), nausea 47(3.0%), headache 39(2.5%), abdominal pain 27(1.7%), dizziness or ataxia 27(1.7%), fever 16(1.0%), malaise 14(0.9%), and other symptoms 10(0.6%). No neurological complications were reported except the dizziness and ataxia (Cairo *et al*., 2008). In an individual case of uncomplicated falciparum malaria, ataxia and slurred speech was reported after treatment with oral artesunate for 5 days (Miller and Panosian, 1997). A similar patient who was treated with oral artemether for 5 days has experienced tremor of the hand and gait disturbance (Elias *et al*., 1999). Consequently the artemisinin derivatives were implicated as cause of neurotoxic effects from these clinical observations.

Some detailed clinical and audiologic examinations were conducted aimed at evaluating neurotoxic effects of artemisinin derivatives to the auditory and vestibular pathways. One of such studies, a retrospective case-control study on 79 cases who had been treated with $\geq$ 2 courses of oral artesunate or artemether or in combination within the previous 3 years for uncomplicated falciparum malaria and 79 age and sex matched controls never having received artemisinin derivative was conducted in western Thailand. Clinical assessments were performed including Romberg's test, gait and balance, fine finger dexterity, tuning fork for hearing acuity, eye movements, nystagmus, and behavior abnormality. Audiometry test was done for each individual at frequencies, 0.25 kHz, 0.5 kHz, 0.75 kHz, 1.0 kHz, 1.5 kHz, 2.0 kHz, 3.0 kHz, 4.0 kHz, 6.0 kHz, and 8.0 kHz. Brainstem evoked response audiometry (BERA) test was performed for both ears to measure latencies at wave forms I-V representing the auditory pathways: Cochlea and acoustic nerve (I), medulla (II), caudal pons (III), rostral pons (IV), and mid brain (V). The test results showed no differences between the cases and controls. No neurotoxicity was identified due to the oral artesunate or artemether treatments (van Vugt *et al*., 2000). A similar case-control study along Thailand-Myanmar border was conducted in 68 subjects who had been treated with AL only at least once for uncomplicated falciparum malaria within the previous five years and 68 age and sex matched controls never having received AL. A series of audiologic examinations, typanometry, audiometry and auditory brainstem response (ABR) were performed for each subject. The results showed no differences between cases and controls and no evidence of brainstem toxicity (Hutagalung *et al*., 2006). Nevertheless these retrospective studies had limitations to an unambiguously demonstrate neurotoxic effects of the artemisinins. First of all due to a lack of baseline assessment of the cases, as a result any consistent pre- and post-treatment differences could not be evaluated and also any transient toxicity would have been missed.

A follow-up or prospective case-control study was conducted to evaluate the ototoxicity of artemether-lumefantrine (AL) among construction site workers in Mozambique. The subjects were 150 cases who acquired uncomplicated falciparum malaria during their term of employment and treated with oral AL and 150 age-, gender-, weight-, ethnic matched control workers being AL-naive. All the subjects were evaluated with physical examinations and audiometry tests at the time of employment that is before AL treatment and at termination of their job contract. The mean interval from AL treatment to the final audiometry test was 163.8

day (range 3-392) for the cases. The audiometry tests are done at frequencies 0.25 kHz, 0.5 kHz, 1.0 kHz, 2.0 kHz, 3.0 kHz, 4.0 kHz, 6.0 kHz, 8 kHz, but no other auditory evaluations were used to investigate neurotoxicity. It was reported that there was significant hearing loss in AL treated group at all frequencies of the audiometry test except for 0.25 kHz and 0.5 kHz. Hence AL was considered ototoxic, which could have been considered irreversible because the mean interval from drug exposure to final audiometry measurement was 163.8 day (Toovey and Jamieson, 2004). However, the impact of noise at the construction site could not be ruled out as a confounding factor for the changes in the thresholds of hearing levels. On the other hand, the methods used to evaluate the neuro-ototoxicity are not comprehensive; such as lack of TEOAE/DPOAE, BERA, in order to evaluate auditory and vestibular pathways to demonstrate where the ototoxicity problem lies.

6. Aim of the Thesis

The aim of the study was to address the controversial reports concerning the neuro-ototoxic effects of artemisinin based treatments of falciparum malaria patients whether it is transient or irreversible. Therefore, we conducted a prospective controlled randomized trial by comparing AL to Q and AP among uncomplicated falciparum malaria patients with detailed clinical examinations and comprehensive auditory and vestibular systems evaluation (using TEOAE, DPOAE, BERA, and Audiogram).

The other purpose of the study was to evaluate and compare the efficacy and general tolerability of the study antimalarials and investigate underlying molecular markers correlated to drug resistance among the parasite isolates. Important parameters were possible side effects, treatment failure rate, clearance time, gametocyte clearance and detection of described and new point mutations in the drug-associated target genes.

7. Overview of Study Outcomes

7.1. Review of Antimalarials Tolerability and Ototoxicity Study

Due to high degree and widespread resistance to the previously used first-line drug, sulphadoxine-pyrimethamine, for uncomplicated falciparum malaria, Ethiopia adopted artemisinin-based combination therapy (ACT) in 2004 in the form of Artemether-Lumefantrine (AL). During the broad introduction of the drug in 2006, we designed and conducted a prospective open-label randomized trial to assess tolerability and address the controversial reports concerning neuro-ototoxicity and resulting hearing loss due to AL by comparing it to Quinine (Q) and Atovaquone-Proguanil (AP) as "positive control" and "negative control", respectively, in the treatment of uncomplicated falciparum malaria. A total of 230 patients $\geq$ 5 years of age were screened; those eligible after complete baseline audio-vestibular evaluations and with microscopy-confirmed uncomplicated falciparum malaria were recruited from April to August 2006 in Jimma University Hospital. Then 97 patients were randomized to receive either AL (n=30) or Q (n=35) or AP (n=32). Tolerability assessments and comprehensive audio-vestibular evaluations were also performed after chemotherapy on follow-up days 7, 28, and 90 for investigation of transient or irreversible adverse effects and ototoxicity. Clinical assessments included otoscopy, Rinne, and Weber tests; neurological examinations (Romberg, Unterberger's stepping, gait, finger-to-nose, nystagmus, Halmagyi test); neuro-otologic evaluation was performed by transitory evoked otoacoustic emissions (TEOAE), distortion product otoacoustic emissions (DPOAE), pure tone audiometry (PTA), and brainstem evoked response audiometry (BERA).

Tolerability was good; no serious adverse effects were observed related to the drugs upon malaria treatment, no vomiting occurred after ingestion of the antimalarial drugs and as a result patient compliance was efficient. However, hearing problems and tinnitus were more common on day 7 with nine of thirty patients complaining of hearing problems in the Q group. In seven of these, audiometry and OAE testing confirmed significant hearing loss. Few patients reporting subjective hearing impairment in the AL group did not have abnormal hearing test results. In the AP group, one patient complained of hearing problem on day 90 that corresponded to significantly impaired audiometry and OAE results; in this patient malaria reinfection was

diagnosed. PTA air conduction hearing thresholds were compared for a standard range of frequencies at 0.125 kHz, 0.25 kHz, 0.5 kHz, 1 kHz, 2 kHz, 3 kHz, 4 kHz, 6 kHz and 8 kHz. Bone conduction thresholds were also measured by PTA at all time points in order to exclude a possible conductive hearing loss. In the Q group, a hearing loss affecting all frequencies was evident on day 7 and has disappeared by day 28. Otherwise, no significant changes of the mean hearing thresholds compared to day 0 were evident. When comparing the mean 4-tone-average (0.5, 1, 2, 3 kHz) as the clinically most significant frequency range, a similar picture emerges. Multivariate analysis of the 4-tone-average revealed a strong interaction between the treatment groups and time on day 7, confirming the temporary threshold shift caused by quinine. Multivariate analysis of the mean average of higher frequencies (4, 6 and 8 kHz) reveals the same effect. There was no evidence of persistent hearing loss in any treatment group from the PTA results. The average DPOAE threshold level of the Q group on day 7 is markedly elevated from baseline, supporting the pure tone audiometry data. Multivariate analysis reveals a significant effect of time on the DPOAE threshold level changes for day 7 and day 28 in the three treatment groups that clearly depicts quinine ototoxicity on day 7. In addition transitory evoked otoacoustic emissions (TEOAE) could be elicited on both ears in all examinations, except for three patients in the Q group on day 7 who suffered a transient hearing loss greater than 30 dB. BERA interpeak latencies (IPL) were calculated for the I–V, I–III and III–V intervals. In all groups, IPL I–V was shorter on day 0 than on later time points of the follow-up, which is described to be impact of fever at the recruitment. The difference in IPL I–III between the AL group and the other two groups on day 28 was limited to the right ear. However, only one patient in the AL group had a potentially clinically relevant interaural difference of IPL I–III greater than 10% on day 28, which disappeared by day 90. In another case IPL III–V was prolonged in one Q treated patient (for left ear) on day 28, but not on day 90. Therefore, no permanent drug-related prolongation of interpeak latencies occurred in all treatment groups.

In conclusion, no neuro-ototoxicity was detected in the auditory and vestibular system of the patients due to AL. No hearing loss was observed in this study except the apparent transient quinine induced cochlear hearing loss due to temporary outer hair cell impairment detected by DPOAE. There are no serious adverse effects caused by the antimalarial drugs. Therefore, the standard oral AL regimen is safe and tolerable for the treatment of uncomplicated falciparum malaria.

7.2 Review of Antimalarials Efficacy and Molecular Markers Study

Patients were examined clinically and parasitologically for uncomplicated falciparum malaria at recruitment and then treated with either Artemether-Lumefantrine [fixed dose of 20 mg artemether/120 mg lumefantrine (children 5–14 kg bwt) or 40 mg/240 mg (children 15–24 kg bwt.) or 60 mg/360 mg (children 25–34 kg bwt.) or 80 mg/480 mg (adults and children ≥35 kg bwt.), at hrs. 0, 8, 24, 36, 48 and 60 (6 doses)] or Quinine sulfate [10 mg/kg (children) or 600 mg (adults and children ≥50 kg bwt.), three times daily for 7 days (21 doses)] or Atovaquone-Proguanil [20 mg of atovaquone/8 mg of proguanil/kg (children < 40 kg bwt.) or 1000 mg/400 mg (adults and children ≥40 kg bwt.) per day for 3 days (3 doses)]. Blood samples were obtained at days 0, 7 and 28 as well as on any day until day 90 in case of re-occurrence of symptoms suggesting malaria and aliquots of 10 µl of capillary blood were spotted to Whatman 3 MM Chromatographic filter paper, air dried and stored at ambient temperature for molecular analysis. Parasite DNA was extracted from blood spots on the filter paper by the Chelex method. Parasite species was determined by nested polymerase chain reaction (PCR). Genotyping of parasite isolates was done by restriction enzymes (RFLP) molecular analysis of *msp-1* and *msp-2* genes to determine clonality and to distinguish between reinfection and recrudescence (resistance). Molecular markers associated with drug resistance were determined by sequencing of *pfserca* and *pfcytb* genes; and by amplification and enzyme digestion of *pfmdr1* and *pfdhfr* genes.

In general there was adequate clinical and parasitological response of all the treatments by day 7, except for few patients having headache in all groups. No early treatment failure occurred in any drug group, but late treatment failures or recrudescences occurred on days ranging from 24 to 70. In the Q group (n=4, 11.4%; on day 24, 28, 28, 40) and AP group (n=2, 6.3%; on day 28, 28). In the AL group, one possible recrudescence (n=1, 3.3%; on day 70) was detected which was further confirmed by sequencing of *msp-1* gene. Rate of gametocyte clearance from the peripheral blood was very fast and efficient with AL compared to the other drugs. The investigation of the molecular markers that are correlated to drug resistance showed a high prevalence of *pfmdr1* mutation at codon 86Y, among 84.5% of the isolates in all treatment groups including the four recrudescent strains in the Q group. There was also high prevalence of polymorphisms at codons N51I (98.8%), C59R (87.6%), S108N (100.0%) of the *pfdhfr* gene,

these triple mutations occurred together in 83.3% of the isolates. Both recrudescent strains in the AP group showed the triple mutations (51I, 59R, 108N). The A16V mutation was only found in one of the recrudescent isolates in the AP group presenting as clinical symptomatic treatment failure. No mutations were detected in the amplified regions of the *pfcytb* gene of those two recrudescent strains in the AP group. The *pfserca* gene was amplified and sequenced from the codons 230 to 463 and 600 to 790 for isolates in AL group. There were twelve SNPs detected in the *pfserca* gene of the AL treatment group, six new and six previously described mutations, but all the carrier strains were treatment-sensitive. The previously reported E431K mutant codon was the most frequent, occurring in seven isolates. Each of the other mutations was detected only once. No sample showed more than two mutations. The previously described mutant codon S769N being associated with *in vitro* resistance in French Guiana was found in treatment sensitive isolate. Other recently published mutations in the *pfserca* gene were not detected in the amplified and sequenced regions of the gene in the isolates. The strain from day 70 recrudescence yielded no mutation in the amplified region. The high degree of mutations observed in the *pfmdr1* and *pfdhfr* are reminiscent of the impact of previously used first-line antimalarials chloroquine and sulphadoxine-pyrimethamine in this area. Those mutations in the *Pfserca* gene are observed in AL sensitive isolates and are considered to have spontaneously developed without resistance-conferring drug pressure. Overall, the distribution of these mutations does not indicate direct correlation of the markers with efficacy/resistance outcomes. However, they may serve as baseline information for further allelic selection that may happen due to long-term use of AL in this area.

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Ototoxicity of artemether/lumefantrine in the treatment of falciparum malaria: a randomized trial

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Abstract

Background: Due to increasing drug resistance, artemisinin-based combination chemotherapy (ACT) has become the first-line treatment of falciparum malaria in many endemic countries. However, irreversible ototoxicity associated with artemether/lumefantrine (AL) has been reported recently and suggested to be a serious limitation in the use of ACT. The aim of the study was to compare ototoxicity, tolerability, and efficacy of ACT with that of quinine and atovaquone/proguanil in the treatment of uncomplicated falciparum malaria.

Methods: Ninety-seven patients in south-west Ethiopia with slide-confirmed malaria were randomly assigned to receive either artemether/lumefantrine or quinine or atovaquone/proguanil and followed-up for 90 days. Comprehensive audiovestibular testing by pure tone audiometry (PTA), transitory evoked (TE) and distortion product (DP) otoacoustic emissions (OAE) and brain stem evoked response audiometry (BERA) was done before enrolment and after seven, 28 and 90 days.

Results: PTA and DP-OAE levels revealed transient significant cochlear hearing loss in patients treated with quinine but not in those treated with artemether/lumefantrine or atovaquone/proguanil. TE-OAE could be elicited in all examinations, except for three patients in the Q group on day 7, who suffered a transient hearing loss greater than 30 dB. There was no evidence of drug-induced brain stem lesions by BERA measurements.

Conclusion: There was no detrimental effect of a standard oral regimen of artemether/lumefantrine on peripheral hearing or brainstem auditory pathways in patients with uncomplicated falciparum malaria. In contrast, transient hearing loss is common after quinine therapy and due to temporary outer hair cell dysfunction.

Background

Resistance to antimalarial drugs is a common challenge in malaria endemic areas worldwide. Due to increasing resistance of *Plasmodium falciparum* strains against chloroquine and sulphadoxine/pyrimethamine, several Asian and African countries have changed their national policy towards first-line treatment with artemisinin-based combination chemotherapies (ACT) as recommended by current WHO guidelines [1,2].

Some case reports [3,4] as well as a recent alarming report on the possibility of irreversible ototoxicity of artemether/lumefantrine in a retrospective evaluation of construction site workers in Mozambique [5] have raised concerns that this potentially serious side effect of ACT has not been addressed thoroughly enough, although no evidence of neurological side effects or ototoxicity has been observed in human safety studies or large-scale field trials [6] and case control studies [7-9].

Ototoxicity has been reported in association with the use of quinoline type antimalarials [10,11], and quinine since long is known to cause reversible hearing loss and tinnitus[12]. Ototoxic effects have not been reported with the use of some other antimalarials in current use, such as atovaquone/proguanil.

To clarify the question of artemisinin-induced hearing loss, this study integrated a comprehensive neuro-otologic assessment into an investigator-initiated, open-label, randomized, controlled study to compare artemether/lumefantrine with quinine and atovaquone/proguanil in the treatment of uncomplicated falciparum malaria.

Methods

Study area and population

The study was carried out at Jimma University (JU) Hospital in the city of Jimma, 1,700 m above sea level and 335 km south west of Addis Ababa, Ethiopia. In this region, malaria transmission is seasonal with peaks from April to June and from September to December during and after the rainy seasons [13]. The first-line treatment of uncomplicated falciparum malaria changed from sulphadoxine/pyrimethamine to AL in Ethiopia in 2004 [14]. Since the drug has not yet been available until recently, oral quinine has mainly been used instead.

Patients over five years of age and suitable for complete audiovestibular testing with parasitologically proven uncomplicated falciparum malaria were recruited from April until August 2006. Uncomplicated falciparum malaria was defined as asexual parasitaemia of less than 100,000/μl blood, acute fever or a history of fever within the preceding 24 hours, and no signs or symptoms suggesting complicated or severe malaria as defined by WHO [15]. Patients with significant hearing loss as determined by failure to detect transitory evoked otoacoustic emissions in either ear, intake of anti-malarial treatment within the previous seven days, severe underlying conditions or concomitant disease masking assessment of response, history of allergy or intolerance against study medications, or pregnancy were excluded. Audiometric testing (duration 1–1.5 h) of eligible patients at enrolment was completed before starting antimalarial treatment.

The study was approved by the Ethical Committee of Jimma University, Ethiopia. Written informed consent was obtained from each patient or the parental guide. Personal subject data are kept confidential. The trial is registered with ClinicalTrials.gov, number NCT00451139. The recommendations guiding physicians in biomedical research involving human subjects issued by the World Medical Association Declaration of Helsinki (Edinburgh, 2000) were applied to this project.

Antimalarial drug regimens

Eligible patients were consecutively stratified according to gender and age and assigned to one of the following treatment groups by stratified random sampling:

1. Artemether/lumefantrine (AL): 20 mg of artemether and 120 mg of lumefantrine (children 5–14 kg bwt.) or 40 mg/240 mg (children 15–24 kg bwt.) or 60 mg/360 mg (children 25–34 kg bwt.) or 80 mg/480 mg (adults and children ≥35 kg bwt.), at hrs. 0, 8, 24, 36, 48 and 60 (6 doses). Patients were instructed to take the doses with high fat food.

2. Quinine sulphate (Q): 10 mg/kg (children) or 600 mg (adults and children ≥50 kg bwt.), according to about 8 mg/kg Quinine base, three times daily for 7 days (21 doses).

3. Atovaquone/proguanil (AP): 20 mg/8 mg/kg (children < 40 kg bwt.) or 1000 mg/400 mg (adults and children ≥40 kg bwt.) per day for 3 days (3 doses).

Artemether/lumefantrine 20/120 mg tablets (Coartem®, Novartis, manifactured by Bejing Novartis Pharma Ltd, Bejing China) and quinine sulphate 300 mg tablets (Remedica Ltd, Limassol-Cyprus-Europe) were obtained from the Ethiopian governmental drug programme through the JU hospital pharmacy. Blister packs of atovaquone/proguanil (Malarone®) were purchased from GSK, Germany.

Procedures

Patients were treated on an outpatient basis and returned on day 7, 28 and 90 and on any day during the follow-up period if symptoms returned. Clinical reassessments covered assessments for potential treatment failure and for potential adverse reactions to the treatment drug including complete audiovestibular testing.

Finger prick blood samples were taken at day 0 for confirmation of *P. falciparum* mono-infection and calculation of parasitaemia (parasites per 200 white blood cells, calculation based on an assumed mean WBC count of 8,000/ml) by microscopic assessment of Giemsa-stained thick and thin blood smears. Aliquots of 10 μl of capillary blood were spotted to Whatman 3 MM Chr filter paper, air dried, and stored at ambient temperature for later molecular analysis. Follow-up blood samples were obtained at days 7 and 28 as well as on any day of potential clinical treatment failure. Patients with falciparum or vivax malaria during the follow-up period were treated according to national guidelines [14]. These patients were excluded from the study after treatment. Patients who completed examinations on day 28 were included in the audiovestibular analysis.

Audiovestibular tests were performed by an examiner blinded to treatment allocation in a separate building specifically designated for this purpose. Although a soundproof chamber was not available, care was taken to reduce ambient noise to a minimum (see below). Clinical audiovestibular evaluation at each visit included the history of specific complaints (i.e., hearing loss, otalgia, tinnitus, vertigo), otoscopy, Weber and Rinne tests, examination for spontaneous and head-shaking nystagmus under Frenzel glasses, and testing the vestibular function by rapid passive head rotation. Physical examination included gait, Romberg and Unterberger test, finger-to-finger test, and hand rapid alternating movements [16].

Pure tone audiometry was performed with a Madsen Midimate 622D diagnostic audiometer (GN Otometrics, Copenhagen, Denmark) and Beyer DT 48 headphones. The thresholds for frequencies from 125 to 8000 Hz were determined via air conduction. In addition, bone conduction thresholds for 250 to 6000 Hz were determined in order to exclude conductive hearing loss.

In contrast to conventional audiometry, the detection of otoacoustic emissions does not rely on the patients cooperation, and is an excellent indicator for physiologic inner ear function. Transitory evoked (TE) and distortion product (DP) otoacoustic emissions (OAE) were measured with a Cochlea Scan® device (Fischer Zoth, Germering, Germany). TEOAE were analysed using a screening protocol giving a pass (detectable TEOAE) vs. fail result. DPOAE levels were measured at frequencies of f_2 = 1.5, 2, 3, 4, 6 kHz with at least three different primary tone levels per frequency between 15 and 65 dB (f_2/f_1 = 1.2). From the resulting DPOAE growth functions, DP thresholds and estimated hearing thresholds are calculated based on normative data [17,18]. The average noise floor during measurements was -1.2/-2.6/-4.1/-3.6/-3.5 dB for 1.5/2/3/4/6 kHz.

Brain stem evoked response audiometry (BERA) examines the velocity of nerve signal conduction along the auditory pathways from the cochlea to the brainstem, and is the gold standard for detection of damage to the participating neural structures. The measurements were done using an evoselect system (Pilot Blankenfelde, Blankenfelde, Germany). The stimulus, a click of alternating polarity, was delivered at a rate of 11.1 Hz at a level of 80 dB HL and contralateral masking at 40 dB HL to patients resting in a supine position. 2000 measurements were averaged and the absolute and interpeak latencies of Jewett waves I, III and V determined.

For molecular typing sample DNA was extracted from filter paper bloodspots using Chelexò (Bio-Rad, Germany) as described elsewhere [19]. Parasite species was confirmed by nested polymerase chain reaction (PCR) [20]. Sequences of parasite genes coding for the polymorphic merozoite surface proteins (MSP) 1 and 2 were amplified by nested PCR and analysed by restriction fragment length polymorphisms (RFLP) technique [21,22]. MSP1 and MSP2 fragment patterns of isolates from patients with parasite re-appearance were compared with those of the respective recruitment isolates to distinguish recrudescences from new infections [23].

Statistical analysis

A sample size of 23 in each group was calculated to have 90% power to detect a difference in means of 5.0 dB (e.g., the difference between a group 1 mean of 5.0 and a group 2 mean of 0.0) assuming that the common standard deviation is 5.0 dB using a two group t-test with a 0.05 two-sided significance level. In order to avoid problems with the assumption of normal distribution and to compensate for possible drop-outs it was decided to increase the sample size to at least 30 per group. Data were analysed using SSPS 14.0 software for descriptive statistical analyses and R package V2.4.0 for multivariate methods. The baseline characteristics of the patients were compared by Kruskal-Wallis test for variables that are measured on a continuous scale or by Pearson Chi-square test for categorial variables. The audiological data in a longitudinal setting were fitted in mixed linear model approaches. Potential factors included in the models were day after enrolment, therapy group and side. Variables not significant on the 5% level were sequentially eliminated from the models with excep-

tion of the therapy group. In case measurements of the left and right side showed no significant differences, both values were treated in the models as repeating measurements. Generally, a p value of < 0.05 was considered significant.

Results

230 patients with suspected malaria were screened and 133 were excluded for various reasons (Figure 1). 97 patients were included and randomized. 30 patients received AL, 35 Q, and 32 AP (Figure 1). Baseline characteristics are given in Table 1. None of the patients had received an artemisinin compound before. Cumulated numbers of patients lost to follow-up were one at day 7, four at day 28, and seven at day 90. Thirteen patients were excluded due to recrudescence or new infection (Figure 1).

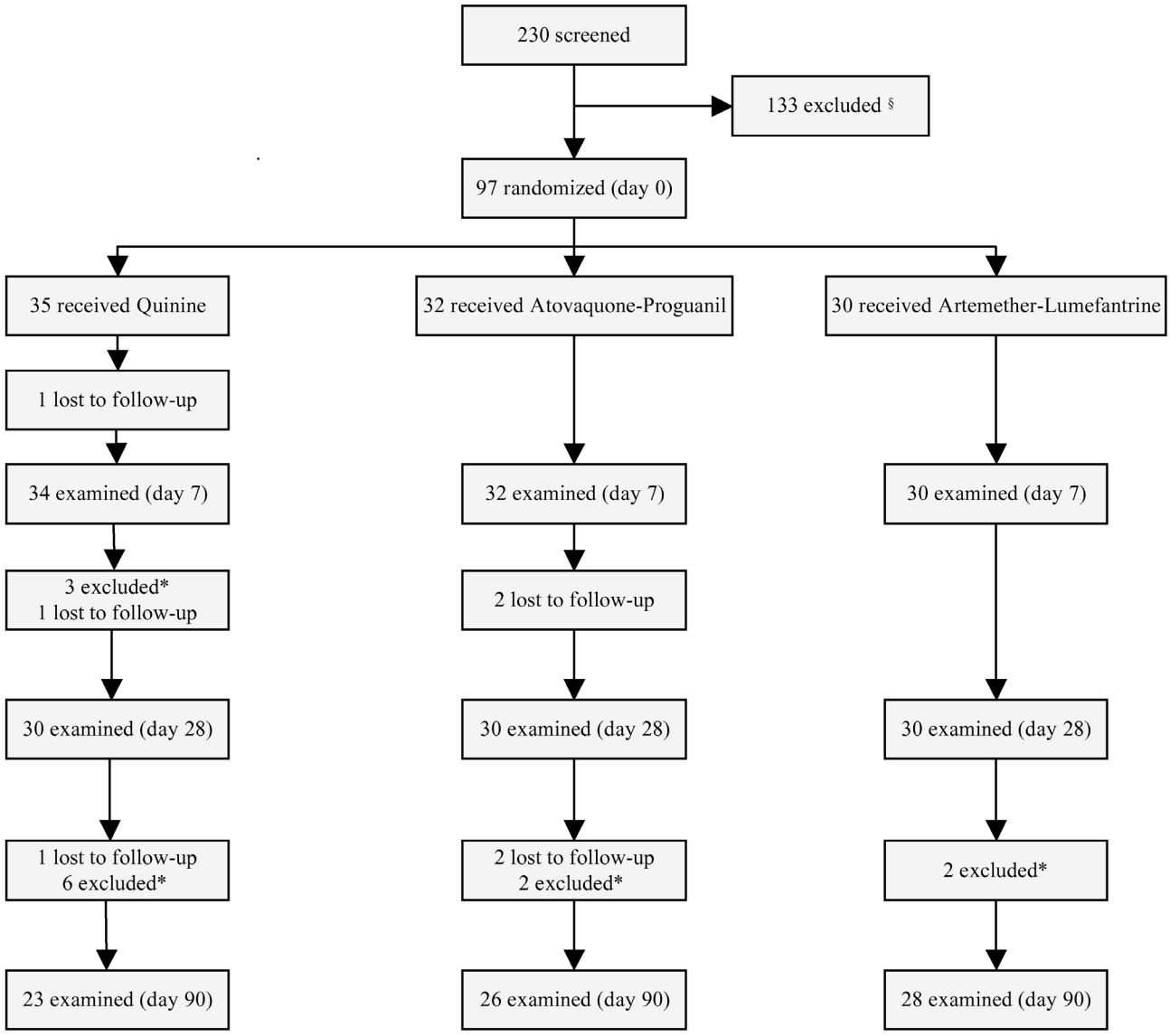

Figure 1

patient flow chart. Enrollment, randomization and follow-up of the patients. § The reasons for exclusion (number of patients) were ear discharge (20), impacted ear wax (18), repeated previous otitis media (14), perforated ear drum (6), negative TEOAE recording (15), Weber test lateralized (10), self-treatment with chloroquine (18), mixed infection (17), pregnancy (15). * Patients treated with a second course of antimalarials because of recrudescence or new infection were excluded from audiovestibular evaluation.

Table 1: Baseline characteristics of the patients who reached the primary end-point (day 28)

		Quinine	Atovaquone/Proguanil	Artemether/Lumefantrine
n		30	30	30
sex	m/f	16/14	16/14	18/12
age	range	6 – 50	6 – 35	8 – 40
	mean	19.2	19.9	18.1
	median	16	18	17
Temperature (C°)	range	36.0 – 39.5	36.0 – 39.3	35.6 – 39.9
	mean	37.4	37.3	37.5
	median	37.2	37.3	37.4
Parasitaemia	range	360 – 63,000	400 – 69,880	480 – 83,600
	mean	15,469	13,028	21,189
	median	6,740	4,960	9,300
Symptoms	Headache	30	30	30
	Nausea/vomiting	21	17	22
	Shivering	23	26	21
	Diarrhea	4	2	3
Actual daily dose (mg/kg)	range	30.5 – 36	15 – 23	2.3 – 4.6
	mean	34.9	19.5	3.4
	stdev	1.6	1.9	0.6

Baseline patients' characteristics of the patients who reached the primary end-point (day 28) were not significantly different between the three treatment groups in respect to age (Kruskal-Wallis-test, p = 0,504), sex (χ^2-test, p = 0,835), parasitaemia (Kruskal-Wallis-test, p = 0,444), body temperature (Kruskal-Wallis-test, p = 0,860). There is also no significant difference in these baseline characteristics of the treatment groups when comparing all 97 treated patients.

Table 2: Clinical and parasitological efficacy

PCR-corrected failure rates	**A/L**	**Quinine**	**A/P**
Clinical failure rate day 7	0/30	0/35	0/32
Parasitological failure rate day 7	0/30	0/35	0/32
Intention to treat failure rate* day 7	0/30	1/35 (~3%)	0/32
Clinical failure rate day 28	0/30	3/35 (~9%)	1/32 (~3%)
Parasitological failure rate day 28	0/30	3/35 (~9%)	2/32 (~6%)
Intention to treat failure rate* day 28	0/30	7/35 (~20%)	4/32 (~13%)
Recurrent parasitaemia			
Number of patients with recrudescence** (day)	1 (70)	4 (24, 28, 28, 40***)	2 (28, 28)
Number of patients with new infection** (day)	1 (73)	4 (34, 40, 40***, 65)	1 (80)
Number of patients with ***P. vivax*** infection (day)	2 (28, 28)	5 (22, 25, 27, 28, 28)	2 (28, 28)
Gametocytaemia			
Number of patients on day 0	1	3	1
Number of patients on day 7	2	10	16
Number of patients on day 28	0	2	0

* lost to follow-up included

***P. falciparum*: Genotyping by PCR and RFLP patterns of the MSP-1 and MSP-2 gene

*** One sample showed two different clones on day 40, one of them corresponded to the clone on day 0.

Clinical and parasitological efficacy

On day 7 no treatment failure was detected in any group (Table 2). Until day 28, three patients in the Q group and one in the AP group presented with falciparum malaria. Another patient with asymptomatic parasitaemia was identified in the AP group. The parasitological failure rate on day 28 was 9% and 6% in the Q and AP group, respectively. There was no treatment failure in the AL group. All treatment failures were recrudescences as confirmed by genotyping.

Between day 28 and day 90 seven patients with falciparum malaria were diagnosed. Five patients had a new infection. A recrudescent and a new strain were found in a patient in the Q group on day 40, and one recrudescence occurred on day 70 in the AL group.

Nine patients (five treated with Q, two with AP, and two with AL) showed *P. vivax* infection during follow-up (Table 2).

Tolerability and ototoxicity assessment

No vomiting occurred after ingestion of the antimalarial drugs, and no serious adverse events were reported during treatment and follow-up. Most symptoms present at the time of diagnosis resolved until day 7 (Table 3). However, hearing problems and tinnitus were more common on day 7 with nine of thirty patients complaining of hearing problems in the Q group. In seven of these, audiometry and OAE testing confirmed significant hearing loss. Patients reporting subjective hearing impairment in the AL group did not have abnormal hearing test results. In the AP group, only the reported hearing loss by one patient on day 90 corresponded to significantly impaired audiometry and OAE results; in this patient malaria reinfection was diagnosed.

Pure tone audiometry

Air conduction hearing thresholds were compared for a standard range of frequencies from 0.125 to 8 kHz. Bone conduction thresholds were also measured at all time points in order to exclude a possible conductive hearing loss. Figure 2 shows similar mean hearing levels at day 0 (baseline) for all groups and all frequencies. In the Q group, a hearing loss affecting all frequencies is evident on day 7 and has disappeared by day 28. Otherwise, no significant changes of the mean hearing thresholds compared to day 0 were evident, except for some slight general improvement in all groups.

When comparing the mean 4-tone-average (0.5, 1, 2, 3 kHz) as the clinically most significant frequency range, a similar picture emerges (Figure 3). Multivariate analysis of the 4-tone-average revealed a strong interaction between the factors group and time on day 7, confirming the temporary threshold shift caused by quinine. Multivariate analysis of the mean average of higher frequencies (4, 6 and 8 kHz) reveals the same effect.

Table 3: Symptoms and clinical signs

Drug Group	Quinine				Atovaquone-proguanil				Artemether-lumefantrine			
n =	30	30	30	23	30	30	30	26	30	30	30	28
day	0	7	28	90	0	7	28	90	0	7	28	90
Hearing Problem	0	9	1	0	0	1	0	1	1	2	1	0
Tinnitus	2	8	3	0	4	6	1	1	6	4	2	0
Vertigo	6	0	1	0	8	1	0	0	4	3	1	0
Imbalance	1	0	0	0	0	0	0	0	0	0	0	0
Spontaneous nystagmus	0	0	0	0	0	0	0	0	0	0	0	0
Provoked nystagmus	0	0	0	0	0	0	0	0	0	0	0	0
Pathologic head rotation test	1	0	0	0	0	0	0	0	1	0	0	0
Impaired coordination	0	0	0	0	0	0	0	0	0	0	0	0
Fever	24	0	5	2	23	2	3	1	26	0	1	2
Shivering	23	0	1	2	26	4	3	1	21	1	1	1
Headache	30	5	5	2	30	10	6	3	30	7	4	2
Nausea	21	1	2	0	17	1	1	0	22	0	0	4
Diarrhea	4	0	0	0	2	1	1	0	3	0	0	0
Vomiting	1	0	0	0	1	0	0	0	1	0	0	0
Anorexia	2	0	1	0	3	0	0	0	0	0	0	0
Abdominal pain	1	0	0	0	0	0	0	0	1	0	0	0
Arthralgia	3	0	0	0	3	0	0	0	1	0	0	0
Myalgia	1	0	0	0	0	0	0	0	0	0	0	0
Chest pain	0	0	0	0	0	1	0	0	0	0	0	0
Cough	0	1	0	0	0	1	0	0	0	0	0	0

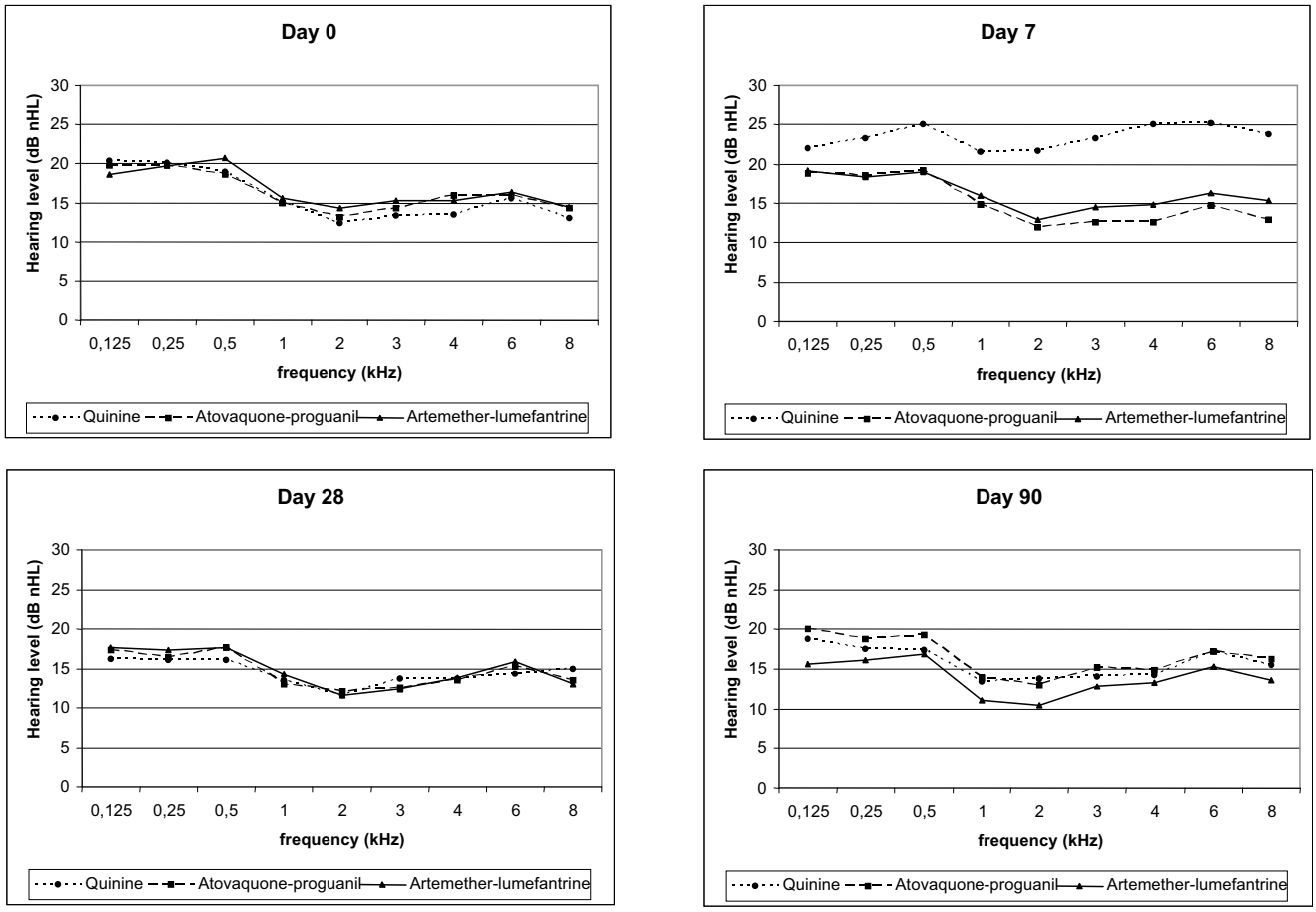

Figure 2

pure tone hearing levels. Audiometrically determined mean hearing levels on day 0, 7, 28 and 90. Transient hearing loss in the quinine treated group is observed on day 7. No permanent hearing loss in either group occurred. Measuring unit of y-axis is dB nHL.

According to the ASHA criteria for ototoxicity [24], there was no evidence of persistent hearing loss in any treatment group.

Otoacoustic emissions

The average DP threshold level of the Q group on day 7 is markedly elevated from baseline, corroborating the pure tone audiometry data (Figure 4). Multivariate analysis reveals a significant effect of time on the DP threshold levels for day 7 and day 28. This is reflected in the general improvement of DP thresholds for these time points when compared to baseline. The three treatment groups do not behave differently, except on day 7 when a significant combined effect of time and group is visible as the Q ototoxicity.

Transitory evoked otoacoustic emissions (TEOAE) could be elicited on both ears in all examinations, except for three patients in the Q group on day 7, in whom TEOAE could not be detected in either ear, and who suffered a transient hearing loss greater than 30 dB.

Brainstem evoked response audiometry

Interpeak latencies (IPL) were calculated for the I–V, I–III and III–V intervals. In all groups, IPL I–V were shorter on day 0 than on later time points (Figure 5). The difference in IPL I–III between the AL group and the other two groups on day 28 was limited to the right ear. However, only one patient in the AL group had a potentially clinically relevant interaural difference of IPL I–III greater than 10% on day 28, which disappeared by day 90. No permanent drug-related prolongation of interpeak latencies occurred.

By comparison of these measurements with normative data (2.49 ms for IPL I–III, 2.16 ms for IPL III–V, 4.45 ms for IPL I–V; [25]), IPL III–V was prolonged in one Q treated patient (left ear) on day 28, but not on day 90.

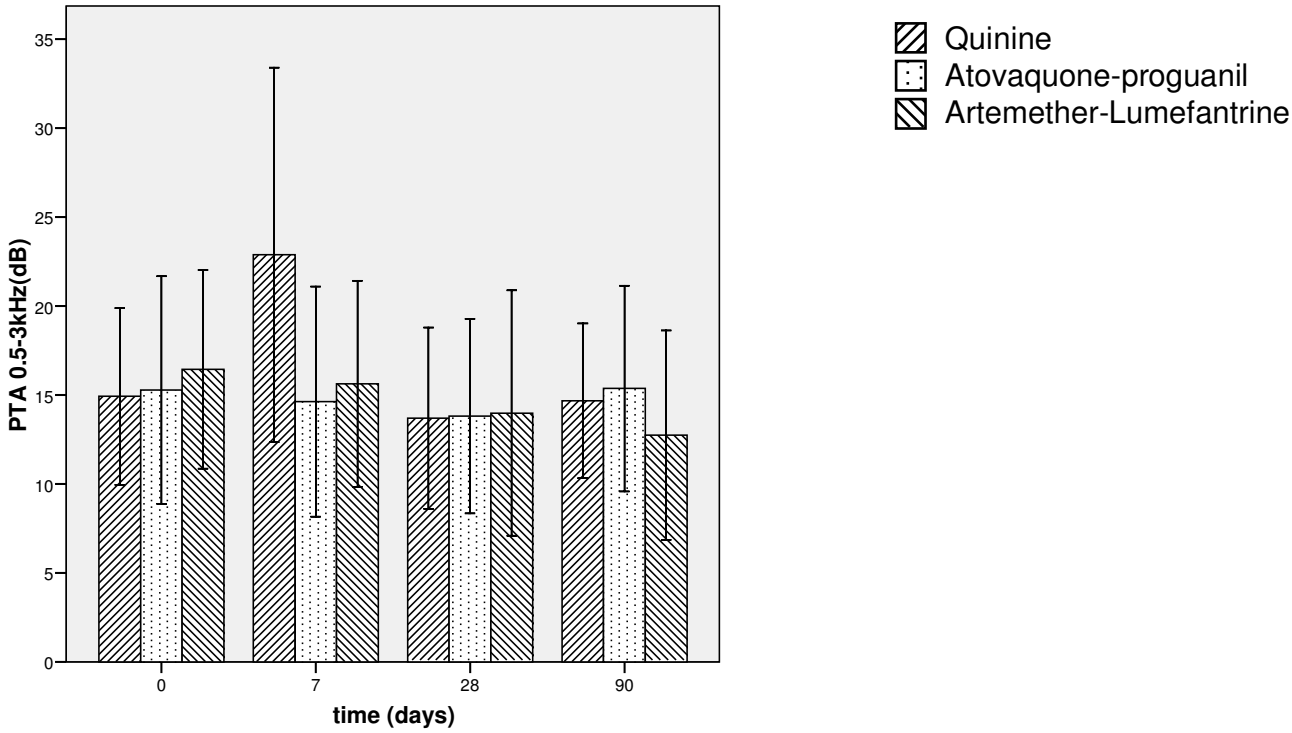

Figure 3

Pure tone average 0,5–3 kHz. Plot of means +/- 1 standard deviation of the pure tone average at 0.5, 1, 2, and 3 kHz for day 0, 7, 28 and 90 stratified by therapy group (solid line: Quinine, dashed line: Atovaquone/proguanil, dotted line: Artemether/lumefantrine). As depicted in the graph a slight improvement of PTA was found during the first 28 days after treatment in the Atovaquone/proguanil and Artemether/lumefantrine group unlike the Quinine group, which experienced a marked but transient hearing loss measured on day 7. On day 90, the differences of means were larger than those on day 28, which can be explained as an effect of the long-term observation. No permanent hearing loss in either group occurred. The results presented in the graph are strongly confirmed by multivariate analysis. Measuring unit of y-axis is dB nHL.

Discussion

The controversy about artemisinins and ototoxicity in humans has only recently been investigated by Toovey *et a*l [5]. The authors compared audiometric data from 150 adult construction site employees who have been treated with AL for uncomplicated malaria with 150 matched controls who neither suffered malaria nor received artemether. Significant hearing loss over the term of their employment was found in frequencies between 1 and 8 kHz. This was judged to be irreversible, because the time between treatment and exit audiogram (mean = 163 days, range 3–392 days) did not correlate with the degree of hearing loss [26]. However, possible confounding factors like the influence of noise exposure in these construction site workers or the lack of a control group of malaria patients treated with other antimalarials, make it difficult to establish a causal relationship between hearing loss and AL therapy from this retrospective evaluation.

Animal studies demonstrated that parenteral admininistration of lipophilic artemisinin derivatives – such as artemether – can induce focal brainstem lesions including auditory and vestibular pathways (reviewed in [27]). Oral preparations, however, have different pharmacokinetics and do not achieve as high plasma concentrations [28], suggesting that the prolonged presence of artemisinin upon slow release from oil-based intramuscular formulations and the relatively high doses used in animal studies are the main cause of neurotoxicity in laboratory animals.

A post-mortem study examined brains of patients who had died of severe malaria and had received either intramuscular artemether (n = 6) or intravenous quinine (n = 15) in doses exceeding currently deployed regimens and found no evidence of the typical artemisinin lesions observed in animal studies [29]. However, since median time from admission to death in the artemether group was

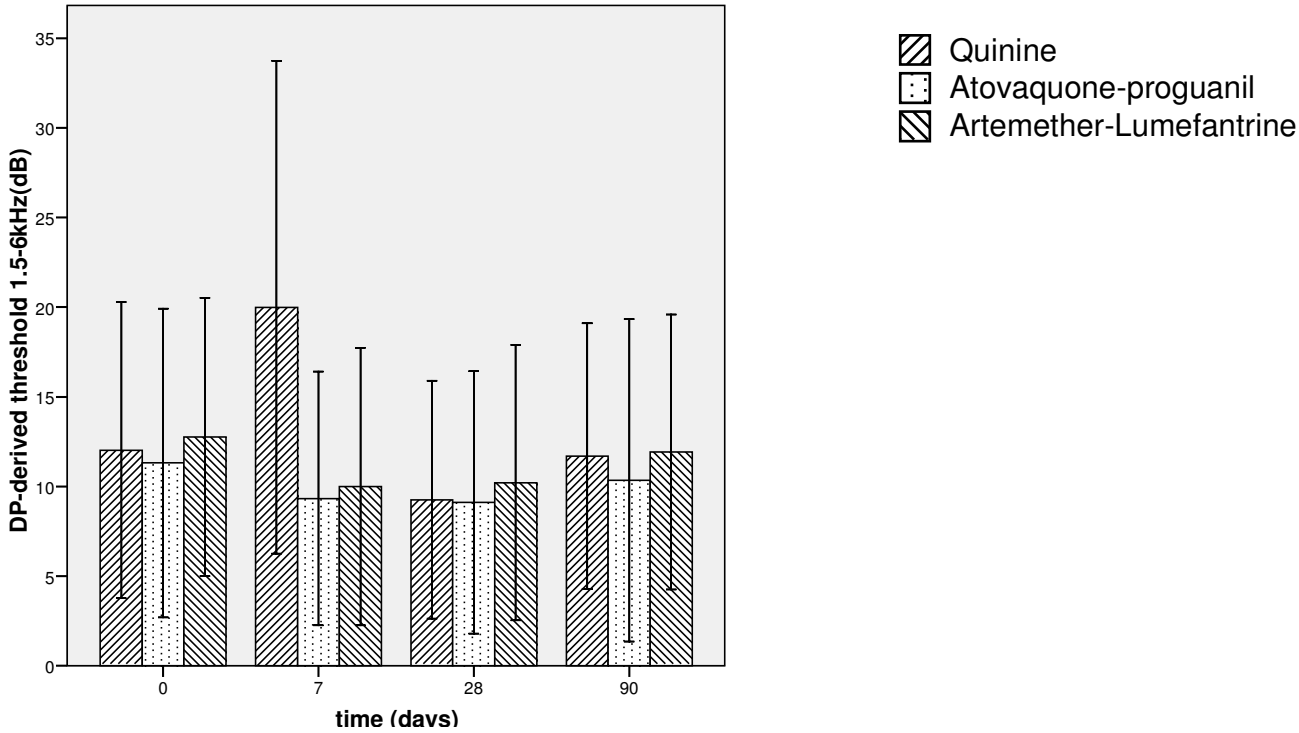

Figure 4
DPOAE level 1,5–6 kHz. Mean DPOAE estimated hearing levels +/- 1 standard deviation at 1.5, 2, 3, 4 and 6 kHz (solid line: Quinine, dashed line: Atovaquone/proguanil, dotted line: Artemether/lumefantrine). Estimated hearing levels are elevated in the quinine treated group on day 7. No permanent elevation in estimated hearing levels in either group occurred, which is confirmed by multivariate analysis. Measuring unit of y-axis is dB nHL.

only 76.5 h, delayed neurotoxicity may not have been detected, as suggested from in vitro studies [30].

Detailed neurological data are provided by Price *et al*: of more than 1,000 patients above five years of age treated with artemether or artesunate (alone or in combination with mefloquine) and examined on days 2, 7 and 28 post-treatment, no patient developed deafness (assessed by tuning-fork test) or permanent neurological abnormalities.

A retrospective study carried out in Vietnam [8] compared 337 subjects who had received from two to 21 courses (median = 2) of either artemisinin or artesunate with 108 controls from the same village. Even though 20% of the subjects had received cumulative doses of ≥ 500 mg/kg artemisinin (or the adjusted equivalent of artesunate), which might be more than in any other group of people in the world, the authors found no evidence of a drug effect on screening audiometry (testing for hearing loss ≥ 40 dB), brainstem evoked auditory potential latencies or neurological examination. Similar results were obtained in two case-control studies from Thailand in 79 subjects treated at least twice with oral artesunate or artemether [9] and in 68 patients who had been treated with AL [7].

A recent study in 15 adult volunteers with experimental falciparum malaria treated with AL could not detect any ototoxicity by using conventional and evoked response audiometry, but did not compare artemisinins to other antimalarials [31].

This study is the first randomized clinical trial directly comparing ototoxicity of AL with other antimalarial drugs. Quinine has since long been known to cause hearing loss and tinnitus (cinchonism). Generally, this side-effect is reported to be reversible within about 24 hours [32], although some case reports have described permanent hearing impairment associated with quinine treatment. In guinea pigs given large doses of quinine, there is degeneration of the organ of Corti which begins with loss of the external hair cells, and may further affect the stria vascularis and inner hair cells.

The Q and AP group can be viewed as a "positive control" and "negative control", respectively. Furthermore, an

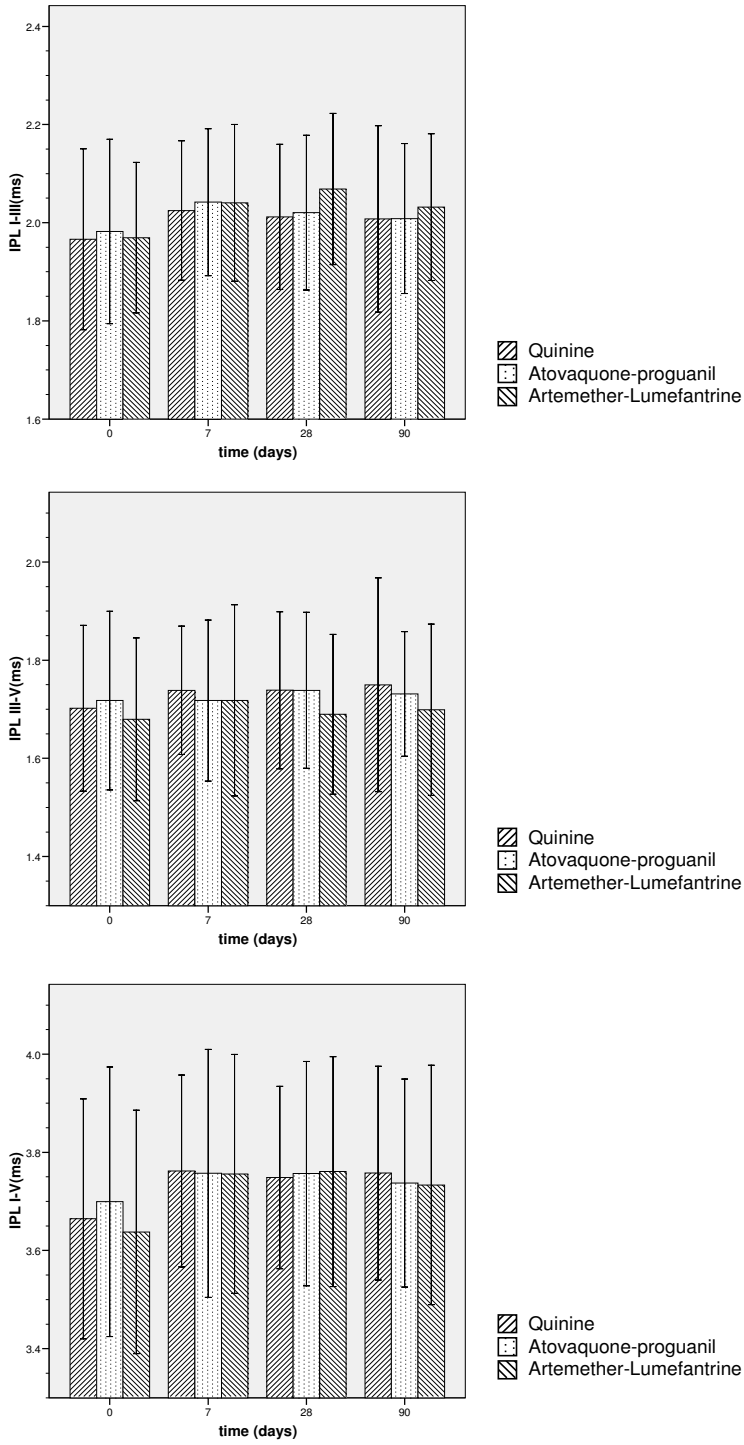

Figure 5

Interpeak latencies I–V, I–III, III–V. Mean interpeak latencies between Jewett waves I, III and V +/- 1 standard deviation (solid line: Quinine, dashed line: Atovaquone/proguanil, dotted line: Artemether/lumefantrine). Interpeak latencies in all groups are shorter on day 0, when patients have elevated body temperature. The difference in IPL I–III between the Artemether/lumefantrine group and the other two groups is limited to the right ear. No permanent drug-related prolongation of interpeak latencies occurs, as confirmed by multivariate analysis.

attempt was made to evaluate the study population's audiovestibular systems as comprehensively as possible, including air and bone conduction audiometry for definite exclusion of conductive hearing loss and otoacoustic emissions for direct objective assessment of cochlear function. In addition to the assessment on day 28, patients were examined on a late time-point (day 90) to check for reversibility of any potential hearing loss. In consequence, day 90 data could be biased by the fact that not all patients were available for the day 90 examinations (7 in the quinine group, 4 in the AP group and 2 in the AL group). However, a separate analysis including only patients who completed follow-up until day 90 showed results similar to those depicted.

The relatively low numbers of positive clinical findings or complaints related to the audiovestibular system (Table 3) makes comparison across groups difficult. Nevertheless, the transient quinine ototoxicity on day 7 is clearly correlated with an elevated number of patients complaining of hearing problems. When comparing the time course in the overall patient population, there is a relatively high proportion of patients who complain of tinnitus on day 0 and of tinnitus and perceived hearing loss on day 7. The symptom vertigo which is prevalent primarily on day 0 does not seem to be of vestibular origin, since the clinical vestibular function tests are not significantly abnormal in either group. There were no relevant neurological impairments detected. For practical reasons, this study did not incorporate objective vestibular function tests. Rotatory and caloric vestibular tests necessitate a considerable amount of specialized equipment. Vestibular evoked myogenic potentials (VEMP) recording is now emerging as a reliable tool to assess otolith function and could be useful in future studies, since this can be done with a standard BERA equipment. The air conduction hearing thresholds of our patients show no detrimental influence of AL in comparison to the other two study drugs. In contrast, a significant temporary hearing loss was observed in quinine treated patients on day 7. The observed general tendency of improved hearing levels on later time points in comparison to day 0 could be due to either a learning process or a direct transient negative effect of malaria on hearing or a lack of concentration in the acutely ill patients on day 0. The relatively high thresholds in the lower frequencies probably reflect the presence of some ambient noise.

Otoacoustic emissions are sound signals resulting from the mechanical action of outer hair cells in the organ of Corti. By stimulating the cochlea simultaneously with two signals of specific frequencies and sound pressure levels, distortion product otoacoustic emissions at a third specific frequency are elicited and can be measured. By plotting stimulus (input) sound pressure levels versus DP (output) levels, a DP threshold value can be calculated, i.e. the lowest stimulus level producing an outer hair cell response. Since outer hair cell responses are needed for physiological hearing perception at the hearing threshold, this DP threshold correlates very well with the subjective hearing threshold. It, therefore, provides an objective estimate of hearing levels in patients with mild to moderate cochlear hearing loss [17].

Overall, the objective otoacoustic emission data largely parallel the subjective audiometric data. It is therefore demonstrated that the elevated hearing levels in audiometric measurements were due to the known transient cochleotoxic effect of quinine.

DP thresholds of later time points are generally lower than those on day 0 (except for the quinine group on day 7). This cannot be due to a lack of concentration on day 0 nor to a learning curve, since this test directly assesses outer hair cell function without the need for patients' cooperation. The fact that the DP threshold levels are slightly lower than the pure tone audiometer thresholds is probably due to some ambient noise that could not be avoided in lack of a truly sound-proof chamber.

From the pattern of artemisinin-induced focal brainstem lesions in animal studies, it can be expected that an analogous lesion in humans would lead to a prolongation of the interpeak latency of Jewett waves III–V. The observed shorter interpeak latencies on day 0 are associated with elevated body temperature, a correlation previously described [33,34]. There was no permanent drug-related latency prolongation in the patients treated with AL. A prolonged interpeak or absolute latency, compared with normative data, occurred in none of the AL-treated patients.

Conclusion

In conclusion, this study could not detect any detrimental effect of a standard oral regimen of AL on peripheral hearing or brainstem auditory pathways in patients with uncomplicated falciparum malaria as assessed by pure tone audiometry, otoacoustic emission recording and brainstem evoked response audiometry. In contrast, this study clearly detects the transient quinine induced hearing loss due to temporary outer hair cell dysfunction. These results therefore support the continued use of oral artemisinine based combination therapy for uncomplicated malaria.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TL initiated and coordinated the study. TE, RG, IBM, YM, and TG participated in undertaking the clinical studies. TE, IBM, and NBR were responsible for microscopy and molecular analysis. RG, EK, and JMH were responsible for the analysis of the data from audiovestibular measurements. MS was responsible for statistical analysis. All authors participated in the design, analysis, interpretation, and writing up of the research work and read and approved the final manuscript.

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Different mutation patterns of *Plasmodium falciparum* among patients in Jimma University Hospital, Ethiopia

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Abstract

Background: The emergence of drug resistance is a major problem in malaria control. Combination of molecular genotyping and characterization of mutations or single nucleotide polymorphisms (SNPs) correlated with drug resistance can provide information for subsequent surveillance of existing and developing drug resistance patterns. The introduction of artemether/lumefantrine (AL) as first-line treatment, never used before in Ethiopia, allowed the collection of baseline data of molecular polymorphisms before a selection due to AL could occur.

Method: 97 patients with uncomplicated falciparum malaria were recruited from April to June 2006 and treated with either AL, quinine (Q) or atovaquone/proguanil (AP) in Jimma University Hospital, Ethiopia. Mutations or SNPs associated with resistance to these drugs were analysed by RFLP (*pfdhfr*, *pfmdr1*) and sequencing of the target genes (*pfcytb*, *pfserca*).

Results: SNPs previously reported to be associated with resistance to the study drugs were identified in recrudescent and treatment sensitive isolates. A total of seven recrudescences were obtained. The *pfmdr1* N86Y mutation was found in 84.5% of isolates. The triple mutation 51I,59R,108N of the *pfdhfr* gene occured in high frequency (83.3%) but no *pfcytb* mutation was detected. Sequencing showed a variety of previously described and new mutations in the *pfserca* gene.

Conclusion: The prevalence of mutations was in accordance with the expected patterns considering recent drug regimens. The broad introduction of AL and the cessation of former drug regimens might probably change the current distribution of polymorphisms, possibly leading to decreased sensitivity to AL in future. Continuous surveillance of molecular patterns in this region is, therefore, recommended.

Background

Malaria is still one of the leading health problems in our time. Most cases and deaths occur in Sub-Saharan Africa. Malaria is endemic in large parts of Ethiopia including the town of Jimma and its surroundings with most cases occurring from September to December and April to June during and after the rainy seasons [1]. High levels of drug resistance of *Plasmodium falciparum* strains against anti-malarials, first chloroquine and later sulphadoxine/pyrimethamine (SP), resulted in new drug policies in Ethiopia. In 2004, the first-line treatment recommendation was switched from SP to artemether-lumefantrine (AL), an artemisinin-based combination therapy (ACT) [1-3]. Due to a shortage in supply, Quinine was used for several months instead. AL was introduced by the underlying study in this region. ACT combines the potential of rapid reduction of the parasite burden and elimination of remaining parasites due to longer-acting partner drugs [4]. The correlation between distinct SNPs and anti-malarial drug resistance or clinical outcome has been widely discussed. Mutations in the *Plasmodium falciparum* multi-drug resistance (*pfmdr1*) gene have been associated with resistance to chloroquine, quinine, mefloquine, lumefantrine and

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artemisinin [5-8]. Increased sensitivity to (Dihydro-) Artemisinin in the presence of wild type codon 86 in *pfmdr1* and *in vivo* selection of *pfmdr* 86N during AL treatment has been reported [9-12].

The target structure for artemisinins was first described by Eckstein *et al* in 2003, abandoning other theories about the mode of action. PfATP6, a SERCA-type ATPase (*pfserca*) of *P. falciparum*, is inhibited by artemisinins [13]. An L263E replacement and other mutations decreased sensitivity to artemisinins [14]. Since then, further resistance-related mutations concerning artemisinins have been detected in laboratory strains and field isolates associated with *in vitro* resistance [15-17]. Recent reports about high *in vivo* tolerance of artemisinin-based combination therapy (ACT) and artesunate monotherapy in Cambodia and Thailand are all the more alarming [18-20].

In this study, polymorphisms related to drug resistance were investigated. Mutations of the genes *pfmdr1* (codon 86) and *pfdhfr* (codon 16, 51, 59, 108, 164), the latter associated with proguanile resistance, were analyzed by RFLP. Regions in the *pfserca* and *Plasmodium falciparum cytochrome B (pfcytb)* gene related to artemether and atovaquone resistance, respectively, were sequenced.

Methods

Study area and population

The study was performed at the Jimma University (JU) Hospital in the city of Jimma, 1,700 m above sea level and 335 km south west of Addis Ababa, Ethiopia. Patients over five years of age (mean 19.1, range 6-50) with parasitologically proven uncomplicated falciparum malaria were recruited from April until June 2006. Written consent was obtained from either the patient or a parent/legal guardian. The study was conducted concomitantly with the previous published work on ototoxicity of artemether/lumefantrine in comparison with quinine and atovaquone/proguanil and was approved by the Jimma University Ethical Committee and is registered with Clinical Trial.gov, Number NCT00451139 [21].

Procedures

Blood samples were obtained at days 0, 7 and 28 as well as on any day until day 90 in case of re-occurrence of symptoms suggesting malaria [22]. Aliquots of 10 μl of capillary blood were spotted to Whatman 3 MM Chr filter paper, air dried, and stored at ambient temperature for later molecular analysis. The definition of the treatment outcome followed the WHO draft protocol for areas with low or moderate malaria transmission [2].

Parasite DNA was extracted from blood spots on the filter paper by the Chelex method [23]. The species was identified by nested polymerase chain reaction (PCR) [24]. Single and multiple clone infections from day 0 were determined by amplification of *msp1* and *msp2* genes. To distinguish between re-infection and recrudescence, genotyping by amplification of *msp1*, *msp2* and enzymatic digestion (RFLP) of the PCR products was performed between the pair of samples [25].

To amplify the *pfmdr1* and *pfdhfr* gene, a nested PCR was used, the polymorphisms were detected by RFLP [26,27]. The PCR products were separated in 2% agarose gels stained with ethidium bromide and visualized under UV light. The regions of interest of the *pfcytb* and the *pfserca* gene were amplified by established PCR procedures [28,29]. The amplified gene fragments were purified from gel using Ultrafree-DNA extraction kit. Cycle sequencing was done with the BigDye Terminator Cycle Sequencing Kit and products analysed on the ABI3730 sequencer. Primers used for sequencing were those from the PCR. Sequences were verified using templates from two independent amplifications of the same DNA sample. Sequencing analysis was performed from both directions for each template. Sequences were analysed using the programme Bioedit and the NCBI blast function for comparison with sequences published in the GenBank database. (Reference strain for *pfserca*: Dd2, accession number: AB121053 and 3D7, acc no.: AL844501.1; for *pfcytb*: 3D7, acc. No.: AF069605)

Results

97 patients were included in the study with 30 patients receiving AL, 35 Q, and 32 AP. Clinical and parasitological efficacy as well as baseline data were described elsewhere in detail [21]. No treatment failure occured before or on day 7 in any treatment group. Until day 28, three patients in the Q group and two in the AP group presented with PCR-confirmed recrudescent falciparum malaria. Later, one recrudescence was detected in a patient in the Q group on day 40, and possibly another on day 70 in the AL group (Table 1).

The overall prevalence of *pfmdr1* mutations was high (84.5%) (Table 2). In the Q group, all four treatment failures showed the *pfmdr1* mutation at codon 86Y, but also 90.0% of the clinical sensitive samples presented with the mutation. In the AL group, four samples showed the wild type, one mixed (wild type and mutation); the remaining 25 samples exhibited only the mutation.

The expected high prevalence of the polymorphisms at codons 51I, 59R, and 108N of the *pfdhfr* gene was found. Both recrudescent strains in the AP group showed the triple mutations (51I, 59R, 108N). The only A16V mutation in the AP group was found in the clinical symptomatic treatment failure (Table 1).

The *pfserca* gene was amplified and sequenced from the codons 230 to 463 and 600 to 790. Sequencing was

Table 1 Clinical and parasitological treatment failures

PCR-corrected failure rates*	AL	Q	AP
Number of patients with recrudescence/total number of patients (day)	1/30 (70)**	4/35 (24, 28, 28, 40)	2/32(28, 28)
Drug resistance associated polymorphisms	1. *pfserca* gene 2. *pfmdr* codon 86N	1. *pfmdr* codon 86N	1. *cytb* gene codon 268 2. *dhfr* triple mutation (51I+59R +108N) 3. *dhfr* codon A16V
Polymorphisms in recrudescent samples n (%)	1. 0 (0) 2. 0 (0)	1. 0 (0)	1. 0 (0) 2. 2 (100) 3. 1 (50)

P. falciparum: Genotyping by PCR and RFLP patterns of the *msp-1* and *msp-2* gene
**Possible Recrudescence

intended for all 30 AL samples including the recrudescent strain, amplification failed due to long storage conditions of the DNA and general problems with these particular PCRs for the day 0 counterpart of the one possible recrudescent sample on day 70 and for many PCRs with the ATP1 and ATP2 primers. All gained sequences could be identified as part of the *pfserca* gene with the reference strain Dd2. 12 mutations or SNPs were identified, six new and six described. The previously reported E431K mutant codon was the most frequent occurring in seven isolates [29,30]. Each of the other genotypes was detected only once. No sample showed more than two mutations. Three of the new mutations were non synonymous and the other three synonymous (Tables 3 and 4). The sample from day 70 yielded no mutation in the *pfserca* gene.

No mutations were detected in the amplified regions of the *pfcytb* gene of 2 recrudescent strains in the AP group.

Discussion

High cure rates of AL have also been reported from other recent studies with 28 days of follow-up in Ethiopia [31,32]. For the detection of late recrudescences, follow-up periods longer than 28 days seem more appropriate. In this study, genotyping indicated a recrudescence on day 70 in one patient treated with AL. The msp-1 gene and especially the RFLP results with different enzymes (Hinf III, Dde I, Rsa I) of the msp-2 gene showed the same molecular pattern for the two samples from day 0 and day 70 [25]. Additionally, a short sequence of the *msp-1* gene was amplified, as the amplification of the *pfserca* sequence failed. The two sequences fully matched, blasting showed a difference between the Ethiopian sequence and other published strains from different regions of 90-96% [33]. Though, re-infection with a very similar clone not to be differentiated by molecular methods is still possible regarding the moderate transmission area. This particular molecular RFLP pattern was found in almost a quarter of all samples. Overall, at least six different patterns in different combinations, four being predominant, were observed. (Data not shown) The two particular samples from day 0 and day 70 showed the tyrosine mutation at codon 86 of the *pfmdr1* gene.

Table 2 Prevalence of *pfdhfr* and *pfmdr1* mutations

Genetic Polymorphism	Overall prevalence rate mutation/total n (%)
DHFR A16V	1/97 (1.1)
DHFR N51I	83/84* (98.8)
DHFR C59R	85/97 (87.6)
DHFR S108N	97/97 (100.0)
DHFR S108T	0/97 (0.0)
DHFR (51I,59R,108N)	70/84 (83.3)
DHFR I164L	0/97 (0.0)
pfmdr N86Y	82/97 (84.5)

*RFLP outcome not distinguishable for 13 samples

Table 3 Prevalence of wild type and mutant codons in the *pfserca* gene

Codon	Wild type n (%)	Mutation n (%)
E237A	6/7 (85.7)	1/7 (14.3)
H243Y	7/7 (100.0)	0/7 (0.0)
L263E	7/7 (100.0)	0/7 (0.0)
L263L	6/7 (85.7)	1/7 (14.3)
L402V	14/15 (93.3)	1/15 (6.7)
E431K	5/12 (41.7)	7/12 (58.3)
N460N	12/12 (100.0)	0/12 (0.0)
A623E	23/24 (95.8)	1/24 (4.2)
A630S	23/23 (100.0)	0/23 (0.0)
R682R	22/23 (95.7)	1/23 (4.4)
N683E	22/23 (95.7)	1/23 (4.4)
N683K	22/23 (95.7)	1/23 (4.4)
K766K	26/27 (96.3)	1/27 (3.7)
K767E	26/27 (96.3)	1/27 (3.7)
K767R	26/27 (96.3)	1/27 (3.7)
S769N	26/27 (96.3)	127 (3.7)
K771E	25/25 (100.0)	0/25 (0.0)
K776N	24/24 (100.0)	0/24 (0.0)

Fragments spanning codons 230 to 463 and 600 to 790, respectively, ref. [29])

Table 4 List of *pfserca* non synonymous and synonymous mutations found

Nucleotide mutation	amino acid (NS; *Syn*)	No. of mutations	Reference
A709C	**E237A**	1	New
C726T	**H243Y**	0	29
T787G, T788A	**L263E**	0	29
A789G	*L263L*	1	New
1204G	**L402V**	1	29
G1291A	**E431K**	7	29
T1380C	*N460N*	0	30
C1867A	**A623E**	1	29
G1887T	**A630S**	0	29
A2045G	*R682R*	1	New
A2046G, T2048A	**N683E**	1	29
T2048A	**N683K**	1	30
A2297G	*K766K*	1	New
A2298G	**K767E**	1	New
A2299G	**K767R**	1	New
G2305A	**S769N**	1	29
A2310G	**K771E**	0	29
G2327T	**K776N**	0	29

Fragments spanning codons 230 to 463 and 600 to 790, respectively, ref. [29])

The previously described mutant codon S769N being associated with *in vitro* resistance in French Guiana was found in a clinical sensitive sample [15,17]. Other recently published mutations were not detected in the amplified regions of the study samples but the *pfserca* gene was only partly sequenced for 31 samples [34]. Overall, 12 different mutations were observed in an AL-naive population, some of these mutations seem to be globally distributed as reports from Asia and South Africa indicate and not to be associated with drug resistance or caused by drug pressure [34]. The next step will be the comparison of the polymorphisms in these isolates with about 350 *P. falciparum* samples, recently collected in Jimma area. Almost four years after the broad introduction of AL in Ethiopia, selection or disappearence of certain mutations may have occured.

A study conducted in southern Ethiopia prior to the introduction of ACTs showed a prevalence of *pfmdr1* 86Y of 81% and of the mutant *pfcrt* 76 of 100% [35]. High prevalence rates of the *pfmdr1* mutation 86Y were therefore expected. Q has been used for decades in Ethiopia as second line treatment and treatment for severe malaria, treatment failures occured sporadically. Exact data were not published. Due to intensive use in Thailand for example, failure rates with Quinine raised dramatically. Attempts to show a clear correlation of drug resistance with molecular patterns has failed so far, mutations in the *pfmdr1* gene were discussed [36]. All treatment failures in the Q group showed the SNP of *pfmdr* 86Y associated with chloroquine resistance but increased sensitivity to quinine in *in vitro* studies. No selection to 86N in the recrudescent samples was observed but the small sample size and few treatment failures allow no conclusions [37].

The high prevalence of *dhfr* mutations is likely caused by wide use of proguanil, active metabolite of sulphadoxine/pyrimethamine, prior to AL introduction in the area. In the above cited study from Ethiopia, the *pfdhfr* mutations N108, I51 and R59 were present in 100%, 97% and 90%, respectively, of all investigated samples, the *pfdhfr* triple mutations (51I+59R+108N) occurred in 87% of the isolates [35]. Another study from Jimma reported 100% prevalence of the 108N and 51I mutations, and 54% prevalence of the *pfdhfr* triple mutation [38]. The occurrence of the triple mutations in both recrudescent isolates of the AP group was therefore very likely. Nevertheless, the idea of these mutations being a necessary but not sufficient cause of resistance to proguanil is supported. The circumstance that the only mutation at codon A16V occurred in the recrudescent sample may have contributed at least to the late treatment failure at day 28.

Although the combination AP has never been broadly used in the study region before, it simply served as negative control in the ototoxicity trial, two parasitological failures occurred, one was clinical symptomatic. Treatment failures have been reported from Africa. Some have been associated with mutations in the *cytb* gene, since *in vitro* resistance to AP was correlated especially with mutations at codon 268 of that gene [39-41]. A molecular survey from Ethiopia and Gabon detected no mutations in the *pfcytb* gene of samples from Ethiopia but several different mutations in 10% of Gabonian samples, although AP was not in use in both regions [42]. Spontaneous mutations are rare but seem to occure independent from drug pressure. Both recrudescent isolates in this study showed no mutations in the amplified sequence containing codon 268 but not the whole gene was sequenced and other mutations are possible. Treatment failures may also be due to limited bioavailability in some patients. However, as AP is highly recommended as prophylaxis for travellers to Ethiopia, a failure rate of 6.3% was quite alarming.

Conclusion

As expected, there were no signs of clinical or parasitological failures in the AL group except for one possible very late recrudescence on day 70 at the time of ACT introduction. The patterns of mutations in general fit with the situation of long-lasting chloroquine and SP usage before the presence of ACT in this area. Q is the national second-line treatment and backup, however its use will be compromised by the degree of resistance

shown in our results. AL seems to be the best treatment option and must be available consistently. Moreover continuous surveillance should be established in the area for AL as data from South East Asia showed decreased susceptibility of *P. falciparum* for ACT several years after introduction. A similar development could possibly be expected in Ethiopia.

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Authors' contributions
TE carried out the clinical study, participated in the molecular genetic studies and helped to draft the manuscript. NBR carried out the molecular genetic studies and the sequence alignment and drafted the manuscript. SF and ZT participated in the clinical studies. RG and MH participated in the design and coordination of the clinical study. TL was PI of the study, participated in its design and coordination. IBM coordinated and designed the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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