Treatment efficacy of artesunate-amodiaquine and prevalence of *Plasmodium falciparum* drug resistance markers in Zanzibar, 2002-2017

May 2019

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(9739 words)
Abstract

**Introduction:** Emergence of resistance to artemisinin-based combination therapy (ACT) is a major threat to combat *Plasmodium falciparum* malaria. Regular therapeutic studies to monitor treatment efficacy is essential, and genotyping of molecular markers is useful for mapping development and spread of resistance.

**Aims:** The study aims are to assess efficacy of artesunate-amodiaquine (ASAQ) and prevalence of molecular markers of drug resistance in Zanzibar in 2017.

**Methods:** Treatment efficacy of the clinical trial conducted in 2017 was compared with efficacies in 2002 and 2005. A total of 142 samples were genotyped for single nucleotide polymorphisms (SNPs) in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) gene, the *P. falciparum* multi drug resistance 1 (*pfmdr1*) gene, and in the *P. falciparum* Kelch 13 (*PfK13*) propeller region. Prevalence of SNPs were assessed during the period 2002-2017.

**Results:** Cure rate was 100% in 2017, compared to 94% and 96%, in 2002-2003 and 2005, respectively. Day 3 fever clearance rate were also high 93% (2002-3), 99% (2005) and 98% (2017) in all studies. Prevalence of *pfcrt* 76T, *pfmdr1* 86Y, 184Y and 1246Y and *pfmdr1* (86Y, 184Y and 1246Y) YYY haplotypes were significantly decreased between 2002-3 and 2017 (p < 0.001). No SNP in the *PfK13* gene related to artemisinin resistance was identified.

**Conclusion:** Efficacy of ASAQ remains high after fourteen years as first-line treatment, despite the wide-scale use of ASAQ, and there is no evidence of selection of resistance markers in Zanzibar. Continuous monitoring of drug efficacy and resistance markers is recommended.

Keywords: *Plasmodium falciparum*, Artesunate-amodiaquine, Therapeutic efficacy studies, Molecular surveillance, Antimalarial drug resistance

Total: 246 words
Acknowledgements

Firstly, I would like to express my gratitude to Andreas for introducing me to KI team to implement both my internship and master project. Secondly, my sincere thanks to Ulrika, Soorej, Mats and Anders who had encouraged and supported me to complete this final milestone in “Global Health” journey. Thirdly, my special thanks to Ulrika who helped a lot not only to finish molecular works but also guidance throughout this project. And I will definitely remember friendly colleagues and lunch time chit chat in the sun at Biomedicum. Also, I am grateful to my peers in Uppsala for giving constructive feedback to improve the draft. Last but not least, my humble thanks to all who had implemented all trials and surveys included in this project. And I would like to extend my thanks especially to Mwinyi Msellem and the Zanzibar Malaria Elimination Team.
# Abbreviations and Glossary

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin-based combination therapy</td>
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<tr>
<td>AL</td>
<td>Artemether-lumefantrine</td>
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<td>AQ</td>
<td>Amodiaquine</td>
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<td>AS</td>
<td>Artesunate</td>
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<td>ASAQ</td>
<td>Artesunate plus Amodiaquine</td>
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<td>CI</td>
<td>Confidence Interval</td>
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<td>CQ</td>
<td>Chloroquine</td>
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<td>DBS</td>
<td>Dry Blood Spot</td>
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<td>DEAQ</td>
<td>Desethylamodiaquine</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>IRS</td>
<td>Indoor Residual Spray</td>
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<td>ITN</td>
<td>Insecticide Treated Net</td>
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<td>PfK13 gene</td>
<td><em>Plasmodium falciparum</em> Kelch 13 gene</td>
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<tr>
<td>LLIN</td>
<td>Long-lasting Insecticidal Net</td>
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<td>mRDT</td>
<td>Malaria Rapid Diagnostic Test</td>
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<td>nPCR</td>
<td>Nested PCR</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>Pfcr t</td>
<td><em>Plasmodium falciparum</em> chloroquine resistance transporter gene</td>
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<td>Pfmdr1</td>
<td><em>Plasmodium falciparum</em> Multi-Drug Resistance 1 gene</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
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<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>SP</td>
<td>Sulfadoxine–pyrimethamine</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>WWARN</td>
<td>Worldwide Antimalarial Resistance Network</td>
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<td>ZAMEP</td>
<td>Zanzibar Malaria Elimination Programme</td>
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<td>ZMCP</td>
<td>Zanzibar Malaria Control Programme</td>
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1. Introduction

1.1 Global Malaria burden

Malaria is a life-threatening disease caused by *Plasmodium* parasites carried through the bite of infected female *Anopheles* mosquitoes [1]. There were five human malaria parasites including *plasmodium falciparum*, *plasmodium vivax*, *plasmodium malariae*, *plasmodium ovale* and *plasmodium knowlesi* [1]. Among them *P. falciparum* and *P. vivax* imposed the greatest threat to humans. In 2017, *P. falciparum* accounted for estimated 99.7%, 62.8%, 69%, 71.9% in the WHO African Region, WHO region of South-East Asia, Eastern Mediterranean and the Western Pacific, respectively. *P. vivax* represented 74.1% cases in the WHO Region of the Americas [2].

Malaria is a major public health issue and remains one of leading causes of morbidity and mortality especially in low and middle income countries [2]. According to world malaria report 2018, an estimated 219 million cases of malaria was reported globally, of which estimated 92% (200 million) of the cases were from WHO African Region [2]. The remaining 5% and 2% of cases were from the WHO South-East Asia Region and the WHO Eastern Mediterranean Region, respectively [2]. Globally, an estimation of 435,000 malaria deaths occurred in 2017, of which 61% (266,000) deaths were in children aged under 5 years. [2].

The earlier world malaria report estimated that 86% (212 out of 247 million) of malaria episodes and 91% (801 000 out of 881 000) of malaria deaths occurred in the Africa Region in 2006 [3]. Therefore, the Africa Region remains suffering from a high burden area of malaria. Malaria burden prevents the countries from economic growth; Gallup and Sachs reported that a 10% reduction in malaria index was associated with 0.3% rise in annual growth [4]. Recent data showed that global malaria incidence rates decreased from 72 cases per 1000 population at risk in 2010, to 59 per 1000 in 2017, 80% of cases were concentrated only in 10 countries from sub-Saharan Africa and one country in Asia, India [2]. Hence, there are countries in Africa that are changing strategies from malaria control to elimination after significant reductions in malaria cases and deaths since 2003-4 [2,3]. However, there was heterogeneity of malaria endemicity in between and within the certain countries in the region.

Malaria control is defined as the “Reduction of disease incidence, prevalence, morbidity or mortality to a locally acceptable level as a result of deliberate efforts. Continued interventions are required to sustain control” [5]. Malaria elimination is defined as “ Interruption of local transmission (reduction to zero incidence of indigenous cases) of a specified malaria parasite in a defined geographical area as a result of deliberate activities. Continued measures to prevent re-establishment of transmission are required” [5]. The previous world malaria report stated that there are 4 out of 41 countries in Africa - Eritrea, Rwanda, Sao Tome and Principe, and Zanzibar
(United Republic of Tanzania) – had the highest impact of malaria control in the region, where malaria burden was reduced 50% or more, between 2000 and 2006-7 with higher population access to antimalarial drugs, insecticidal nets, and high coverage of indoor residual spraying (IRS) [3]. Consistently, Björkman et al. reported that malaria burden in Zanzibar has started to decreased with first deployment of artemisinin based combination therapy (ACT) in 2003 together with free universal distribution of long lasting insecticidal nets (LLIN) in 2005 and IRS in 2007 [6]. Therefore, maintaining the impact of reduction is beneficial not only to Zanzibar but also other countries in the world.

1.2 Malaria Elimination in History

1.2.1 Wars and Development of Malaria Control Tools

During the World War I (1914 -1918), the third relentless foe that weakened both allied and axis military forces, was malaria [7]. Available published data and military records indicate that at least 1.5 million soldiers were estimated to be infected with malaria, and case fatality was ranging from 0.2 -5.0% within a four-year period [7]. In the First World War, Quinine, the oldest antimalarial drug, was mainstay treatment for malaria [7]. During World War II (1939 – 1945), the United States Army did extensive research to combat malaria at home and fronts [8]. As an outcome of this effort, dichloro-diphenyl-trichloroethane (DDT) and Chloroquine were developed which in turn revolutionized malaria control and even framed the global malaria eradication strategy after the end of the Second World War [8].

1.2.2 Global Malaria Eradication Program

The World Health Assembly held in Mexico approved the Global Malaria Eradication Program (GMEP) in 1955 [9]. By employing DDT mainly as malaria vector control tool, which killed specifically indoor resting adult mosquitos, led to raise high hopes among national malaria control programs to eliminate malaria globally [9]. During the period 1955 to 1987, in total of 22 countries and 2 territories were certified as malaria-free by WHO, but the GMEP was not successful in reaching its goal to eliminate malaria globally owing to the development of drug resistance and insecticidal resistance [9–11]. The lessons learnt from GMEP can be summarized as the ingredients for a successful public health program are not only sustainable financial and political support, but also effective malaria surveillance and continuous research, active community participation, and flexibility of the program to cope with changing disease epidemiology [9].
1.2.3 Early Development of Resistance

Although IRS with DDT was initially a successful vector control intervention, early signs of the mosquito vector resistance to DDT was first reported in Greece in 1951 [12]. Not only did the vector development resistance to insecticides, the malaria parasite also developed resistance to the main antimalarial of choice, resulting in major challenges in the GMEP [9]. According to WHO definition, malaria drug resistance is defined as “the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than usually recommended but within the limits of tolerance of the subject” [5]. Quinine resistance was first reported from the railway construction site in the Brazilian Amazon in 1910 and later reported from Thailand, Asia in 1987 [13,14]. Moreover, the emergence of \textit{P. falciparum} chloroquine resistance imposed a new burden to the elimination of malaria; resistance was confirmed along the Cambodia-Thailand border and in Cambodia by 1960 [15]. Payne reported that wide-scale use of Chloroquine added in table salt led to faster development of chloroquine resistance to \textit{P. falciparum} [16].

1.2.4 Development of the Nobel Prize Winning Drug

After the emergence of resistance to Quinine, bark from the cinchona tree as early as in 1910, and another resistance to synthetic compound, Chloroquine in 1960, urgent development of new alternative potent drug to fight against malaria was crucial [13,16]. Antimalarials drugs have been used in the world for centuries. The therapeutic use of cinchona appeared in 17th century in the West but in the East, the alternative, qinghaosu, was used in Chinese herbal medicine even earlier [17]. The exploration of Chinese herbs with antimalarial activity was started in 1969 and artemisinin was discovered in 1979 [18]. Professor Youyou Tu later reported that the hope in search of a new drug lay in a quote mentioned in “A Handbook of Prescriptions for Emergencies” by Ge Hong (284–346 CE) [18]. The quote stated that the use of qinghao as “A handful of qinghao immersed with 2 liters of water, wring out the juice and drink it all” [18].

1.2.5 Artemisinin-based Combination Therapy

In the mid-1990s, the artemisinin-based combination therapies (ACT) were introduced to ensure the maximum therapeutic life to replace the failing drug such as chloroquine and sulfadoxine–pyrimethamine (SP) [19]. WHO recommended to use ACT “to reduce the number of parasites during the first 3 days of treatment (reduction of parasite biomass), while the role of the partner drug is to eliminate the remaining parasites (cure)” [20]. The combination of short acting artemisinin derivatives with long acting partner drug aids the rapid resolution of clinical symptoms, for instance, fever and fast reduction of parasite biomass and prevent development of gametocytes [20].
1.2.6 Prevention of ACT Resistance
The widespread use of ACTs has significantly contributed to reduced malaria transmission and a reduction in malaria related deaths over the last two decades [21]. Unfortunately, emergence of resistance to artemisinin-based combination therapy was first reported in western Cambodia and Thai-Cambodia border in 2008 and 2009 [22,23]. Therefore, continuous surveillance of drug resistance plays an important role in hindering the spread of artemisinin resistance and maintaining the therapeutic efficacy of ACTs [24].

1.3 Genetic Studies in Malaria
Molecular studies of malaria has certain role not only in diagnosis of malaria by PCR methods but also monitoring of specific genetic markers that were related to drug resistance [25].

1.3.1 Molecular Markers and Drug Resistance
A review on malaria drug resistance reported that single nucleotide polymorphisms (SNPs) in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene and *P. falciparum* chloroquine resistance transporter (*pfcrt*) were associated with decreased sensitivity to quinine [26]. Moreover, both *in vitro* and *in vivo* studies, have shown that the *pfcrt* 76T and the *pfmdr1* 86Y SNPs are associated with resistance to chloroquine and amodiaquine [27–34]. Amodiaquine and its active metabolites desethylamodiaquine (DEAQ) has similar mechanism to chloroquine and the *pfmdr1* 86Y, 184Y, and 1246Y haplotype has been found to be involved in resistance/treatment failures of amodiaquine (DEAQ) [35,36]. In addition, mutations in the *P. falciparum* Kelch 13 (*PfK13*) propeller region have been associated with delayed parasite clearance after treatment with artemisinin [37]. The combined analysis of *PfK13*-propeller sequence polymorphisms drew the conclusion that the most common African allele (*PfK13 A578S*) was not associated with artemisinin resistance, and there was no evidence of artemisinin resistance outside South East Asia and China to date [37,38]. In 2012, a single synonymous SNP (*PfK13 C469C*) in 5 out of 23 isolates was reported in Fukayosi, Tanzania which has no association with drug resistance (parasite clearance half-life is less than 5.5 hour) [37]. Recent study in mainland Tanzania found seven non-synonymous *PfK13* mutations (L463S, G496S, M476I, V510M, E556K, M562T and E602D), none of which were associated with day 3 parasitemia [39]. The most prevalent *PfK13* alleles (F446I, R539T, I543T, P574L and C580Y) that are associated with artemisinin resistance (delayed parasite clearance) in South East Asia region have not been detected in the sub-Saharan Africa to date [37,40]. In contrast, *PfK13* plays an important role in determining artemisinin resistance in South East Asia [41].
1.4 Study Context: Zanzibar

Geographically, Zanzibar is an archipelago consisting of several islands located in Indian Ocean, adjacent to mainland Tanzania (Figure 1). Administratively, Zanzibar is a semi-autonomous region of Tanzania composed of two main islands with a population of approximately 1.3 million people [42]. According to the 2012 census report, the population in Unguja, the largest island, was about 900,000 and in Pemba over 400,000 people [42]. In 2017, the world bank’s poverty assessment of Zanzibar showed that there was the steady improvement in urban areas, and although overall the poverty rate remained as 30.4%, the rate in Pemba island was increased by 6.9% from 48.5% in 2010 to 55.4% in 2015 [43]. The interrelatedness of malaria and poverty has been recognized for long time, yet the causality is multiple and complex. [44]. Even though the causality between malaria and poverty is fairly debatable, a review article revealed that there was direct or indirect association between socio-economic status and uptake of preventive and treatment measures [45]. Since the 2008-9 fiscal year, the health care budget in Zanzibar has increased throughout the years, however, out of pocket spending is still 30% of the total household expenditure [46].

1.4.1 GMEP Era (1955–1969)

Zanzibar was one of the participating countries in the Global Malaria Elimination Program [9]. Owing to effective vector control interventions, there was significant reduction of malaria prevalence from as high as 74.2% before 1960 to as low as 6.3% in the GMEP era [11]. However, suppression of malaria to very low levels, did not imply that malaria was no longer a threat, instead malaria resurged to critical levels by the end of 1990 [11,47].

Figure 1. A geographical map of Mainland Tanzania and Zanzibar. (Source: Malaria Indicator Survey Report 2017 [48])
1.5 Malaria Epidemiology in Zanzibar

1.5.1 Malaria Epidemiology and Control Measures in Zanzibar

According to WHO, uncomplicated malaria patient is defined as “a patient who presents with symptoms of malaria and a positive parasitological test (microscopy or RDT) but with no features of severe malaria” [20]. The first line ACT for uncomplicated falciparum malaria has been artesunate-amodiaquine (ASAQ) since 2003 [20]. In contrast, the first ACT in mainland Tanzania is Artemether-Lumefantrine since January 2007 [49]. The wide-scale deployment of ACT, free of charge for all ages at public health facilities, together with strengthened vector control with long lasting insecticidal nets (LLIN) and IRS has contributed to the remarkable reduction of malaria in Zanzibar [6].

The predominant human malaria parasite is P. falciparum in both mainland and Zanzibar [50]. The findings of entomological surveillance showed that there was the major shift in primary vector population – Anopheles gambiae (95%) to An. arabiensis (89%) - after wide-scale use of IRS and LLIN in Zanzibar between 2005 and 2010 [6,50]. In addition to that, human biting rates per person night was also decreased 98% (12.44 to 0.27) between 2002-3 and 2010 when An. arabiensis becoming the predominant vector with outdoor biting behavior [6]. In 2015, coverage of the two key vector control interventions for indoor biting malaria such as IRS and ITNs were approximately 80% in Zanzibar [6].

1.5.2 Progress During MDGs (1990-2015) and Beyond

In September 2000, heads of state and government from 189 countries around the world came together to United Nations Headquarter in New York to sign United Nations Millennium Declaration at General Assembly [51]. The Millennium Development Goals (MDGs) involve the global effort to alleviate poverty and improve health and well-being of all individuals [52]. There were in total of 8 goals, 21 targets and 60 indicators for measuring progress between 1990 and 2015 [53]. Among them, MDG Goal 4. “To reduce child mortality” and Goal 6. “To combat HIV/AIDS, malaria, and other diseases” are directly linked to reduction of malaria burden [52]. The MDGs progress reports has shown that the MDG Goal 4 the Under 5 mortality rate (USMR) declined from 202 per 1,000 live births in 1990, to 101 per 1,000 in 2005, and declined again to 79 per 1,000 in 2010 in Zanzibar, where the communicable diseases including malaria remains the leading cause of under 5 mortality [54]. The continuation of progress in malaria control has been proven that the community malaria prevalence was reduced from 10.3% to 0.43%, and under 5 mortality from 1.01% to 0.36% between 2003 and 2015 [6]. The main reductions occurred after malaria control activities were initiated, with the endorsement of ASAQ as first line antimalarial to treat P. falciparum in 2003 [6]. The microscopy/RDT positivity rate was reduced significantly
Resistance Markers to Artesunate and Amodiaquine

(a mean reduction of 94.2%) between 2002 and 2015 [6]. Annual decreasing of positivity rate from 2003 to 2015 can be found in Appendix 1. Moreover, the estimation of annual parasite incidence (APIs) was reduced from 3.6 to 2.0 per 1000 inhabitants between 2003 and 2008. Zanzibar is approaching to become a “very low transmission” area with lower APIs (<5 cases per 1000 population) and malaria prevalence (> 0 but < 1%) according the malaria elimination framework by WHO [55]. Therefore, the implementation strategies of the Zanzibar Malaria Control Program (ZMCP) were adapted to a pre-elimination setting [6]. The deployment of implementation strategies over the past decade in Zanzibar can be found in the Appendix 2. In August 2013, ZMCP changed its name to the Zanzibar Malaria Elimination Program (ZAMEP) [56]. At the end of MDGs era, sustainable development agenda were developed based on achievement of MDGs [57]. In 2015, world leaders adopted 17 Sustainable development goals (SDGs) to further improvement of inequalities, alleviate poverty and combat climate change [58]. SDGs has 17 goals, 169 targets and 230 indicators in which SDG goal 3 “Ensure healthy lives and promote well-being for all at all ages” is related mainly to health [59]. SDG target 3.3 is to “fight communicable diseases” in which SDG indicator 3.3.3 is to reduce malaria incidence to 9 or fewer cases per 1,000 population by 2030 [53,60]. Despite the achievement has been made in the MDGs era, Zanzibar still have final milestones to reach in the SGDs era until achieving malaria elimination.

1.6 Rationale of the Study

Resistance to artemisinin-based combination therapies, first line antimalarial drugs to treat P. falciparum malaria, is one of the greatest challenges in both malaria control and elimination [2]. In order to combat the growing resistance, WHO recommends conducting therapeutic efficacy (TES) studies at regular intervals in all endemicity (high-moderate-low) to update and validate in vivo therapeutic drug efficacy [40]. TES studies involved initial treatment with ACT accompanied by follow-up period of 28 to 42 days [40]. In addition, genotyping of molecular markers has been a useful tools for monitoring drug resistance overtime and to track development and spread of resistance alleles in malaria parasites [24]. Since 2003, ASAQ has been the first line treatment for uncomplicated P. falciparum malaria in Zanzibar. Therefore, continuous monitoring of drug efficacy is crucial to track the development of resistance markers after fourteen years deployment of ASAQ. This study will assess the efficacy of ASAQ combined with single low-dose primaquine (PQ) and the prevalence of SNPs that are associated with artesunate and amodiaquine resistance in a clinical trial conducted in 2017 in Zanzibar. The findings of genotyping of specific markers including pfcr7 76T, pfmdr1 86Y, Y184 and 1246Y associated with tolerance/resistance to
amodiaquine, and SNPs in the PfK13 propeller domain associated with resistance to artemisinin will be compared with previous studies conducted during the period 2002-2013.

1.6.1 Main Research Question
What is the efficacy of ASAQ and the prevalence of molecular markers associated with resistance to both artesunate and amodiaquine, after fourteen years of use as first-line treatment?

1.6.2 Additional Aims and Objectives
1. To assess the treatment outcome in 2017, in comparison with treatment outcomes in 2002-3 and 2005
3. To examine the prevalence of SNPs in PfK13 propeller domain in 2017 compared to 2013

2. Methodology

2.1 Study Design
The samples analyzed in this study were collected during a one-armed clinical trial with the aim to assess the efficacy of ASAQ given together with single low dose PQ (0.25mg/Kg). Evans described the single-arm design as “…the simplest trial design where a sample of individuals with the targeted medical condition is given the experimental therapy and then followed over time to observe their response” [61]. In this single-arm trial, all enrolled individuals were given same aforementioned antimalarial drugs with a 28-day follow-up period to assess the clinical and parasitological response. Adequate clinical and parasitological response (ACPR) defined as the “absence of parasitemia on day 28, irrespective of axillary temperature, in patients” [55]. During the follow-up period, clinical and laboratory examinations were conducted on scheduled visits on days 1, 2 and 3 and after treatment visits on days 7, 14, 21 and 28. According to the clinical and laboratory assessments, the patients were classified into therapeutic failure (early or late) or adequate therapeutic response group. The study protocol was based on the WHO guidelines for surveillance of antimalarial drug efficacy [24].

2.1.1 Study Sites
The clinical trial involved 14 primary health care (PHC) facilities in three regions including Micheweni on Pemba Island, and Bububu and Uzini on Unguja Island (Figure 2). These 14 PHCs served as “Satellite Sites” where participants were screened for malaria using Rapid Diagnostic Test (mRDT). Only uncomplicated P. falciparum positive malaria patients diagnosed by mRDT, and those who meet the inclusion criteria (see below) were eligible for the study. Patients were
referred to the “Recruitment Sites” to be enrolled in the study. The “Recruitment Site” was set up with microscope, haemaque machine for hemoglobin measurements, and well-trained technicians. The responsive candidates were then oriented to the study protocol.

![Map of Zanzibar indicating study sites in circles](image)

**Figure 2. A geographical map of Zanzibar indicating study sites in circles.** The study involved three districts - West and Central on Unguja Island and Micheweni on Pemba Island. Source: Barnes 2018 [62]

2.1.2 Study Population

In total of 9062 patients of all ages presenting at the satellite study sites with sign and symptoms compatible with uncomplicated *P. falciparum* malaria were screened and 146 patients were enrolled in the study. Informed consent was obtained before the study; consent forms were signed prior to study enrollment.

2.1.3 Timing and Duration of the Study

The study was conducted for six months from April to October 2017.

2.1.4 Sample size Calculation

Sample size was calculated for an efficacy rate of 95% with 95% CIs and margin of error <5%. To achieve this precision, at least 90 patients were required where attrition rate was set for 20% of patient enrolled.
2.1.5 Participant Selection

Uncomplicated *P. falciparum* positive malaria cases were recruited according to following inclusion and exclusion criteria.

### Inclusion Criteria

- Age 3 months and above;
- *P. falciparum* infection detected by mRDT and confirmed by microscopy;
- Presence of *P. falciparum* malaria asexual parasitemia (any level, regardless of high or low parasitemia)
- Presence of axillary $\geq 37.5^\circ C$ or history of fever during the past 48 hours
- Ability to swallow oral medication;
- Ability and willingness to comply with the study protocol for the duration of the study and to comply with the study visit schedule; and
- Informed consent from the patient or from a parent or guardian in the case of children.

### Exclusion Criteria

- Presence of general danger signs in children aged under 5 years or signs of severe falciparum malaria according to the definitions of WHO (Appendix 3);
- Mono-infection with a Plasmodium species other than *P. falciparum* detected by microscopy;
- Presence of febrile conditions other than malaria (e.g. measles, acute lower respiratory tract infection, severe diarrhea with dehydration) or other known underlying chronic or severe diseases (e.g. severe malnutrition, cardiac, renal and hepatic diseases, HIV/AIDS);
- Regular medication, which may interfere with the study drugs; (Appendix 4)
- History of hypersensitivity reactions or contraindications to any of the study medicines; and
- Pregnancy

Individuals who were excluded from study also received standard health care services. Details information regarding ethical perspective can be found under “Ethical considerations” section.
2.1.6 Treatment and Follow-up
Artesunate + amodiaquine was administered orally as a fixed dose combination, i.e. Artesunate/Amodiaquine Winthrop®, prequalified by the WHO, at a dose of approximately 4 mg/kg artesunate + 10mg/kg amodiaquine once daily for 3 consecutive days. PQ was administered orally, i.e. ® Sanofi Aventis prequalified by the WHO, as a single dose (0.25 mg/kg) together with the first dose of ASAQ. All doses of antimalarial was administered under direct supervision by a researcher or clinic staff. The study participants were observed for 30 minutes to monitor any adverse reactions or vomiting. If the patients vomited within 30 minutes, he/she was re-treated again and observed for another 30 minutes. Patients who did not tolerate the treatment were given rescue therapy and withdrawn from the study. Every effort was made to schedule a follow-up visits for those who failed to return to the study site. A participant had the right to withdraw consent at any time without losing access to health care services. Detailed information regarding “patient discontinuation or protocol violation” can be found in the Appendix 5.

2.1.7 Clinical and Laboratory Examination
The following clinical and laboratory examinations were conducted during scheduled visits after being admitted to the study. The assessment timepoints in relation to following procedures are mentioned in the Table 1.

Table 1. Laboratory tests and clinical examinations performed during the study.

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<td>Clinical history + Physical Examination</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Filter paper sample</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Physical Examination – A complete medical history and demographic information and contact details were taken at baseline.

Measuring Body Weight – To assess the nutrition status, and for determining treatment dose (number of tablets).

Body Temperature – axillary temperature was measured for all the participants.

Microscopic Blood Examination – Giemsa-stained thick films were taken by qualified microscopists for asexual and gametocyte counts. Malaria species was identified at screening on day 0 to confirm adherence in line with the inclusion and exclusion criteria.

Filter Paper Blood Samples – Two to three drops of blood (50-100µL) were collected from finger pricks on 3MM (Whatman) filter paper for genotyping of malaria parasites.

Pregnancy Test for women of reproductive age (15 -49 years) – woman found to be pregnant at screening were excluded from this study. Pregnant women in the first trimester were treated with oral quinine 10 mg/kg body weight, 8 hourly for 7 days. Pregnant women in the second and third trimester received artemisunate 4 mg/kg + amodiaquine 10 mg/kg treatment once daily for 3 days according to national treatment guidelines. During the entire study period, female study participants of child-bearing age, defined as above (menstruating women above 49 years were also considered), were encouraged to use barrier methods for contraception, and the possible risks of the study drugs to the fetus during pregnancy was explained.

Assessment of Hemoglobin Level – Assessment of anemia and to monitor possible adverse effects of PQ in G6PD deficient patient.

2.2 Molecular Analysis
Molecular analysis regarding the study SNPs were performed to assess the existence of markers that are related to artemisunate + amodiaquine and artemisinin resistance. A total of 142 samples from Day 0 and another 9 recurrent samples detected by quantitative PCR (qPCR) at Day 28 [62] were examined in this study. The analysis of SNPs and gene amplifications associated with tolerance/resistance to ACT was performed according to established protocols at Karolinska Institutet, Stockholm, Sweden.

The genotyping of the SNPs pfcrt K76T, pfmdr1 N86Y, Y184F and D1246Y were analyzed according to established nested PCR – restriction fragment length polymorphism (RFLP) protocols [63]. Firstly, DNA extraction from “Dried Blood Spots” (DBS) collected on filter paper (3MM; Whatman) was conducted following the Chelex-100 resin (Bio-Rad Laboratory, USA)
extraction protocol (adapted from (Hsiang et al., 2010)) [64]. Then the extracted DNA were run for nested PCR followed by RFLP [65]. The nest PCR reaction involved 1× Taq polymerase reaction buffer, 2.5–3 mM magnesium chloride, 0.2 mM dNTP, 0.5–1 μM of each primer and 1.25 units of Taq DNA polymerase (Promega Corporation, USA). The RFLP reactions involved 1 × NEBuffer 1/3, 0–1 × BSA and 10 U/reaction of ApoI, Tsp509 I or EcoR V restriction enzymes following manufacturers’ instruction (New England Biolabs, UK). Positive and negative controls (3D7, Dd2 and 7G8) and a no template control were included in each PCR-RFLP procedure. Extracted DNA was stored at -20 ºC until use. Finally, the product of PCR-RFLP were run by gel-electrophoresis on 2–2.5% agarose gel stained with GelRed. Then the visualization of the genotypes was done under UV transillumination (GelDoc 2000, BioRad, HerculesW, CA, USA).

PCR-RFLP method was used to determine the existence of polymorphisms using restriction enzymes, for instance, point mutation in genotype pfmdr1 codon 86 resulted in changing the amino acid from wild-type (Asparagine) to mutant (Tyrosine) [66]. However, PCR-RFLP gel electrophoresis results can either be wild-type alone, mutant alone or both, i.e., mixed infection. A mixed infection was defined if the PCR-RFLP results contain both undigested and digested PCR amplicons. The PCR-RFLP results were reviewed by two independent reviewers. Any inconsistent findings were evaluated by third person for confirmation. Regarding analysis of haplotypes, all isolates presenting as a mixed infection for more than one SNP were excluded from the analysis.

In addition, polymorphisms in the 850bp fragment of the kelch 13 propeller domain were analyzed by nested PCR amplification with Q5 high-fidelity polymerase (New England Biolabs, UK), follow by bidirectional direct Sanger sequencing of the PCR amplicon (Arrey et al., 2014) [67].

2.3 Brief Descriptions of Previous Studies

The two previous clinical in vivo trials were conducted in 2003/3 [68] and 2005 (unpublished). They were both open labelled, randomized two-arm studies comparing in vivo efficacy of ASAQ and artemether-lumefantrine in children (age ≤5 years) with uncomplicated P. falciparum malaria (2000-200 000 parasites/µl). Study procedures embracing the standard ethical requirements in two previous studies were similar to those in 2017 study. The molecular analyses were also similar except the sequencing of PfK13 gene which were only performed for 2017 study samples. Baseline characteristics of the study participants are described in Table 2.

In addition, samples collected in 2010 and 2013 were included in the trend analysis of SNPs. The study conducted in 2010 was a health facility-based survey conducted in twelve primary health care facilities, six each in North A (Unguja Island) and Micheweni (Pemba Island) districts. In total of 121 RDT positive samples collected from febrile patients were genotyped for SNPs in pfcr and pfmdr1 [69]. The samples from 2013 were also collected from febrile patients attending
health facilities. mRDT positive fever patients at PHC facilities were recruited during January–July 2013 in the same two sentinel study districts as above. Total 113 patients were included for genotyping of SNPs in 2013 [65].

2.4 Statistical Analysis
Data from the clinical trials were entered by two independent data clerks in Microsoft Excel sheets and cleaned using GSPro. Statistical analysis was performed in Stata. 95% Confidence Intervals (CIs) were calculated for proportions, fever clearance rate, parasite clearance rate by microscopy, proportions of patients cured on Day 28, as well as for geometrical mean parasite densities on Days 1-3. Trend analysis of SNPs 2002-2017 was performed by comparing the frequencies of genotypes in-between the study years by Chi square test or fisher exact test for smaller frequencies. Statistical significance was defined as p < 0.05. The sequencing output of P/K13 was analyzed by using Sequencher 5.1 software to identify polymorphisms.

2.5 Ethical Considerations
2.5.1 Informed Consent – Consent forms were translated from English into Swahili, and then explained to the patient, parent or guardian. Details about the trial and its benefits and potential risks were explained. Participants over 18 years of age were asked to sign a consent form after any questions had been answered; written assent form was taken from children under the age of 18. If the patient was illiterate, a literate witness was asked to sign; if possible, the signatory with no connection to the research team was selected by the participant. Consent statement for the pregnancy test was also required for female participants of child-bearing age who were sexually active.

2.5.2 Confidentiality - All information regarding patients remained confidential and shared only by the study team. Unique identifiers were used for computer-based data entry and blood samples. In all cases, the principal investigator checked that screening forms, the case report form, and the completed identification code list were kept in locked files.

2.5.3 Free Health Care - throughout the follow-ups, health care services were provided to the study patients presented with any illness related to malaria regardless of treatment outcome. Any person who decided not to participate or who did not meet the criteria were referred to the health facility staff. If a patient was withdrawn from the study before he/she has completed the full course of the treatment, the clinician provided all necessary arrangements to receive the full dose of the medicine.

2.5.4 Compensation – Transportation costs for all visits to the health center were covered and reimbursed in this study.
2.5.5 Ethical Approvals – All studies involved in this analysis were conducted in accordance with the principles stated in the latest version of the Declaration of Helsinki and Good Clinical Practice (World Medical Association Declaration of Helsinki, 2013) [70]. For the present study (2017), ethical approval was obtained from the Zanzibar Medical Research and Ethics Committee [ZAMREC/0001/January/2016] and the Zanzibar Food and Drugs Board [No.ZFDB/M.M:B:L:Z/16]. Ethical approval for molecular work at Karolinska Institutet was granted by the Regional Ethics Committee in Stockholm, Sweden [2013/836-32].

2.6 Clinical Trial Registrations
ClinicalTrials.gov Identifiers: NCT03764527 (study performed 2002/3), CT03768908 (study performed 2005), NCT01002066 (study performed 2010) and NCT03773536 (present study 2017).
3. Results

3.1 Study Participants

A total of 9062 febrile patients were screened at the fourteen health centers from May to September 2017. Out of 233 mRDT positive candidates, 146 met all inclusion criteria and were enrolled at the three “Recruitment Sites”.

Baseline patient characteristics are presented in Table 2, along with the data from the previous clinical trials in 2002/3 [68] and 2005 (Unpublished). Despite differences in patients age the geometrical means of the parasite density at study enrollment were quite similar. There was high compliance to the study protocol, and only two patients were withdrawn from the study due to “repeated vomiting on day 1” and “itching on day 3” and another two were lost to follow-up due to “long travel distance” and “travel to mainland Tanzania during follow-up”. Hence a total of 142/146 (97.3%) fulfilled the study follow-up to Day 28. Below study flow chart describes the number of individuals screened, enrolled and included in final analysis (Figure 3).

![Study Flow Chart](image)

**Figure 3 – Study Flow Chart**
### Table 2. Patient characteristics at enrollment who received artesunate-amodiaquine (ASAQ) for treatment of malaria for uncomplicated *P falciparum* malaria infections

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>2002/3</th>
<th>2005</th>
<th>2010</th>
<th>2013(^1)</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screened</td>
<td>2097</td>
<td>2076</td>
<td>20423</td>
<td>ND</td>
<td>9062</td>
</tr>
<tr>
<td>Enrolled</td>
<td>207</td>
<td>177</td>
<td>121</td>
<td>113</td>
<td>146</td>
</tr>
<tr>
<td>Sex, no. of patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>104</td>
<td>81</td>
<td>65</td>
<td>ND</td>
<td>101</td>
</tr>
<tr>
<td>Female</td>
<td>103</td>
<td>94</td>
<td>56</td>
<td>ND</td>
<td>45</td>
</tr>
<tr>
<td>Age, months or years, median (range)(^2)</td>
<td>24 (4 – 72) months</td>
<td>28 (4 – 60) months</td>
<td>12 (0 - 92) years</td>
<td>21.5 (1.5 - 50) years</td>
<td>16 (2 - 56) years</td>
</tr>
<tr>
<td>Parasite count, parasites/mL, geometric mean (range)</td>
<td>19,731 (2000–198,440)</td>
<td>20890 (2000–176,000)</td>
<td>7699 (6 - 782400)</td>
<td>ND</td>
<td>7610.46 (55-304000)</td>
</tr>
<tr>
<td>Temperature, °C, arithmetic mean ± SD</td>
<td>38.7 ± 1.2</td>
<td>37.8 ± 1.2</td>
<td>38.3 ± 1.4</td>
<td>ND</td>
<td>37.8 ± 1.4</td>
</tr>
<tr>
<td>Hemoglobin level, g/dL, arithmetic mean ± SD</td>
<td>8.5 ± 1.6</td>
<td>9.2 ± 1.4</td>
<td>ND</td>
<td>ND</td>
<td>11.7 ± 2.2</td>
</tr>
</tbody>
</table>

\(^1\)Baseline data not collected for samples collected in 2013.

\(^2\)Only under 5 children were recruited in 2002/3 and 2005 studies.
3.2 Treatment Outcome

Parasite clearance rate up to Day 3 by microscopy was similar in all three clinical trials (2002-3, 2005 and 2017) (Figure 4). In 2017 study, only one patient remained malaria positive by microscopy by day 3, apart from that all participants were negative up to Day 28 in contrast to the findings in 2002/3 and 2005 (Table 3). The cure rates were 94% (95% CI 89.4-96.5), 96% (95% CI 91.7-98.3) and 100% (95% CI 97.4-100.0) for 2002-3, 2005 and 2017 studies, respectively. The cure rate in 2017 is higher than that of PCR adjusted cure rate (358/378); (94.7%; 95% CI 91.9-96.7) for 2002/3 and 2005 combined (p = <0.001). The total number of recurrent parasitemia – new infections during 28 days of follow-up – were 44 (22%), 16 (9%) and zero (0%) in 2002/3, 2005 and 2017 respectively.

![Figure 4. Mean parasite clearance by microscopy from Day 0 to Day 3](image)

*Figure 4. Mean parasite clearance by microscopy from Day 0 to Day 3* – representing mean parasite density determined by microscopy comparing clearance in 2002-3, 2005 and 2017 in Log scale. Note: There is no parasitemia at day 3 in 2005 study.
Table 3. ASAQ treatment outcome after 28 days follow-up

<table>
<thead>
<tr>
<th>Year of study</th>
<th>Total patients (i)</th>
<th>Positive Day 3 (ii)</th>
<th>Parasite recrudescence (ii)</th>
<th>Recurrent new infection (ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002/3</td>
<td>206/208</td>
<td>1 (0.5%)</td>
<td>13 (6%)</td>
<td>44 (22%)</td>
</tr>
<tr>
<td>2005</td>
<td>172/177</td>
<td>0</td>
<td>7 (4%)</td>
<td>16 (9%)</td>
</tr>
<tr>
<td>2017</td>
<td>142/146</td>
<td>1 (1%)</td>
<td>0 (0%) (iii)</td>
<td>0 (0%) (iii)</td>
</tr>
</tbody>
</table>

(i) Number completed follow-up as per protocol/number enrolled  
(ii) Number of recrudescence after PCR correction  
(iii) MSP1 and GLURP genotyping were not done since there was no positive case by microscopy on day-28 in 2017

The fever clearance rates were also high (<37.5 °C by Day 3) in all studies - 93% in 2002/3, 99% in 2005, and 98% in 2017. Hemoglobin levels were higher at enrollment (Day 0) in 2017 than in 2002/3 and 2005 (Table 2). The average decline by Day 7 after treatment with ASAQ + PQ was mean 1.1 g/dL (-3.3 to 6.6) in 2017, compared to mean 0.2 g/dL (-3.4 to 4.1) after treatment with ASAQ alone in 2002-3.

3.3 Molecular Analysis

The prevalence of the SNPs in pfcrt K76T, pfmdr1 N86Y, Y184F and D1246Y (2002/3 - 2017) are presented in Figure 5. Overall, the decreasing trend of isolates associated with amodiaquine were identified. There was significant decline in frequencies of pfcrt 76T (98% to 4.9%, p < 0.001), pfmdr1 86Y (86.7% to 2.1%, p < 0.001), pfmdr1 Y184 (99.5% to 61.3%, p = 0.005, and pfmdr1 1246Y (36% to 1.4%, p < 0.001) between 2002-3 and 2017. In the present study, the enrolled patients who had overnight travelled to mainland Tanzania was 21% (30/144). Human migration between Zanzibar island and Mainland could increase the importation of P. falciparum infection from Tanzania where AL is prescribed as first-line antimalarial drug. Therefore, present of travel history assuming imported malaria infections are compared with locally acquired cases in Zanzibar (Table 4). However, parasite genotype profiles are similar and there was no significant association with regards to the travel history in all alleles.
Figure 5. Molecular genotyping of single nucleotide polymorphisms in *P. falciparum* infections. This bar chart showing the frequency of polymorphisms associated with amodiaquine resistance. The solid (black) represents the resistance alleles, pattern (dotted) - mixed infection and white – wild-type, respectively. The error bars represent the 95% confidence interval of the proportions of resistance alleles (either alone or mixed infections). The total number of genotyped samples are shown in the bracket next to study year.

Table 4. Proportions of *P. falciparum* infections with the genotypes/associated with tolerance/resistance to amodiaquine (*Pfcr* and *Pfmdr1* genes) in 2017 according to travel history to mainland Tanzania

<table>
<thead>
<tr>
<th>2017</th>
<th>Pfcr n/N (%)</th>
<th>Pfmdr1 n/N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>76T</td>
<td>86Y</td>
</tr>
<tr>
<td>Yes travel</td>
<td>2/30 (6.6%)</td>
<td>1/30 (3.3%)</td>
</tr>
<tr>
<td>No travel</td>
<td>5/114 (4.4%)</td>
<td>2/114 (1.8%)</td>
</tr>
<tr>
<td>All</td>
<td>7/144 (4.9%)</td>
<td>3/144 (2.1%)</td>
</tr>
</tbody>
</table>

(1) All mixed infections (i.e. mutant + wildtype) were also counted as resistance alleles.
A total of nine samples positive by qPCR on Day 28 were also processed for nested PCR to examine the status of SNPs. However, these samples have shown very low (<1 p/µl) parasite density by quantitative PCR, and they were all consistently negative in the nested PCR used for genotyping SNPs. Therefore, the Day 28 samples were not successfully genotyped.

The pfmdr1 YYY and YYD are the most frequent haplotypes in 2002-3, in contrast, the NYD and NFD haplotypes were most frequent in 2017. There was zero YYY and only three YYD in 2017 study. There was significant decline of YYY (p < 0.001) and YYD (p <0.001) between 2002-3 and 2017 (Figure 6).

![Figure 4. Haplotypes shift from YYY to FDN between 2002 & 2017](image)

Figure 4. Haplotypes shift from YYY to FDN between 2002 & 2017, representing the frequencies of pfmdr1 haplotypes. (N86Y, Y184F, and D1246Y). Fisher exact test was performed to compare the frequencies between all years. Asterisk (*) and (**) indicate p value below 0.05 and 0.001 respectively.

A PfK13 pilot study conducted in 2013 identified no SNPs in the PfK13 propeller region in Zanzibar, yet the finding still needs validation due to small sample size (N=34) (unpublished). However, regarding genotyping of PfK13 gene, 98% (139 out of 142) samples were successfully sequenced in 2017. Among them, 5 (3.6%) samples had the previously reported synonymous SNP cysteine to cysteine at amino acid position 469 (CYS 469 CYS) which was previously reported from Fukayosai, Tanzania in 2012 [37]. One additional synonymous SNP serine to serine at amino acid position 477 (SER 477 SER) was also identified in a single (0.7%) sample Table 5. In 2017, only 1 out of 6 isolates reported history of travelling to mainland, Tanzania in the past one month. Moreover, the association between PfK13 and presence of travel history cannot be proven due to small sample size. Nevertheless, the SNPs identified in 2017 study are not associated with artemisinin resistance [37].
Table 5. Representing *PfK13* single nucleotide polymorphisms identified in 2017 according to travel history to mainland Tanzania

<table>
<thead>
<tr>
<th>Study Site</th>
<th>Age (years or month)</th>
<th>Sex (M/F)</th>
<th>Travel (Y/N)</th>
<th><em>PfK13</em> SNP$^1$</th>
<th>Previous Reported SNP (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bububu</td>
<td>22 yr</td>
<td>F</td>
<td>N</td>
<td>Synonymous SNP: CYS 469 CYS</td>
<td>Y</td>
</tr>
<tr>
<td>Bububu</td>
<td>6 yr</td>
<td>M</td>
<td>N</td>
<td>Synonymous SNP: CYS 469 CYS</td>
<td>Y</td>
</tr>
<tr>
<td>Uzini</td>
<td>7 yr</td>
<td>M</td>
<td>Y</td>
<td>Synonymous SNP: CYS 469 CYS</td>
<td>Y</td>
</tr>
<tr>
<td>Bububu</td>
<td>21 mth</td>
<td>F</td>
<td>N</td>
<td>Synonymous SNP: CYS 469 CYS</td>
<td>Y</td>
</tr>
<tr>
<td>Bububu</td>
<td>15 yr</td>
<td>M</td>
<td>N</td>
<td>Synonymous SNP: CYS 469 CYS</td>
<td>Y</td>
</tr>
<tr>
<td>Bububu</td>
<td>41 yr</td>
<td>F</td>
<td>N</td>
<td>Synonymous SNP: SER 477 SER</td>
<td>N</td>
</tr>
</tbody>
</table>

$^1$Synonymous mutation within *PfK13* alleles C469C was previously reported in Fukayosi, Tanzania in 2012 [37].
4. Discussion

Zanzibar is one of the countries in the Africa Region with the most remarkable decrease in both malaria morbidity and mortality since 2005 [6,50]. To achieve malaria elimination, maintaining the drug efficacy and prevention of development of resistance is critical. The aim of this study is to assess the drug efficacy of ASAQ and track the development of molecular markers that are known to be associated with amodiaquine and artesunate resistance. The following sections discussed the evidence provided as the result of this study in relation to the drug efficacy of ASAQ and polymorphisms associated with resistance to amodiaquine and artesunate.

4.1 Efficacy of Artesunate-Amodiaquine

Efficacy studies conducted in Zanzibar (2002-3, 2005 and 2017) consistently showed that the first line ACT (ASAQ) treatment for uncomplicated *P. falciparum* malaria remains effective. Malaria diagnosis by microscopy is the conventional tool to monitor drug resistance in therapeutic efficacy studies, and revisions of national malaria treatment guideline can be considered if day 3 parasitemia and day 28 cure rates are more than 10% and less than 90%, respectively [71]. Microscopic examination of malaria parasites showed that parasitemia on day 3 were as low as 0.5%, 0% and 1%, and cure rates on day 28 remains as high as 94%, 96% and 100% in 2002-3, 2005 and 2017 respectively. In addition, the proportion of recurrent infection on day 28 (after PCR correction) decreased during the period from 2002-3 to 2017. Findings regarding treatment efficacy could provide as an evidence to maintain ASAQ as a first line treatment in Zanzibar.

4.2 Single Nucleotide Polymorphisms

Polymorphisms in *pfcrt* and *pfmdr1* gene have been associated with reduced sensitivity to ASAQ and AL, yet the characteristic of these alleles are not fully understood [72]. The selection of *pfcrt* 76T, *pfmdr1* 86Y, 184Y and 1246Y alleles have been associated with reduce sensitivity to ASAQ, whilst in reverse, selection of wild types alleles are related to reduced sensitivity in AL [29–31,33,35,73]. Hence, *pfmdr1* 86N, 184F and 1246D alleles were found to be associated with high proportion of treatment failure in Tanzania where AL was the first line drug to treat falciparum malaria [36]. Similarly, a study conducted in Burkina Faso reported that there was significant increase in *pfcrt* 76K and decreased in *pfcrt* 76T after AL treatment whereas increased in *pfcrt* 76T and decreased in *pfcrt* 76K after ASAQ treatment [73]. Regarding *pfmdr1* N86Y alleles, 86Y has been associated with increased with ASAQ treatment failure, but the evidence is less convincing for *pfmdr1* Y184F [73].

On the one hand, the mechanism of higher selection of wild types in this study SNPs - *pfcrt* 76K, *pfmdr1* 86N, 184F and 1246D alleles - are not fully understood. On the other hand, continuous wide-scale use of ASAQ is more likely to select mutant type due to presence of sustain drug
pressure at population level [27,29,30,35,36]. However, in 2012, Froberg et al. reported the first observation of decreased prevalence of SNPs that were associated with ASAQ resistance in Zanzibar and suggested three possible underlying causes of selecting pfcr76K, pfmdr1 86N and 1246D alleles were genetic dilution caused by imported cases from mainland Tanzania, selection by artemunate per se, and fitness cost of the parasite itself. [74]. Parasite fitness seems to have advantage over selection of circulating resistant parasites [75]. The prevalence of mutants pfcr76T and pfmdr1 86Y found to be decreased in low transmission areas when drug pressure is diminishing [65,75,76]. However, information regarding mediating factors of resistance polymorphisms on parasite fitness is not known sufficiently [76]. In 2015, Morris et al. also reported that significant further decline in pfcr76T and pfmdr1 86Y alleles in Zanzibar [65]. Current study (2017) has shown further reductions in markers associated with tolerance and resistance to ASAQ since the last time report in 2015. The molecular findings of 2017 study are consistent with two previous studies, hence, continuous monitoring of genetic changes in SNPs have strong implication for shaping malaria treatment policy in Zanzibar.

4.2.1 Haplotypes

In the study, pfmdr1 (86Y, 184Y, 1246Y) haplotypes YYY and YYD are the most frequent in 2002-3, but interestingly, NYD and NFD haplotypes were most frequent in 2017. NFD haplotypes were associated with reduced sensitivity to AL [77]. There was only three YYD and zero YYY haplotypes that are otherwise known to be the risk factor for treatment failure in patients treated with ASAQ in African settings [35,77,78]. There was a significant decline of YYY (p < 0.001) and YYD (p <0.001) between 2002-3 and 2017. A study conducted in Western Kenya reported that when the drug pressure of AQ is decreased, there was significant increase in selection of pfmdr1 and pfcr wild type alleles within three year period (between 2008 – 2010) of replacing AQ with ACT as a first line treatment [79]. This provides additional evidence that ASAQ remains an effective first line treatment in Zanzibar.

4.3 Generalizability

This study was a follow-up study to monitor efficacy of local first line ACT in Zanzibar with adaptation of WHO TES guideline to surveillance of artemisinin resistance. Findings of this paper may contribute to reaching the elimination target in Zanzibar by ensuring the effectiveness of ASAQ plus single low dose PQ. The aim is to eliminate local malaria transmission by 2023, according to the Zanzibar Malaria Elimination Programme. Moreover, this study can contribute to pooled analysis of resistance data initiated by such as “Worldwide Antimalarial Resistance Network” (WWARN) and WHO world artemisinin status reports. Although the findings in this study are specific to the Zanzibar setting, and are not generalizable to other settings with varied
malaria endemicities, and applicable to other ACTs, the study protocol involving satellite sites and recruitment centers in order to achieve a large enough sample size to power a clinical trial can be applicable to other low-transmission settings.

4.4 Study Limitations

4.4.1 Limitation of Study Designs
The original studies involved in this analysis are three clinical trials and two surveys. Two surveys conducted in 2010 and 2013 were health facility based, providing a snap-shot measurement of the prevalence of drug resistance markers, and did not assess treatment efficacy. Regarding three clinical trials, follow-up period in 2017 study was 28 days, whilst it was 42 days for the two previous studies (2002-3 and 2005). Although WHO recommends 28 days follow-up period for antimalarials including amodiaquine - which have elimination half-lives of less than 7 days, longer duration of follow-up up to 42 days in clinical trials is beneficial to evaluate the true cure rate [24]. The study conducted in Zanzibar (2002-3) reported that high proportion of recrudescence after day-28 follow-up [68]. Additionally, laboratory analysis to differentiate re-infection or recrudescence was not done for day-28 in 2017 study since there was no parasite positivity by microscopy on day-28.

4.4.2 Participants Characteristics
Despite the patient’s demographic and clinical information such as age, sex, history of fever, hemoglobin level, treatment history, parasite and gametocyte carriage and parasite density are available for clinical trials, there was no comprehensive data for two surveys conducted in 2010 and 2013. Therefore, no complete data was available for comparison of fever clearance, parasitemia and hemoglobin level assessments for all studies.

4.4.3 Molecular Methods
Overall, the limitation of highly sensitive PCR is risk of contamination which can lead to false positive results. However, even highly sensitivity PCR can still give negative results if the parasite density approaches to the detection limit. For such very low density sub-microscopic infection can also give inconsistent results owing to the lower reproducibility [80]. Moreover, the sensitivity of RFLP assay is low to detect mixed infection. Regarding running of gel-electrophoresis, it is moderately time consuming and requires minor adjustments to get a better visualization. Repetition is unavoidable until the DNA bands are clear enough for identification of interest.
5. Conclusion

Despite the wide-scale use of ASAQ as first-line drug to treat uncomplicated *P. falciparum* malaria, efficacy remains high after fourteen years of first deployment in 2003. The molecular markers associated with ASAQ resistance - SNPs *pfcrt* 76T, *pfmdr1* 86Y, 184Y and 1246Y alleles and *pfmdr1* (86Y, 184Y and 1246Y) YYY haplotypes were significantly decreased between 2002-3 and 2017. Although two synonymous SNPs in the *PfK13* gene (five CYS469CYS and one SER477SER isolates) were identified, these SNPs have not been associated with artemisinin resistance. Continuous monitoring of drug efficacy and resistance markers is warranted to track the development and characteristics of artemisinin resistance in Zanzibar.
References


Annex

Appendix 1. Showing the remarkable decreasing community prevalences of asexual *P. falciparum* parasitemia by microscopy or RDT; all age groups in May/June in two study districts

<table>
<thead>
<tr>
<th>Year</th>
<th>Micheweni district</th>
<th></th>
<th>North A district</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>Positive</td>
<td>Positivity rates (95% CI)</td>
<td>Tested</td>
</tr>
<tr>
<td>2003</td>
<td>1189</td>
<td>172</td>
<td>14.5% (12.5–16.6)</td>
<td>2167</td>
</tr>
<tr>
<td>2005</td>
<td>1241</td>
<td>135</td>
<td>10.9% (9.2–2.7)</td>
<td>1503</td>
</tr>
<tr>
<td>2006</td>
<td>1182</td>
<td>56</td>
<td>4.7% (3.6–6.1)</td>
<td>1433</td>
</tr>
<tr>
<td>2007</td>
<td>1575</td>
<td>15</td>
<td>1.0% (0.5–1.6)</td>
<td>1499</td>
</tr>
<tr>
<td>2008</td>
<td>2091</td>
<td>10</td>
<td>0.5% (0.2–0.9)</td>
<td>1746</td>
</tr>
<tr>
<td>2009</td>
<td>1539</td>
<td>0</td>
<td>0.0% (0–0.2)</td>
<td>1163</td>
</tr>
<tr>
<td>2011*</td>
<td>1271</td>
<td>10</td>
<td>0.8% (0.4–1.4)</td>
<td>1561</td>
</tr>
<tr>
<td>2013*</td>
<td>1579</td>
<td>7</td>
<td>0.4% (0.2–0.9)</td>
<td>1447</td>
</tr>
<tr>
<td>2015*</td>
<td>1515</td>
<td>9</td>
<td>0.6% (0.3–1.1)</td>
<td>1497</td>
</tr>
</tbody>
</table>

Note: *Malaria diagnosis by RDT instead of blood slide microscopy (Source: Björkman et al. [6])
### Appendix 2. Implementation of malaria control tools/strategies in Zanzibar between 2002 and 2016

<table>
<thead>
<tr>
<th>Year, month</th>
<th>Interventions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002, November</td>
<td>New antimalarial treatment policy: ACT; 1st line: ASAQ, 2nd line: AL</td>
</tr>
<tr>
<td>2003, September</td>
<td>ACT deployment in all public health facilities.</td>
</tr>
<tr>
<td>2004</td>
<td>ITN distribution, geographically focused Intermittent preventive treatment in pregnancy (IPTp)</td>
</tr>
<tr>
<td>2005, September</td>
<td>LLIN universal distribution to all children &lt; 5 years and pregnant women</td>
</tr>
<tr>
<td>2006, July</td>
<td>IRS (pyrethroid) aiming at annual universal coverage, in March before the main transmission season (after 2006)</td>
</tr>
<tr>
<td>2006</td>
<td>RDT provision to all public health facilities. LLIN provision initiated to all pregnant women and infants (9 months old) in MCH clinics</td>
</tr>
<tr>
<td>2008</td>
<td>LLIN universal distribution—two nets per household</td>
</tr>
<tr>
<td>2009</td>
<td>New antimalarial treatment policy: 1st line: ASAQ, 2nd line: quinine Weekly reporting of malaria cases by mobile phone from health care facilities (MEEDS)</td>
</tr>
<tr>
<td>2012</td>
<td>LLIN universal distribution—two nets per household IRS policy change: targeting hotspots only (carbamate 2012–2014, pirimiphos-methyl 2015-) Malaria case investigation and reactive household RDT screening and LLIN distribution</td>
</tr>
<tr>
<td>2015</td>
<td>RDT and ACT provision to private health facilities (AMFm programme) Intermittent screening and treatment in pregnancy (ISTp) replacing IPTp</td>
</tr>
<tr>
<td>2016</td>
<td>Larviciding in few selected sites New antimalarial treatment policy: ACT + PQ (single low dose)</td>
</tr>
</tbody>
</table>

ACT artemisinin-based combination therapy, ASAQ artemunate-amodiaquine, AL- artemether-lumefantrine, ITN - insecticide-treated net, IRS - indoor residual spraying, RDT - rapid diagnostic test, LLIN - long-lasting insecticidal net, MEEDS - malaria early epidemic detection system, AMFm - affordable medicines for malaria, MCH - mother and child health (Source: Björkman et al. [6])
Appendix 3. Definition of severe *falciparum* malaria

**Clinical manifestations**

- prostration,
- impaired consciousness,
- respiratory distress (metabolic acidosis),
- multiple convulsions,
- circulatory collapse,
- pulmonary edema (radiological),
- abnormal bleeding,
- jaundice,
- hemoglobinuria.

**Laboratory findings**

- severe anemia (hemoglobin < 5 g/dL, hematocrit < 15%),
- hypoglycemia (blood glucose < 2.2 mmol/l or 40 mg/dl),
- acidosis (plasma bicarbonate < 15 mmol/l),
- hyperlactatemia (venous lactic acid > 5 mmol/l),
- hypercarotenemia (> 4% in non-immune patients),
- renal impairment (serum creatinine above normal range for age).

**Classification of severe malaria in children**

**Group 1: children at increased risk for death**

- prostration
- respiratory distress

**Group 2: children at risk for clinical deterioration**

- hemoglobin < 5 g/dL, hematocrit < 15%
- two or more convulsions within 24 h

**Group 3: children with persistent vomiting**
Appendix 4. Medications (with antimalarial activity) that should not be used during the study period

**List of medications**

- chloroquine, amodiaquine;
- quinine, quinidine;
- mefloquine, halofantrine, lumefantrine;
- artemisinin and its derivatives (artemether, artether, artesunate, dihydroartemisinin);
- proguanil, chlorproguanil, pyrimethamine;
- sulfadoxine, sulfalene, sulfamethoxazole, dapsone;
- primaquine;
- atovaquone;
- antibiotics: tetracycline*, doxycycline, erythromycin, azythromycin, clindamycin, rifampicin, trimethoprim;
- pentamidine.

* Tetracycline eye ointments can be used.
Appendix 5. Patient discontinuation or protocol violation

Study patients who meet any of the following criteria will be classified as withdrawn.

- Withdrawal of consent. A patient may withdraw consent at any time, without prejudice for further follow-up or treatment at the study site.
- Persistent vomiting of the treatment. A patient who vomits the study medication twice will be withdrawn from the study and given rescue treatment.
- Failure to attend the scheduled visits during the first 3 days.
- Serious adverse events necessitating termination of treatment before the full course is completed. A patient can be discontinued from the study if the principal investigator decides so due to an adverse event of adequate nature or intensity. In this case, information on the adverse event and symptomatic treatment given must be recorded on a case report form. If the adverse event is serious, the principal investigator must notify the sponsor or its designee immediately and follow the reporting procedures

- Enrolment violation:
  - severe malaria on day 0; or
  - erroneous inclusion of a patient who does not meet the inclusion criteria.
- voluntary protocol violation: self- or third-party administration of antimalarial drug (or antibiotics with antimalarial activity) (Appendix 4)
  - involuntary protocol violation:
  - occurrence during follow-up of concomitant disease that would interfere with a clear classification of the treatment outcome;
  - detection of mono-infection with another malaria species during follow-up; or
  - misclassification of a patient due to a laboratory error (parasitemia), leading to administration of rescue treatment.

Patients who are withdrawn will be followed up until recovery or to the end of follow-up, no treatment outcome will be assigned to these patients, and they will be censored or excluded from the analysis. The reasons for discontinuation or protocol violation will be recorded on the case report form.