Artemisinin resistant falciparum malaria in Myanmar

Kyaw Myo Tun

Green Templeton College

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy in Clinical Medicine

Michaelmas Term 2015

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Abstract

Artemisinin-based combination therapy (ACT) is first-line treatment for *Plasmodium falciparum* malaria globally but artemisinin resistance is now prevalent across Southeast Asia. Myanmar has the highest malaria burden in the region, and determining the prevalence of artemisinin resistance and current therapeutic efficacy of first-line antimalarial drugs is critical for both clinicians and policy makers planning malaria control and elimination programmes. The aim of this research was to study the geographical extent, prevalence, degree and optimum treatment of artemisinin-resistant falciparum malaria in Myanmar through a countrywide molecular survey and two multicentre clinical trials supported by parasitological and pharmacological investigations.

In a molecular survey of clinical falciparum malaria cases carried out in 55 sites across 10 administrative regions and border sites in neighbouring countries 39% of cases (371/940) were associated with parasites carrying a kelch13 propeller mutation. Kelch13 mutation prevalence exceeded 10% in much of the east and north of the country and was 47% in an area 25 km from the border with India.

In a trial conducted in central and northern Myanmar treatment efficacy of dihydroartemisinin-piperaquine (DP) was 100% but there was delayed parasite clearance associated with the kelch13 mutation F446I (median clearance half-life 4.7 hours, IQR, 3.7 to 6.2).

In a randomised controlled trial of 3-days versus 5-days artemether-lumefantrine (AL) treatment efficacy was 100% (95%CI, 94.9-100) and 97% (95%CI, 90-99.7) respectively and the two arms showed equal clearance rates (measured by an ultrasensitive quantitative polymerase chain reaction assay, uqPCR)..There was no association between the presence of kelch13 propeller mutations and residual parasite density at day 21, measured using uqPCR. Gametocyte carriage rates were high reinforcing the need to implement single low-dose primaquine (0.25 mg/kg) with ACTs to kill gametocytes in this area of artemisinin resistance.

In conclusion, artemisinin resistant falciparum malaria is widespread in Myanmar. While DP and AL remain efficacious, the partner drugs are vulnerable and if resistance develops treatment efficacy is likely to decline rapidly. Greater efforts are urgently needed to monitor treatment efficacy of first-line antimalarial drugs and develop alternative treatment regimens.

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List of Abbreviations

3MDG	Three Millennium Development Goals Fund
ACT	Artemisinin-based combination therapy
AE	Adverse event
AL	Artemether-lumefantrine
AS	Artesunate
AS-MQ	Artesunate-mefloquine
AUC	Area under the curve
CRF	Case report form
CRT	Chloroquine resistant transporter
CTSG	Clinical Trials Support Group (MORU)
DHA	Dihydroartemisinin
DHA-PIP	Dihydroartemisinin piperaquine
DHPS	Dihydropteroate synthase
DHFR	Dihydrofolate reductase
DP	Dihydroartemisinin-piperaquine
EDTA	Ethylene diaminetetraacetic acid
EIR	Entomological inoculation rate
G6PD	Glucose-6-phosphate dehydrogenase
GCP	Good Clinical Practice
GLURP	Glutamate-rich protein
GMS	Greater Mekong Subregion
Hb	Haemoglobin
Hct	Haematocrit
IC	Inhibitory concentration

ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IM	Intramuscular
IV	Intravenous
K13	Kelch13
Kg	kilogram
MDR	Multi drug resistance
mg	milligram
MFT	Multiple first-line therapies
MOCRU	Myanmar Oxford Clinical Research Unit
MORU	Mahidol Oxford Tropical Medicine Research Unit
MSP	Merozoite surface protein
NMCP	National Malaria Control Programme
PCE	Parasite clearance estimator
PCR	Polymerase chain reaction
PCT	Parasite clearance time
PD	Pharmacodynamic
*Pf*PI3K	*Plasmodium falciparum* phosphatidylinositol-3-kinase
PI	Principal investigator
PK	Pharmacokinetic
PQ	Primaquine
QA	Quality assurance
QC	Quality control
RSA	Ring-stage survival assay
SAE	Serious adverse event

SEA	Southeast Asia
SEARO	South East Asia Regional Office
SNP	Single-nucleotide polymorphism
SOP	Standard Operating Procedure
SP	Sulphadoxine-pyrimethamine
TDR	Special programme for research and training in tropical diseases
TRAC	Tracking Resistance to Artemisinin Collaboration
uqPCR	Ultrasensitive polymerase chain reaction
WHO	World Health Organisation
WWARN	Worldwide Antimalarial Resistance Network

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Author's contribution

The author's contribution to the original research performed for this thesis involving: (1) in the design and conducting the molecular epidemiological survey from 2013 to 2014 (2) protocol development and patient recruitment of the DP trial from 2013 to 2014 (3) protocol development and patient recruitment of the 3 vs. 5 AL trial from 2013 to 2015 (4) statistical analysis of all studies. Kyaw Myo Tun was not involved in doing kelch13 sequencing, highly sensitive PCR in the molecular and genetic laboratory and geostatistical modelling.

1 Introduction

1.1 Aim

The primary objective of this thesis is to study the spread of resistance of *Plasmodium falciparum* to artemisinin derivatives in Myanmar, through epidemiological, clinical, parasitological and pharmacological investigations, to determine the geographical extent, prevalence, severity and optimum treatment of artemisinin resistance.

The specific objectives were:

- To examine the spread of artemisinin resistance molecular markers of *P. falciparum* in field isolated from patients in Myanmar and neighbouring countries (chapter 3)
- To determine the *P. falciparum* parasite clearance rate after treatment with dihydroartemisinin-piperaquine in two different geographical regions of Myanmar, and to identify parasite genetic determinants of the slow *P. falciparum* clearance phenotype in Myanmar (chapter 4)
- To compare a 5 day regimen of artemether-lumefantrine with the standard 3 day regimen for the treatment of uncomplicated falciparum malaria, by assessment of adequate clinical and parasitological response as well as parasitocidal efficacy using ultrasensitive molecular detection methods on days 5 and 7 (chapter 5)
- To determine the spatial and temporal distribution in Myanmar of kelch13 mutations, day 3 positivity rate, uqPCR positivity and gametocytaemia following treatment (chapter 6)

1.2 Background information

Myanmar, also known as Burma, is situated in the Greater Mekong Sub-region of Southeast Asia. It has a total land area of 676,578 square kilometres. It has land borders with Thailand and China to the east and Bangladesh and India to the west. Myanmar is composed of 14 states and regions, with Naypyitaw the new capital and Yangon (Rangoon) the largest city. The country is further subdivided into 65 districts and 325 townships, 59 sub-townships, 2,759 wards, 13,729 village tracts and 64,986 villages.

Myanmar is home to 135 ethnic groups speaking over 100 languages and dialects. The major nationalities are Kachin, Kayah, Kayin, Chin, Mon, Bamar, Rakhine and Shan. The 2014 census revealed a population of 51.4 million (1). The majority of the population live in malaria endemic areas, with 37% of the total population reside in high transmission areas, 23% in low transmission area, and the remaining 40% in malaria free areas (2).

Myanmar is a tropical country with three seasons: the hot season (March to June); the rainy season (July to October); and the cold season (November to February). The temperature, humidity and rainfall during the rainy season provide favourable conditions for malaria vector mosquitoes. *Anopheles dirus* and *An. minimus* are the most common vectors but *An. aconitus*, *An. annularis*, *An. culicifacies, An. sundaicus and An. maculatus* are also found throughout the country (3-7).

During the course of the clinical research carried out for this thesis a cross sectional molecular survey was done in ten states and regions of Myanmar and two clinical trials were done in three states and regions. (Figure 1.1)

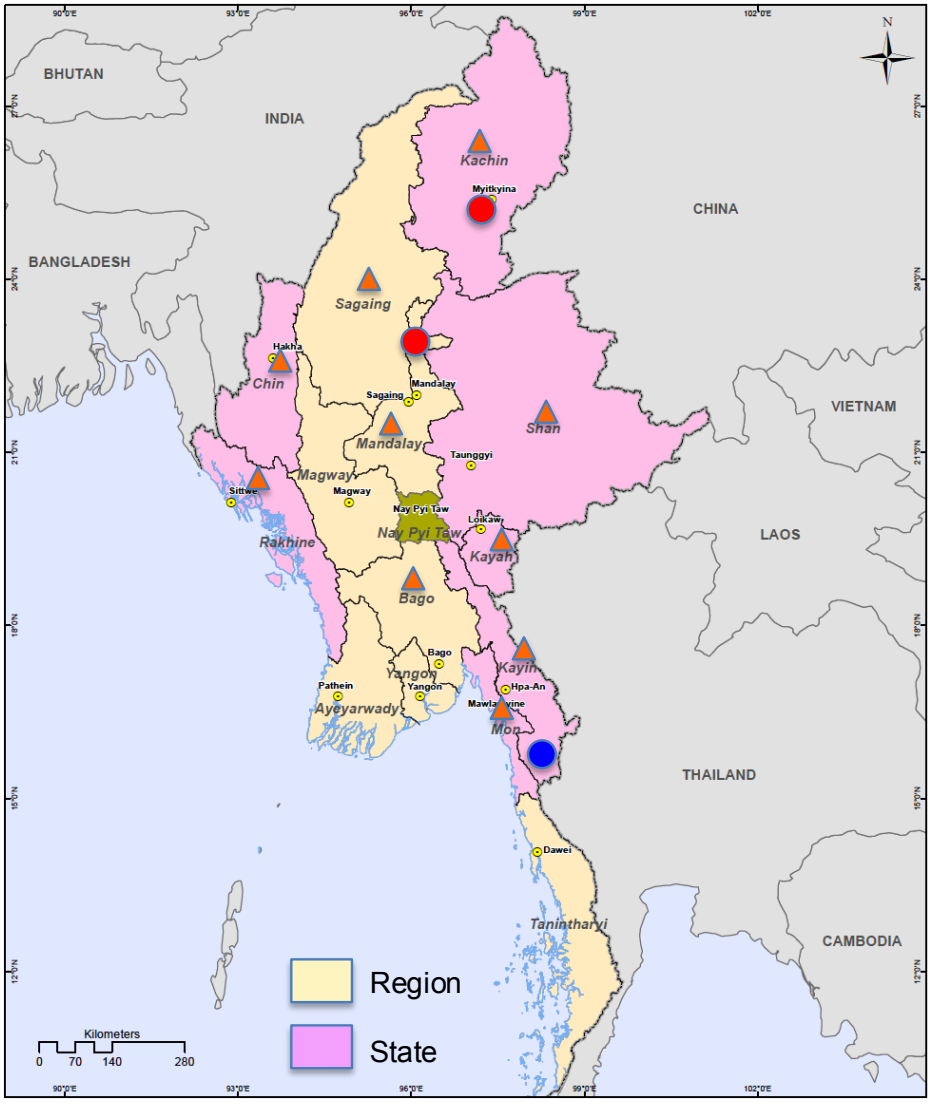


Figure 1.1 Myanmar map showing states and regions. Red circles represent DP trial sites and blue 3 vs. 5 AL trial sites. Orange triangles represent cross sectional molecular survey sites. (© Myanmar Information Management Unit)

1.3 Global malaria burden

Malaria is the most important parasitic disease of man, and remains a major public health problem in the world today. In 2013 there were 3.2 billion people living in malaria endemic areas, with 1.2 billion judged to be at high risk of being

infected (2). Among these, 198 million (range from 124 to 283) people became infected. An estimated 584,000 of these cases resulted in death (range from 367,000-755,000) (2). The highest burden of malaria morbidity is in Africa (82%), followed by Southeast Asia (12%), and the Eastern Mediterranean Region (5%)(Figure 1.2) (2).

Human malaria is caused by five different parasite species, namely *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*. An estimated 92% of cases are due to *P. falciparum*, accounting for 99%, 84%, 67% and 54% of malaria infections in Africa, the Western Mediterranean Region, the Eastern Mediterranean Region and Southeast Asia respectively. *P. vivax* causes 8% of total malaria cases worldwide and the other three species are relatively rare (2).

1.4 Malaria in Myanmar

Myanmar is situated in the west of Southeast Asia and to the east of the Indian subcontinent and it is estimated to have 1 to 10 confirmed cases of malaria per 1,000 of the population. All five human malaria species are present in Myanmar, but of these *P. falciparum* and *P. vivax* are the most prevalent. In 2014, 74% of government reported cases were caused by *P. falciparum* and the remaining 26% were caused by *P. vivax* (2).

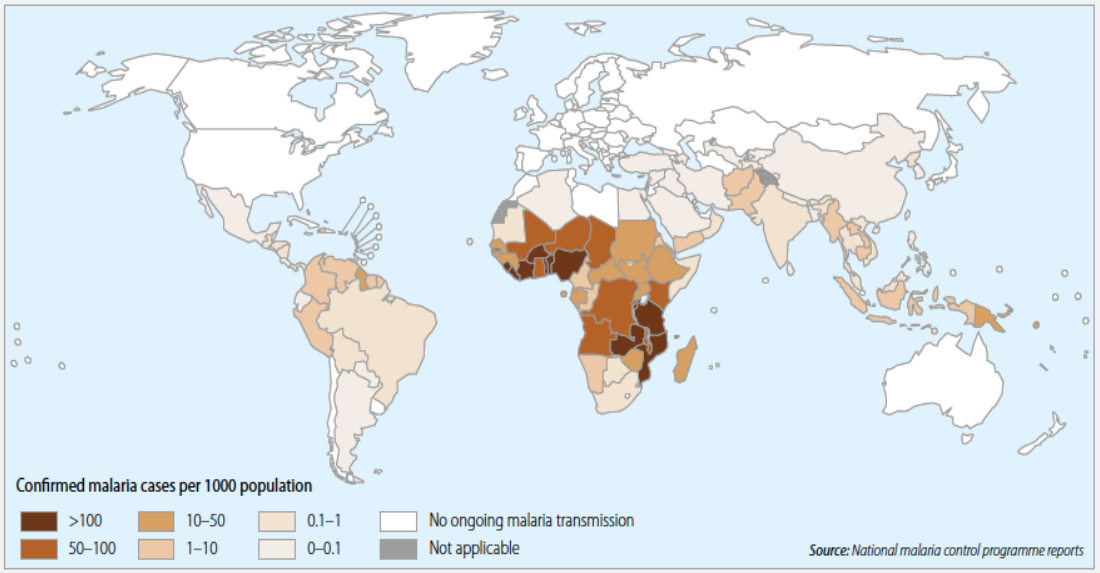


Figure 1.2 Malaria transmission in different geographical regions.

© World malaria report 2014, World Health Organization, ISBN:9789241564830, “Countries with ongoing transmission of malaria, 2013” (Page 2) (2)

Between 2000 to 2013 the number of confirmed cases of malaria in the Southeast Asia region fell from 2.9 to 1.5 million. However Myanmar is one of the top three countries which together contribute 96% of the cases in WHO SEARO (South East Asia Regional Office) (Myanmar 22%, India 58% and Indonesia 16%) (2).

Both upward and downward trends were observed in the reported malaria incidence in Myanmar from 2000 to 2013 (Figure 1.3A). In 2000, the annual malaria incidence was 12 per 1,000. There was a dramatic decrease in 2005 but this was followed by an increase in annual incidence to 13.18 per 1,000 in 2009. Since 2009 the trend has been downward, and by 2013 the incidence was 6.44 per 1,000.

The mortality from malaria has gradually decreased from 2000 to 2013. The reported number of malaria deaths was 5.0 per 100,000 in 2000 and 0.48 per 100,000 in 2013.

1.4.1 Malaria trends around the sites of clinical studies reported in this thesis: Kachin, Kayin States and Mandalay Region

1.4.1.1 *Kachin State*

Kachin State, with a total area of 89,038 square kilometres, is situated in the north of Myanmar. It shares international borders with Yunnan province and Tibet in China, and India and within Myanmar it borders the Sagaing region and Shan state. It is composed of four districts and 18 townships, and the state capital is Myitkyina. The total population in 2014 according to the government census was 1,689,654 with 108 males for every 100 females. Kachin State has a population density of 19 per square kilometre (1). Thirty-six percent of the population lives in

urban areas and 64% in rural areas. Kachin, Lisu, Shan, Bamar and other minor ethnic groups reside in this state. The main occupations of the local people are farming and working in the jade mines.

Myitkyina district is served by two 300-bed hospitals, and primary care is provided by a network of rural health centres. In addition a number of faith-based organisations and international non-governmental organisations (NGOs) provide preventive and primary health care.

There was a steady decline in the annual malaria incidence in Kachin State from 35.8 per 1,000 in 2000 to 22.8 per 1,000 in 2007 (Figure 1.3B). However the malaria incidence rose to 68.1 per 1,000 in 2010 and then fell sharply to 16 per 1,000 in 2013. A possible explanation for the dramatic increase in 2010 was population movement from peripheral areas of Kachin State to the cities because of conflict in the area. Another possible reason is an increase in case finding activity using rapid diagnostic tests provided by international NGOs. The mortality rate declined markedly from 2000 to 2013, with reported malaria deaths falling from 16.6 per 100,000 in 2000 to 1.9 per 100,000 in 2013. The number of malaria cases and deaths in Kachin State is one of the highest in Myanmar.

1.4.1.2 *Mandalay Region*

Mandalay Region is situated in the centre of Myanmar and covers 29,686 square kilometers. It shared borders with Shan state, and the Naypyitaw, Magway and Sagaing regions. It is composed of 7 districts and 28 townships and the regional capital is Mandalay. The population in the 2014 census was 6,145,588, with 91 males for every 100 females and a population density of 207 per square kilometre (1). Thirty-five percent of the population lives in urban areas and 65% in

rural areas. One of the clinical trials was conducted in the Thabekkyin township of Mandalay Region. Most of the people residing in Thabeikkyin area work in the gold mines or in the forest, and the rest are farmers. Shan, Bamar and Lisu ethnic groups reside within Mandalay Region. Two hospitals (100 beds) and two station hospitals (25 beds) serve for curative care of the population in Thabekkyin. Apart from these hospitals, there are two rural health centres and twenty sub centres run by midwives providing primary health care including malaria diagnosis and treatment.

There has been steady decline in annual malaria incidence in Mandalay Region from 6.27 per 1,000 in 2000 to 2.98 per 1,000 in 2006 (Figure 1.3C). However, the malaria incidence increased to 4.67 per 1,000 in 2011 and then fell sharply to 2.2 per 1,000 in 2013. A possible explanation for the increase in 2011 is the increase in case finding activity using rapid diagnostic tests provided by international NGOs. The annual malaria mortality declined markedly from 2000 to 2013. The reported number of malaria death was 2.78 per 100,000 in 2000 and 0.03 per 100,000 in 2013.

1.4.1.3 *Kayin State*

Kayin State is situated in the east of Myanmar and covers 30,383 square kilometers. It share borders with Bago region, Mon and Kayah states, and it has a long international border with Thailand. The capital city is Hpa An and composed of 3 districts and 7 townships. The population in the 2014 census was 1,502,904 with a population density of 52 per square kilometre, and there were 97 males for every 100 females (1). Twenty-two percent of the population lives in urban areas and 78% in rural areas. One of the clinical trials was conducted in Kyainseikgyi township in Kayin state. Most of the people residing in the

Kyainseikgyi area are farmers and rubber-plantation workers. The population of Kayin State comprise Kayin, Bamar and Shan ethnic groups. One hospital (50 beds) and two station hospitals (25 beds) serve for curative care, and primary health care is provided by rural health centres. Numerous faith-based organizations and international NGOs provide alternatives for both curative and preventive health care in many parts of Kayin state. Considerable cross border movement occurs between Kayin state and Thailand for economic reasons and because of the long history of ethnic conflict in the area.

Figure 1.3D shows the upward then downward trend in malaria morbidity from 2001 to 2013 in Kayin State. The annual malaria incidence in Kayin State was highest in 2011 (16.5 per 1,000), but decreased to 8.39 per 1,000 in 2013. The mortality rate has steadily declined from 2001 to 2013, with reported malaria deaths 7.38 per 100,000 in 2001 and 1.5 per 100,000 in 2013.

However, the routine data collection system in Myanmar has many weaknesses. Mid-wives or health assistants working in the sub-centres or rural health centres have to report basic health care data, vaccination data, birth and death reports, and all activity in vertical programmes including malaria to the next level (township) every month. This administrative burden is in addition to their routine health care service delivery work, and the figures presented above are likely to be considerable underestimates. There was a twenty to thirty percent discrepancy in the number of malaria cases reported by the National Malaria Control Programme (NMCP) and those identified in our research clinic records for the year we conducted research in Thabeikkyin and Myitkyina.

According to the NMCP, malaria incidence and mortality is high especially in the states and regions bordering Thailand and China, as malaria diagnosis and

treatment are difficult to access. The movement of the people in the border areas is driven by armed conflict, cross border business, employment opportunities and social reasons (8).

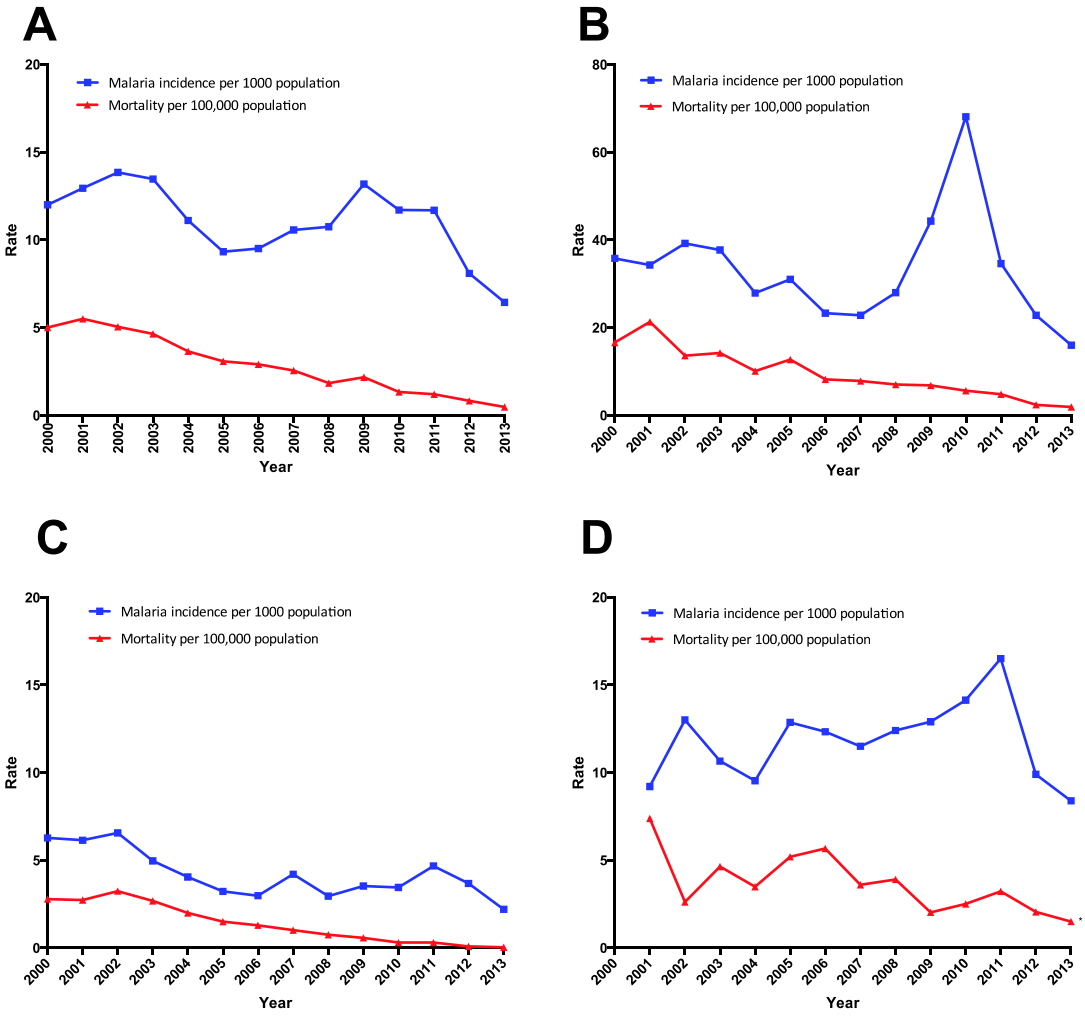


Figure 1.3 Malaria morbidity and mortality trends in (A) Myanmar (the whole country), (B) Kachin State, (C) Mandalay Region, and (D) Kayin State (2000-2013). Source: National Malaria Control Programme (9)

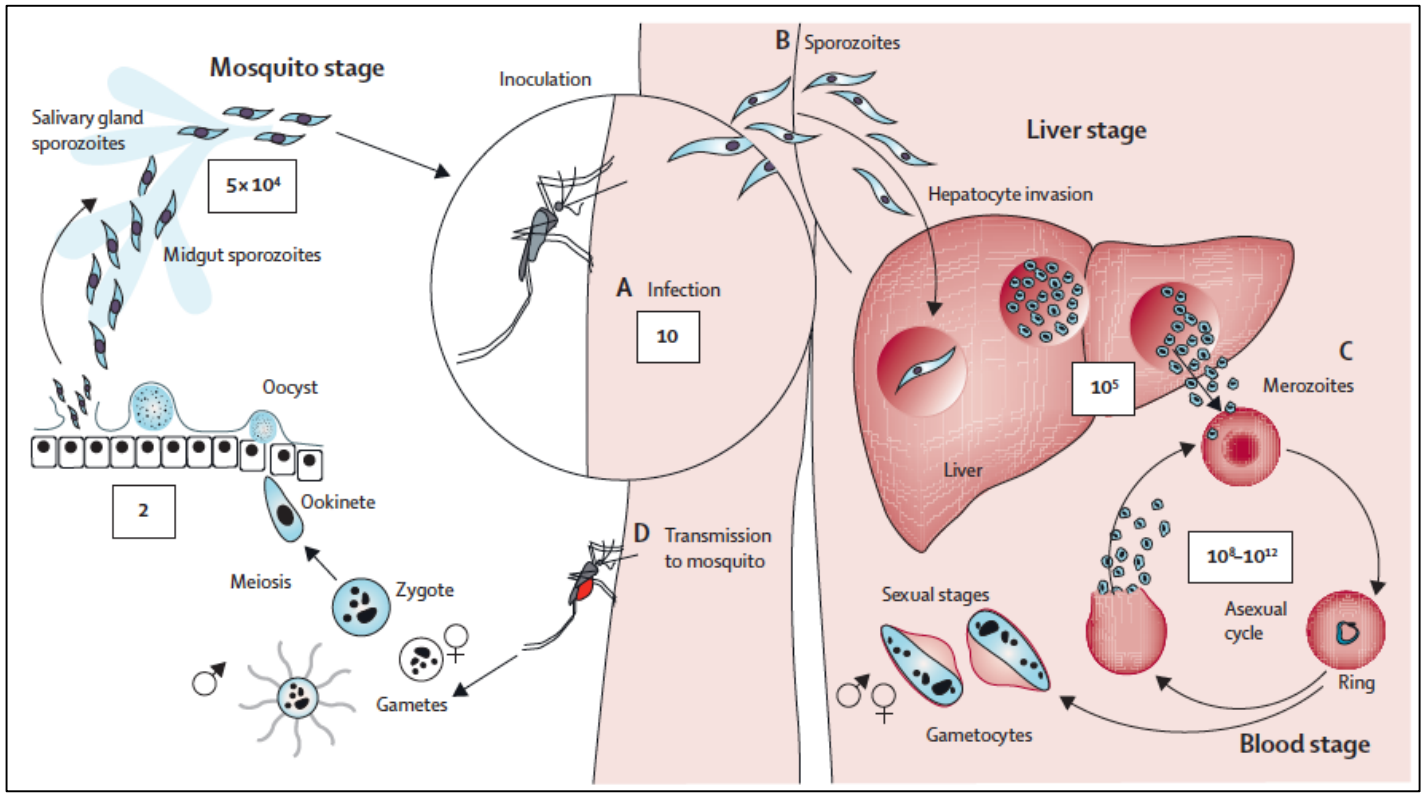


Figure 1.4 The life cycle of *Plasmodium falciparum* (10)

1.5 Life cycle of *Plasmodium falciparum*

P. falciparum is a eukaryote with a complex life cycle in mosquitoes and humans. The whole genome of *P. falciparum* has been sequenced, revealing a 23 mega base nuclear genome consisting of 5,300 genes on 14 chromosomes (11). The life cycle of *P. falciparum* has four distinct phases, namely, asexual reproduction phase, gametocyte development phase, sporozoite formation phase and a liver-stage developmental phase (Figure 1.4) (12-14). Many serial mitoses occur during the human part of the life cycle, with the parasite population capable of enormous expansion during an infection.

1.5.1 Development in the mosquito

The malaria parasite is transmitted to mosquitoes in the blood meal of female Anopheline mosquitoes. After the blood meal, the ingested male gametocyte, which is a haploid cell, undergoes three rounds of mitosis and then exflagellates to release 8 haploid male gametes (12). One of these fertilises the female gamete and diploid zygotes are created (14). The zygotes then develop into ookinetes and oocysts, following two rounds of meiosis. Depending on ambient temperature, an oocyst then undergoes 10 to 13 rounds of DNA synthesis and mitosis and produces 2,000 to 10,000 sporozoites (12, 14). The sporozoites then migrate to the mosquito salivary glands in preparation for injection into the human host when the mosquito next takes a blood meal. This whole process, sporogony, takes 8-35 days depending on temperature and mosquito species (15). The mosquito is the only place in which genetic recombination (meiosis) takes place (Figure 1.4) (10).

1.5.2 Development in the human host

The infected female Anopheline mosquito transmits sporozoites into the human host during a blood meal. The sporozoites are injected into capillaries in the dermis and then invade hepatocytes where they replicated into hepatic schizonts, which then rupture releasing merozoites into the blood stream. The exoerythrocytic (hepatic) part of the life cycle takes approximately 5.5 days (15). One mature hepatic schizont contains roughly 30,000 to 50,000 merozoites (13, 15). Merozoites released into the systematic circulation invade red blood cells, starting the asexual erythrocytic cycle. Within the erythrocytes the immature ring forms of the parasite begin haemoglobin digestion, develop into tropozoites and then, following cell division, schizonts consisting of 12 to 20 merozoites. These merozoites are released into the blood stream on schizont rupture, initiating another erythrocytic cycle. For *P. falciparum* the erythrocytic cycle takes approximately 48 hours (13, 15). Rather than developing into schizonts after erythrocyte invasion some merozoites become either male or female gametocytes (gametocytogenesis), a process that takes 7-12 days (13, 15). The protein synthesis process is shut down in mature gametocytes and they are therefore less susceptible to antimalarial drugs compared to asexual forms (14). The life cycle in the mosquitoes begins when a female anopheles mosquito bites an infected human and gametocytes are ingested into the mosquito's mid gut.

1.6 Pathophysiology

In falciparum malaria disease is caused by the destruction of infected red blood cells in the spleen, the host reaction to the liberation of parasites and red blood cells material in to the blood stream, and by microvascular obstruction caused

mainly by sequestered parasitized erythrocytes (14, 15). All species of malaria induce acute illness but *P. falciparum* (and rarely *P. vivax*) infection can lead to severe malaria (14). In falciparum malaria, erythrocytes containing maturing parasites adhere to the endothelium in a process known as sequestration. The young ring-stage parasites circulate in the peripheral circulation, but trophozoites transport molecules to the red cell surface (principally erythrocyte membrane protein 1 (*Pf*emp1) which bind to adhesion molecules on the endothelial cell surface (cytoadherance) leading to sequestration. Thought to have evolved to avoid the "filtering" function of spleen, sequestration is one of the survival strategies of the parasite (16). Sequestration occurs mainly in small capillaries and venules and, together with other phenomena including rosetting and increased red cell deformability (of both infected and uninfected cells), leads to microvascular obstruction (10, 14). Occlusion of blood flow causes reduced oxygen delivery to the vital organs. Sequestration in the brain is believed to be one of the causes of coma in cerebral malaria (17) but the full pathophysiology of coma is not yet fully understood (15). Other complications of severe malaria include acute renal injury, pulmonary oedema, anaemia, metabolic acidosis, haemodynamic shock, gastrointestinal bleeding, liver dysfunction and hypoglycaemia (15, 18).

1.7 Clinical manifestations

The clinical features of malaria range from asymptomatic submicroscopic malaria, through acute febrile illness to severe and complicated malaria, and if untreated may lead to death. The clinical manifestations in an individual patient depends on host immunity, host genetics and intensity of transmission. Malaria infection has a classic periodic febrile attack pattern, which varies with species.

Age, nutritional status, and genetic factors can also influence the severity of disease. In high transmission areas it is mainly younger children who develop severe malaria, as older children and adults have sufficient immunity to control the infection. However in areas where transmission is low or unstable severe and fatal malaria infection can occur in any age group, with the demographics of severe disease more driven by exposure (for instance young men working in the forest). Pregnant women are particularly vulnerable to severe malaria, and malaria infection also causes low birth weight in the new born infant, prematurity, stillbirth and abortion. The symptoms of malaria in pregnancy vary according to immunity, and transmission intensity (19).

The most common clinical features of uncomplicated falciparum malaria are fever, malaise, anorexia, nausea, dizziness, myalgia, backache and headache. The malaria 'paroxysm' of fever and shivering with profuse sweating is more common in vivax malaria and rarely seen in *P. falciparum* infection (14). The World Health Organization definition of severe falciparum malaria is based on clinical and laboratory diagnosis (18, 20). The most common clinical manifestations of severe malaria are prostration, impaired consciousness, respiratory distress, multiple convulsions, circulatory collapse, pulmonary oedema, abnormal bleeding, jaundice and haemoglobinuria (14, 20). Acidosis, caused by renal failure, lactic acidosis and other unknown acids, is a common finding on laboratory analysis. In Southeast Asia, the number of deaths from malaria has decreased from 5,500 in 2000 to 776 in 2013 (2). However, uncomplicated falciparum malaria is still a public health problem in this region, and if inadequately treated can lead to severe malaria and death. This thesis explores the diagnosis and treatment of uncomplicated falciparum malaria and

molecular epidemiology of artemisinin resistance markers in Myanmar. I aimed to assist policy makers by determining the distribution and molecular and clinical nature of drug resistance malaria infections, and the therapeutic efficacy of first line drugs.

1.8 Diagnosis of malaria

Patients with suspected malaria should be tested with rapid diagnostic tests (RDTs) or by microscopic examination of the peripheral blood smear (20). RDTs are based on histidine-rich protein 2 (HRP2) or parasite lactate dehydrogenase (LDH). RDTs provide similar sensitivity to a trained microscopist, but false positives may arise as *Pf*HRP2 may remain in a patient's blood stream for up to one month after an episode of acute malaria (15). The reference standard for diagnosis is currently malaria microscopy. The thin and thick films can be stained with Giemsa's or Field's stain and counted by using oil immersion microscopy. The detection limit of microscopy is roughly 10^8 total parasites in the circulation of a human host. Molecular detection by highly sensitive qPCR methods based on 18S RNA can detect parasitaemias as low as 10 parasites per millilitre, which is more than 1,000 times more sensitive than conventional microscopy (15, 21).

1.9 Antimalarial drugs and mode of action

The three broad groups of antimalarial drugs are the antifols, the quinoline related compounds, and artemisinin and its derivatives (15).

1.9.1 Drugs targeting parasite folate synthesis

Antifols and sulphas consists of pyrimethamine and the biguanides and these drugs interfere with folic acid synthesis (15). Pyrimethamine inhibits the enzyme

dihydrofolate reductase (DHFR) and sulphonamides inhibit dihydropteroate synthase (DHPS) (Table 1.1). Sulphadoxine-pyrimethamine (SP) is used as intermittent preventive treatment of malaria in pregnancy in moderate to high transmission areas (20). Multiple target enzyme gene mutations for sulphadoxine and pyrimethamine have been identified. Proguanil is a biguanide compound which is converted into the active metabolite cycloguanil in vivo, which inhibits dihydrofolate reductase (20). Specific gene mutations encoding for resistance of proguanil have been identified.

1.9.2 Quinolines and related drugs

Chloroquine and amodiaquine (4-Aminoquinolines) and piperaquine (Bisquinolines), quinine, mefloquine, lumefantrine and primaquine are included in this group. The exact mode of action of this group is not yet clear (15). The quinoline related compounds are weak bases and act on acid vacuoles of malaria parasite (15). For example, chloroquine inhibits intraparasitic haem detoxification and interferes with the biosynthesis of nucleic acids (20). Chloroquine resistance is associated with mutations in the *pfMDR* and *pfCRT* genes.

1.9.2.1 ***Piperaquine***

Piperaquine is a bisquinoline compound with molecular mass 535.5 (Figure 1.5D). The hydroxyl derivative is effective against chloroquine resistant malaria parasites (15, 20). It is co-formulated with dihydroartemisinin as an artemisinin combination therapy for uncomplicated falciparum malaria. The molecular marker of piperaquine resistance remains to be discovered.

1.9.2.2 *Lumefantrine*

Lumefantrine, previously known as benflumetol, is a fluorine derivative of the aryl amino-alcohol group of antimalarials. Lumefantrine has a molecular mass of 528.9 and is lipophilic and hydrophobic. It is co-formulated with artemether in the globally most commonly used artemisinin-based combination therapy. The mode of action is prevention of haem detoxification in the parasite food vacuole (20)(Figure 1.5C).

1.9.2.3 *Primaquine*

Primaquine, molecular mass 259.4, is an 8-aminoquinoline active against a broad range of malaria stages including the pre-erythrocytic stages (including hypnozoites) and circulating mature gametocytes (Table 1.1)(Figure 1.5E). It is used as a gametocytocidal drug in *P. falciparum* and for the radical cure of *P. vivax*. The elimination half life is 8 hours (15). Primaquine is bio-transformed into the inactive metabolite carboxyprimaquine via the mono-amine oxidase pathway. A second route of metabolism, via the P450 (CYP) 2D6 pathway, generates reactive intermediates (20, 22). It is thought that one of these, as yet unidentified, is responsible for primaquine's antimalarial efficacy. Human genetic polymorphisms of P450 (CYP) 2D6 play an important role in determining primaquine efficacy (23), and further work is needed to describe these polymorphisms in Myanmar. Primaquine administration may lead to haemolysis in individuals with glucose 6 phosphate dehydrogenase (G6PD) deficiency. The degree of haemolysis depends on the G6PD mutation and the dose and duration of treatment (20). It is thought that the primaquine metabolite responsible for haemolytic complications is also generated by the CYP 2D6 pathway (22).

G6PD deficiency gene frequencies in Myanmar typically range from 10-20% (24-26) and G6PD Mahidol (487G>A) is the most common variant in general Myanmar population (24, 25) but G6PD Mahidol and Viangchan is the two most common in Kayin population (27). The use of single low dose (0.25 mg/kg) primaquine in G6PD deficiency (including Mahidol and Viangchan) is safe with a very low risk of haemolysis (28).

Table 1.1 Antimalarial drugs and possible drug targets*

Drugs	*Location/ function*	*Life stage/ Target*
Sulfadoxine	Parasite cytoplasm	Blood stage/ DHPS
Pyrimethamine Proguanil	Parasite cytoplasm	Blood stage/ DHFR
Chloroquine Amodiaquine	Food vacuole	Blood stage/ heme polymerization
Mefloquine Quinine	Food vacuole	Blood stage/ heme polymerization
Atovaquone	Mitochondria	Blood stage and liver stage / cytochrome bc_1 complex
Primaquine	Unknown	Liver stage and gametocytes / target not known
Artemisinins §	Food vacuole	Blood stage and gametocyte / heme polymerization #

*Adapted from Kappe S. *et al.* (29), DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase.

§ exact drug target is not known. #possible drug target.

1.9.3 Artemisinin and derivatives

1.9.3.1 *History of artemisinin*

The artemisinins were developed by Chinese scientists working on project 523, a secret military-initiated project to find new antimalarial drugs which began on the 23rd May 1967 in the middle of the Chinese cultural revolution (30). The project scientists were divided into two groups, one focused on Chinese traditional medicine and the other on synthetic compounds (30). In the early 70s, artemisinin was successfully extracted from *Artemisia annua*, a plant used in traditional Chinese medicine for the treatment of fever, and shown to have antimalarial activity. A water soluble derivative, artesunate, and a methyl ether derivative, artemether, were also developed (30). Both artesunate and artemether are pro-drugs that are hydrolysed to dihydroartemisinin in the human body (30). The findings were first published, in English, in 1979 (31-33). However the US army, Special Programme for Research and Training in Tropical Diseases (TDR), and pharmaceutical companies wanted to develop synthetic derivatives and not be reliant on Chinese supplies. This led to much time and money being spent on the development of arteether and artelinic acid, compounds with no material advantages over artemether and artesunate (31). Most subsequent clinical and pharmaceutical development has focused on artemisinin and the original Chinese derivatives, artemether, artesunate and their hydrolysis product dihydroartemisinin.

For the discovery and development of the artemisinins Zhou Yiqing and his team received the “European Inventor Award” in 2009 for “Co-artem” and Youyou Tu (controversially) received the “Lasker-Debakey Clinical Medical Research Award” in 2011 (34) and the “Nobel prize in Physiology or Medicine” in 2015 (35).

Artemisinin is soluble in aprotic solvents and is thermo-stable (36). The mechanism of action of artemisinin is not yet clear but it is associated with reduction of its endoperoxide bridge (31, 37, 38). Other models invoke the production of free radicals following activation by heme in the malaria food vacuole (36). The antimalarial stage-specific action of artemisinin is broad, ranging from young rings to stage IV gametocytes (31, 39). However it has no activity against pre-erythrocytic stages or against mature stage V gametocytes.

1.9.3.2 ***Artemisinin***

Artemisinin is a naturally occurring sesquiterpene lactone endoperoxide obtained from *Artemesia annua* by ethyl ether extraction. It kills all asexual stages of the intraerythrocytic cycle as well as gametocyte stages I to IV (31). It is unique in rapidly clearing young ring-stage parasites, an attribute probably responsible for the relative mortality reduction compared to other antimalarial drugs when used to treat severe malaria. Because of its short half-life, artemisinin needs to be given for at least 7 days if used as monotherapy.

In patients with *P. falciparum* infection, the artemisinins act potently on the young ring-stage unsequestered parasitized erythrocytes, killing these parasites before they can sequester (40). In addition, a study carried out in 1972 described the process of erythrophagocytosis or “pitting” of parasitized red cells by the spleen (41). The macrophages in the spleen envelope the parasites within red cells by fragmentation or pitting, and after that the pitted red cells (or once-infected red cells) are returned to the circulation (41, 42). The rapid clearance of parasites by artemisinin is due to rapid pyknosis and pitting of artemisinin-sensitive parasites

(43). Therefore, the rate of parasite clearance is a good metric to use when assessing the efficacy of ACT.

1.9.3.3 *Artemether*

Artemether is a methyl ether derivative of artemisinin (Figure 1.5A). It can kill the malaria parasite from ring stage to schizonts. Oral and intramuscular forms are available. It is lipid soluble and prepared as an oil-base injection (20). It is well tolerated and in some cases, hypersensitivity reactions, dizziness and elevated liver enzyme are side effects. In a mouse model, high doses of artemether injection caused neurotoxicity (44, 45).

1.9.3.4 *Artesunate*

Artesunate is a hemisuccinate derivative of artemisinin. Intravenous, intramuscular, rectal and oral forms are available and the intravenous form is recommended as first line therapy for severe malaria globally. Compared to quinine for the treatment of severe malaria, artesunate was associated with relative reductions in mortality of 35% in Southeast Asia and 22.5% in Africa (46, 47). Artesunate has intrinsic antimalarial activity and like artemether is hydrolysed *in vivo* to dihydroartemisinin. It is well tolerated and adverse effects are same as other artemisinin derivatives including hypersensitivity reactions, dizziness, arthralgia, gastrointestinal disturbances but neurotoxicity has not been observed in clinical trials at therapeutic doses (20) .

1.9.3.5 *Dihydroartemisinin*

Dihydroartemisinin is the active hydrolysis product of artesunate and artemether (Figure 1.5B).

Pharmacokinetic parameters of the antimalarial drugs used in the current studies are summarised in Table 1.2.

Table 1.2 Pharmacokinetic parameters and molecular weight of the antimalarial drugs used in the studies reported in this thesis (20)

Drugs	*C_{max} (ng/mL)*	*T_{max} (h)*	*Elimiation$T_{1/2}$ (h)*	*Molecular mass*
Artemether	171-540	1.5-10.0	5.7-7.0	298.4
Lumefantrine	4,456-28,300	2-66.3	32.7-275	528.9
Dihydroartemisinin	366-698	0.97-2.8	0.85-1.40	284.4
Piperaquine	71.6-730	1.48-5.7	13.5-28[§]	535.5
Primaquine	65-295	1.8-4.0	3.5-8.0	259.4

C_{max}, the maximum concentration; T_{max}, the time taken to reach maximum concentration; $T_{1/2}$, Half-life. [§] in days.

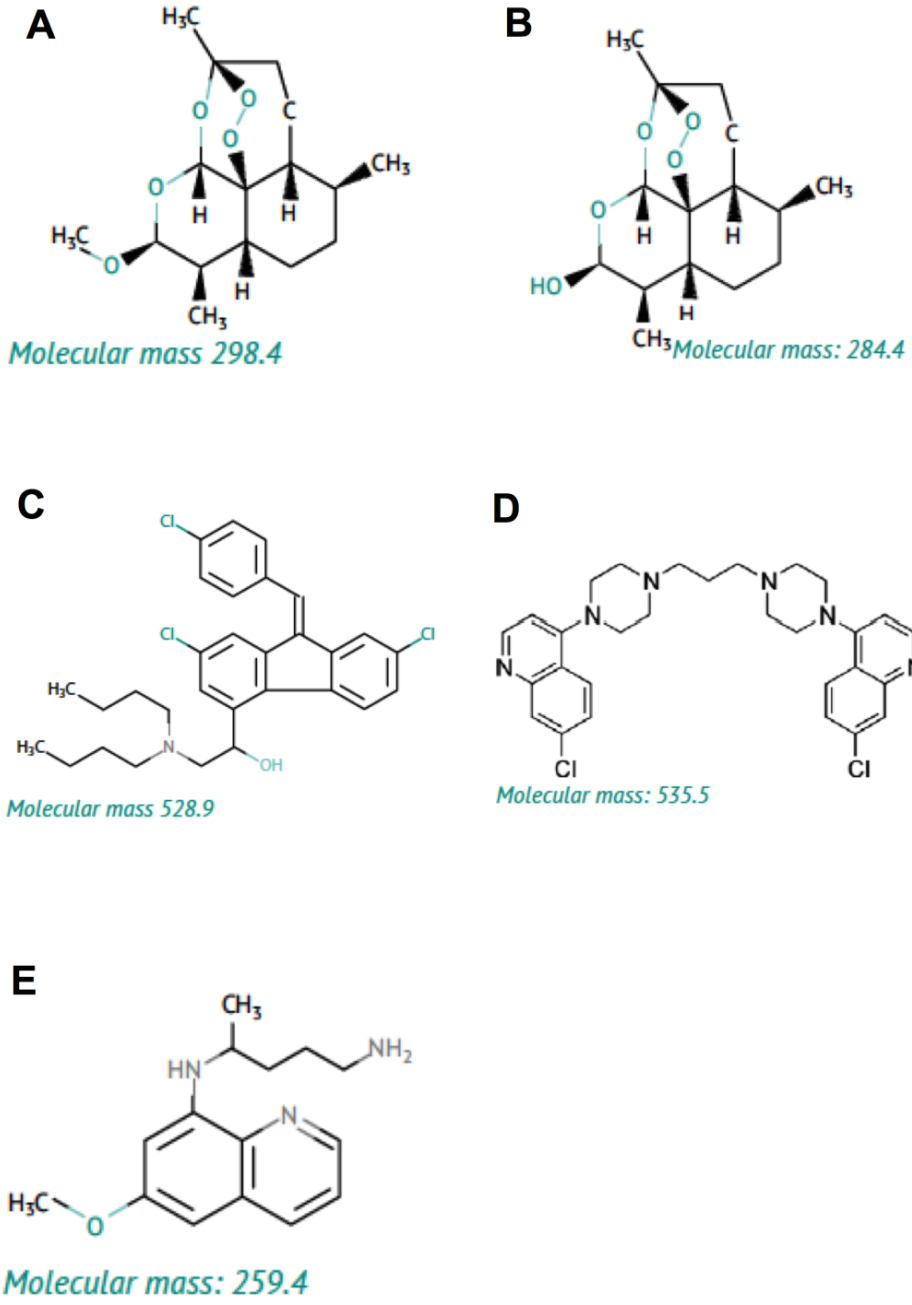


Figure 1.5 Chemical Structures of the antimalarial drugs used in the studies reported in this thesis. A) Artemether B) Dihydroartemisinin C) Lumefantrine D) Piperaquine and E) Primaquine.

(Reproduced from Guidelines for the treatment of malaria by the World Health Organization (20))

1.9.3.6 *Reasons for combination drugs for the treatment of malaria*

Chloroquine was first introduced in 1945 and was widely used in the 1950s and 1960s. However, chloroquine resistant *P. falciparum* was first reported in 1957, and has since spread across all of Southeast Asia and through most of Africa (48). Chloroquine resistance emerged spontaneously in more than 4 geographical regions, but it was the parasites from Western Cambodia, which spread to Africa (49-51). Clinically significant resistance of pyrimethamine emerged only twice and again resistance started in Asia and spread to Africa (49, 52, 53). The flanking microsatellite markers revealed a minimum of four or five independent origins of sulphadoxine-pyrimethamine resistance (53, 54). Another potent antimalarial drug, mefloquine, introduced in 1977 and reported resistance in 1982 (55, 56).

After introducing artemisinins monotherapy for malaria treatment, cases of recrudescence were observed and the duration of treatment necessary to prevent this is long, e.g. artesunate monotherapy for 7 days (57). Because artemisinin dervatives are short acting, combination with long-acting antimalarials is beneficial. The concept of combination therapy in leprosy, tuberculosis and HIV infection is well established and successful in clinical practice. Concurrent use of two or more drugs with different modes of action and different biological targets give additive potential (58). In general, there are non-artemisinin based antimalarial combinations and artemisinin-based combinations. The latter have some advantages because of the unique properties of the artemisinin component. Some advantages are rapid resolution of symptoms, rapid clearance of parasites, reduction of gametocyte levels and effectiveness against multidrug resistant *P. falciparum* (58). To obtain high treatment efficacy with ACTs,

adherence to treatment is crucial. The short-acting artemisinins eliminate parasite biomass quickly and the long-acting partner drug kill the remaining parasites, and the combination reduces the chance of emergence of resistance to both drugs (20, 59). The most commonly used ACTs in Southeast Asia are artesunate-mefloquine, artemether-lumefantrine, and dihydroartemisinin-piperaquine (20, 60). The pharmacokinetics and pharmacodynamics of artesunate and mefloquine are shown in Figure 1.6. If combination therapy is given, area B represents the number of parasites exposed to the partner drug mefloquine alone after the artesunate has been eliminated, and if artesunate is not given, area A is the (much greater) number of parasites exposed to mefloquine alone (20). With three days of ACT two erythrocytic cycles of *P. falciparum* are covered, leading to an eight log reduction in biomass, with the remaining parasites cleared by the long acting partner drug (61). Recently, the most potent antimalarial drug artemisinin derivatives failed to clear parasites rapidly and delayed in parasite clearance in western Cambodia (62). It was found that pfmdr1 Tyr86 variant and/or increased numbers of copies of pfmdr1 were associated with high treatment failure in artesunate-mefloquine combination therapy (56, 62).

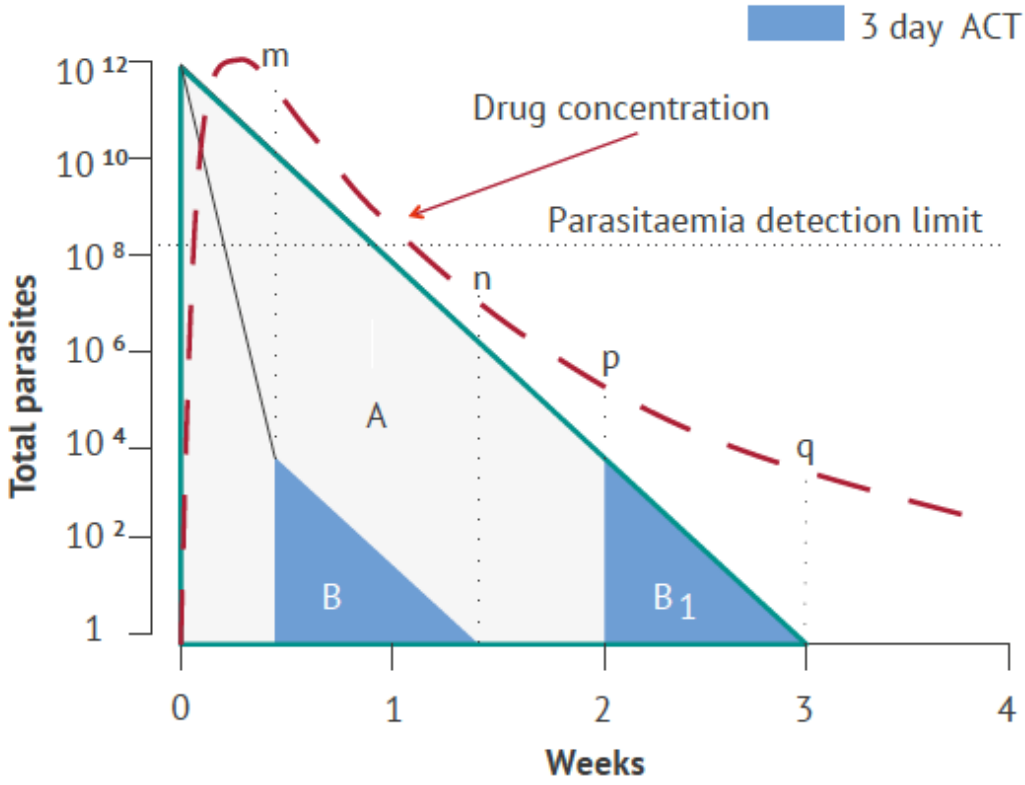


Figure 1.6 Pharmacokinetics and pharmacodynamics of artemisinin-based combination therapy (20)

1.9.3.7 *Artemisinin combination therapy (ACTs) and role of Primaquine in P. falciparum treatment*

In the WHO malaria treatment guidelines and many national guidelines ACT is combined with primaquine to reduce *P. falciparum* transmission. Successfully treated patients with *P. falciparum infections* are still infectious for more than one week. Smithuis *et al.* (63) reported that in Myanmar ACT and primaquine 0.75 mg/kg reduced gametocyte carriage in patients as much as 12 fold more than without primaquine treatment. Studies have looked at the effect of different dosages of primaquine on *P. falciparum* gametocyte clearance. Eziefula *et al.* (64) found that 0.4 mg/kg was as good as 0.75 mg/kg dose but doses between 0.1 and 0.4 mg per kilogram needed further evaluation (65). Primaquine drug plasma concentration will increase if co-administered with dihydroartemisinin-piperaquine (20).

1.10 Treatment of malaria

Treatment regimes vary according to severity of disease and national treatment guideline policy.

1.10.1 Uncomplicated *Plasmodium falciparum* malaria

The World Health Organization (WHO) defined uncomplicated malaria 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 treatment for uncomplicated Pf malaria is artemisinin-based combination therapy (ACTs) except for pregnant women in first trimester (20). ACT currently includes an artemisinin derivative and one slowly eliminated partner drug. The parasite

clearance rate with artemisinin derivatives is rapid, and compared with other antimalarial drugs the artemisinins are highly effective against the younger ring forms of the parasite (in the absence of artemisinin resistance) (66). The partner drug clears the remaining parasites because of its slow elimination half-life. According to WHO guideline, the currently recommended first line ACTs are artemether-lumefantrine, dihydroartemisinin-piperaquine, artesunate-amodiaquine, artesunate-sulfadoxine-pyrimethamine and artesunate-mefloquine (20). In the Greater Mekong Subregion, Cambodia and Thailand use a single first line therapy but Myanmar uses multiple first line therapies (60). Two modelling studies have supported the notion that to prevent the development of resistance the use of multiple first line therapies (MFT) is superior to sequential use of single first line antimalarial drugs (67, 68). However, using a population genetics approach Antao and Hastings have suggested that the effect of multiple first line therapies on the spread of artemisinin resistance genotypes is negligible compared to that of sequential single first line antimalarial drugs (69). Recent work by Nguyen *et al.* found that MFT strategies are significantly superior to single first line and can delay artemisinin resistance especially in low transmission setting (70).

1.10.2 Antimalarial drugs usage in Myanmar

ACTs have been used as first line antimalarial drugs since 2005. Before 2005, artesunate monotherapy was widely used for the treatment of falciparum malaria especially in malaria endemic border areas like Myanmar-Thailand and Myanmar-China borders. The three ACTs recommended by the Myanmar NMCP for the treatment of uncomplicated falciparum malaria are artemether-lumefantrine, artesunate-mefloquine and dihydroartemisinin-piperaquine. The

NMCP provides artemether-lumefantrine (Coartem®, Novartis, Switzerland) to all health facilities for the treatment of uncomplicated malaria cases throughout the country. However, drug usage in the private sector is only loosely controlled by the government. A recent cross-sectional survey showed that 33% of self-reported antimalarial dispensing from retailers was artemisinin monotherapy. 63% reported cutting up blister packages, so were not selling full course of drug to consumers. Those selling antimalarial drugs in the private sector include drug vendors, informal pharmacies, and general purpose retailers. There is currently no regulation of this practice; a case can be made for regulation in the context of containment efforts to counter the emergence of artemisinin resistance (71).

In low intensity transmission settings such as Myanmar, WHO recommends usage of single low dose primaquine (0.25 mg/kg body weight) as a gametocytocidal drug in combination with ACTs without G6PD testing prior to treatment (20). The Myanmar national treatment guidelines still recommend the older primaquine 0.75 mg/kg single dose for the interruption of malaria transmission, but with testing for G6PD deficiency (72).

1.11 Antimalarial drug resistance

Antimalarial drug resistance is defined as "*the ability of a parasite strain to survive and/or multiply despite administration and absorption of an antimalarial drug given in doses equal to or higher than those usually recommended, provided that drug exposure is adequate*" (20). Quinine has been used widely to treat malaria since the early 17th century, and low level resistance developed after 278 years. However this is the exception, as for other drugs such as choloroquine resistance has developed within 10-15 years of introduction (56).

Drug resistance is generally first identified in low and moderate transmission settings (73), possibly because in these areas immunity is generally low, malaria symptomatic, and a higher proportion of infecting parasites are exposed to drug treatment. However the recent finding of widespread very low level asymptomatic carriage in endemic 'low transmission' areas of Southeast Asia has challenged this hypothesis (74).

For some antimalarial drugs, such as the antifols and atovaquone, the drug target is known and mutation in the gene encoding the target protein identified. For sulfadoxine-pyrimethamine for example, the principal mutations associated with resistance are in codons 108, 51, 59 and 164 of the DHFR gene and codons 436, 540, 581, and 623 of the DHPS gene. For most other antimalarial drugs the exact mechanism of action and therefore of resistance is unknown, but in many cases molecular markers associated with resistance have been identified. For chloroquine resistance associated mutations include *pf*CRT Thr76 and *pf*MDR Tyr86, Phe184, Cys1034, Asp1042 and Tyr1246 (15, 29, 56) (Table 1.3).

1.11.1 Artemisinin resistance and the discovery of kelch13 gene

In 2006, Denis et al (75) performed surveillance of the efficacy of artemisinin-based combination drugs in Cambodia, an epicentre of drug resistance, and found that cure rate was decreased in the Pailin area. The WHO criteria for drug resistance are based on a threshold of 10% treatment failure, and this work signalled and alarming finding warranting further investigation. At the same time, Alker *et al.*(76) published a paper in which they described *in vivo* resistance to artesunate-mefloquine in falciparum malaria and one possible molecular marker; pfmdr1 copy number was high among treatment failure cases on the Cambodian-Thai border. Artemisinin resistance was first recognised in western Cambodia as

prolonged parasite clearance times in clinical cases by Noedl *et al.*(77) and Dondorp *et al.* (62).

Table 1.3 Antimalarial drugs and resistance (gene; mutation positions in corresponding resistance alleles)*

Resistant to drug	*Low to intermediate-level resistance*	*High-level resistance*
Sulfonamides, sulfones	DHPS; 436, 437, 540, 581, 613	
Pyrimethamine	DHFR; 108 then 51 and 59	DHFR; 108 + 51 + 59 + 164
Cycloguanil, Chlorcycloguanil	DHFR; 16 + 108	DHFR; 108 + 51 + 59 +164
Chloroquine	CRT; 76	CRT;76 and other mutations, MDR; 86, and other undefined gene products
Mefloquine, halofantrine, lumefantrine	MDR; amplification of wild type allele	
Atovaquone	Not documented	Cytochrome b; 133 ± 280
Artemisinins and derivatives	Kelch13 gene; C580Y, I543T, R539T, Y493H, P441L, F446I, G449A, N458Y, P553L, R561H, V568G, P574L, A675V	

CRT, chloroquine resistance transporter; MDR, multidrug resistance p-glycoprotein pump; DHFR, dihydrofolate reductase ; DHPS, dihydropteroate synthase. (Adapted from White NJ (78) and WHO (60))

In 2010, using microsatellite markers, Anderson *et al.* reported high heritability in parasite clearance phenotype in parasites from patients in western Cambodia, with 56-58% of the variance in clearance rate determined by parasite genetics (79). In 2012, Cheeseman and co-workers demonstrated that a selective sweep on chromosome 13 of *P. falciparum* is strongly associated with slowed parasite clearance (80). The heritable component of slowed parasite clearance was the cardinal sign of artemisinin resistance (80). In 2013, heritability of parasite clearance phenotypes was assessed by Takala-Harrison *et al.* (81). In a genome wide association study they showed that two alleles at SNPs MAL13-1718319 and MAL10-688956 on chromosome 13 and 10 were strongly associated with clearance rate in Western Cambodia (82). Nevertheless, the puzzle of finding definitive molecular markers of artemisinin resistance remained.

In 2014, Ariey and colleagues reported an in vitro experiment in which they cultured an artemisinin-resistant parasite line from the F32-Tanzania artemisinin sensitive clone under artemisinin drug pressure for five years (83). Using a whole genome sequencing approach, they successfully identified eight single-nucleotide mutations in seven genes, including one (M476I in the propeller domain of the *PF3D7_1343700* kelch gene on chromosome 13. Validation in artemisinin resistant Cambodian samples revealed 17 different SNPs in *PF3D7_1343700*, with C580Y the most prevalent (83). Straimer *et al.* conducted transfection studies in which they genetically modified the *P. falciparum* kelch13 locus using Zinc-finger nucleases and assessed impact using a ring stage survival assay (0-3hr) (84). Introducing common kelch13 propeller mutations had a major impact on ring stage survival in laboratory strains, and even more so in 'sensitive' Cambodian field isolates – suggesting an additional contribution from

currently unknown genetic factors. A genome-wide association analysis carried out by Miotto *et al.* identified, in addition to kelch13, artemisinin resistance-associated nonsynonymous polymorphisms in the *fd* (ferreodoxin), *arps10* (apicoplast ribosomal protein S10), *mdr2* (multidrug resistance protein 2) and *crt* (chloroquine resistance transporter) genes (85)(Figure 1.7). Thus the role of kelch13 propeller mutations in conferring artemisinin resistance has been proven, though other known and unknown genetic polymorphisms may contribute to the artemisinin resistance phenotype. *Pf* kelch13 propeller polymorphisms can be used as molecular markers for tracking artemisinin resistance falciparum malaria and made possible the countrywide molecular survey of artemisinin resistance in Myanmar carried out in this thesis.

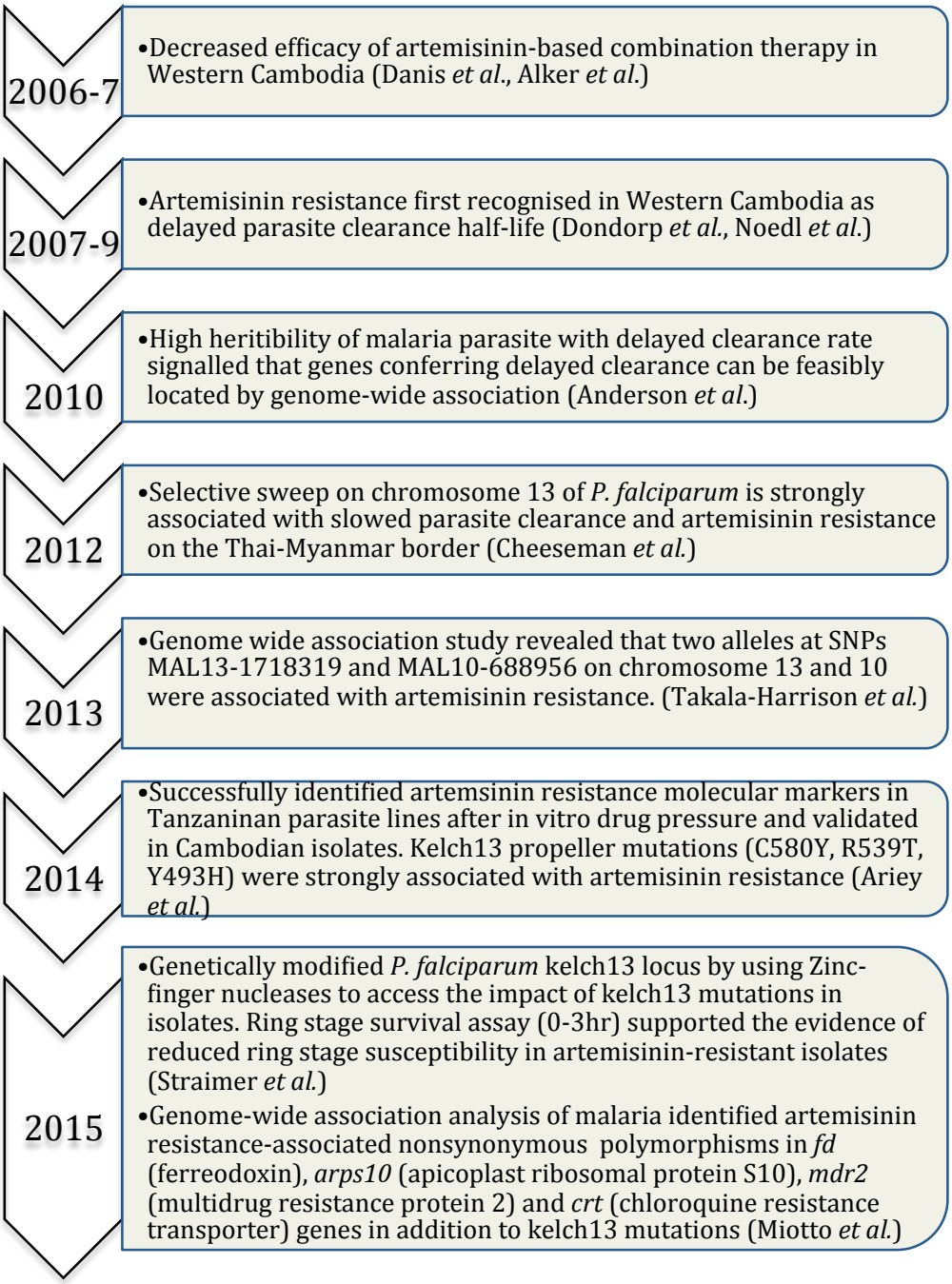


Figure 1.7 Milestones in the search for artemisinin resistance molecular markers

1.11.1 *Pf*kelch13 gene

The kelch13 gene encodes a protein, homologous to the human KEAP 1 protein (Kelch-like ECH associated protein), containing 726 amino acids and composed of three domains, namely, N-terminal (plasmodium specific sequence), BTB/POZ (BTB- Broad-Complex, Tramtrack and Bric a brac and POZ- Poxvirus and Zinc finger) and six C terminal propeller domains (86) (Figure 1.8). There is no functional motif on the plasmodium specific sequence (87). The BTB/POZ domain consists of 120 amino acids, is highly conserved throughout metazoan evolution, and is thought to act as a specific protein-protein interaction interface (88). The kelch propeller motif is found in most organisms and contains 44-56 amino acids, characterized by single four-stranded (β_1-β_4) antiparallel β sheets (89, 90). The exact function of the kelch motif in falciparum has not been fully elucidated, though in other organisms it is involved in a wide variety of protein-protein interactions (87).

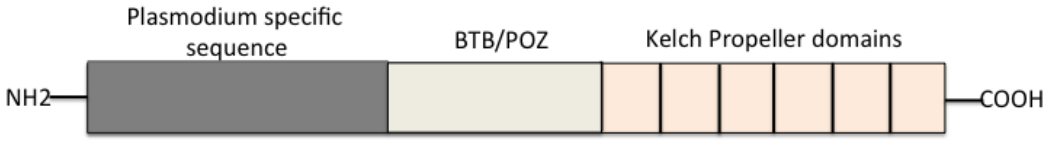


Figure 1.8 Schematic diagram of *Plasmodium falciparum* kelch13 gene (BTB-Broad-Complex, Tramtrack and Bric a brac and POZ- Poxvirus and Zinc finger) (83)

1.11.2 Artemisinin and its derivatives

Artemisinin (qinghaosu) and its derivatives are crucial components of the currently recommended first line treatment of malaria. Artemisinins kill asexual as well as having an effect on immature sexual stages of *P. falciparum*. However the utility of the artemisinins is threatened by drug resistance parasites in Southeast Asia. The WHO redefined current status of artemisinin resistance into suspected and confirmed. The suspected artemisinin resistance defined as “a high prevalence of the delayed parasite clearance phenotype, or high prevalence of kelch13 mutants” and the confirmed artemisinin resistance defined as “a combination of delayed parasite clearance and kelch13 resistance-associated mutations in a single patient” (60). Artemisinin resistance was first recognised in 2007-9 in Western Cambodia (62) and then appeared in northwest of Thailand (91, 92). Resistance has emerged or spread throughout in Cambodia, Thailand, Laos, Vietnam and Myanmar (93-99).

1.11.3 Monitoring of artemisinin derivatives by clinical methods

Artemisinin resistance is characterised by slow parasite clearance time or rate following artemisinin treatment (100). Because artemisinin derivatives are given in combination treatment, recrudescence rates are not a very useful indicator for resistance of artemisinin in the presence of partner drug sensitivity (20). *In vivo* measurement of artemisinin resistance requires frequent measurement of parasite counts, from 6 hourly to 8 hourly for the first 24 to 48 hours. By using the worldwide antimalarial resistance network (WWARN) tool parasite the clearance half life can be calculated from serial parasite count data (100). Malaria is a disease mostly occurring in remote and rural settings in Africa, Asia and South

America, and therefore ideally monitoring of drug efficacy should be by simple and affordable methods, such as serial malaria microscopy. The decline of malaria parasites in the peripheral blood can be assessed serially by blood smear, and the parasite clearance time in hours can be a useful indicator of drug efficacy. Nevertheless, the initial parasitaemia and other factors such as host genetics and immunity, type and dosage of antimalarial drugs, and stages of asexual parasites can affect the clearance time. The frequency of sampling of blood smears may affect the calculation of parasite clearance (101). There are errors in counting parasitaemia by microscopy and variation between microscopists. Some other factors may play a role like poor staining, the amount of blood on slide and distribution of parasites within the blood films (100). At the beginning and end of the curve, there are numerous sources of errors as shown in the Figure 1.9. In an effort to standardise methodology across studies and parasite clearance estimator (PCE) was devised to provide consistent estimates of log-parasitaemia over time (100, 102). However, if the initial parasitaemia is too low, the PCE cannot perform well. It has been recommended that initial parasitaemia should be at least 10,000 parasites per μL.

The sampling schedule of blood smears plays an important role in the estimation of parasite clearance half-life (101). The standard recommended schedule is 6 hourly until two consecutive slides are negative, though in many remote clinical settings this is a difficult and punishing schedule for study nurses and research physicians. A modelling approach has been used to determine the optimal sampling design for parasite clearance (101), leading to the proposal that in areas of unknown resistance status, a more intense schedule for the first two days and then daily blood smears until two blood slides are negative. Therefore,

in one of the studies in this thesis (the DP trial) a sampling schedule of blood smears 6 hourly for 48 hours then daily was used in order to get accurate and reliable estimations of clearance half-life.

Artemisinin resistance is currently defined as increased parasite clearance half life, calculated from the slope of the log-linear portion of the log parasite clearance curve, with a 5 hour cut-off value (20). However, the distributions of sensitive and resistance parasite clearance half-lives are bimodal and overlap and a single cut-off value often does not fit well (103). If possible, the drug level of the artemisinin derivative should be measured to ensure that adequate absorption has occurred, but this is not easy to perform.

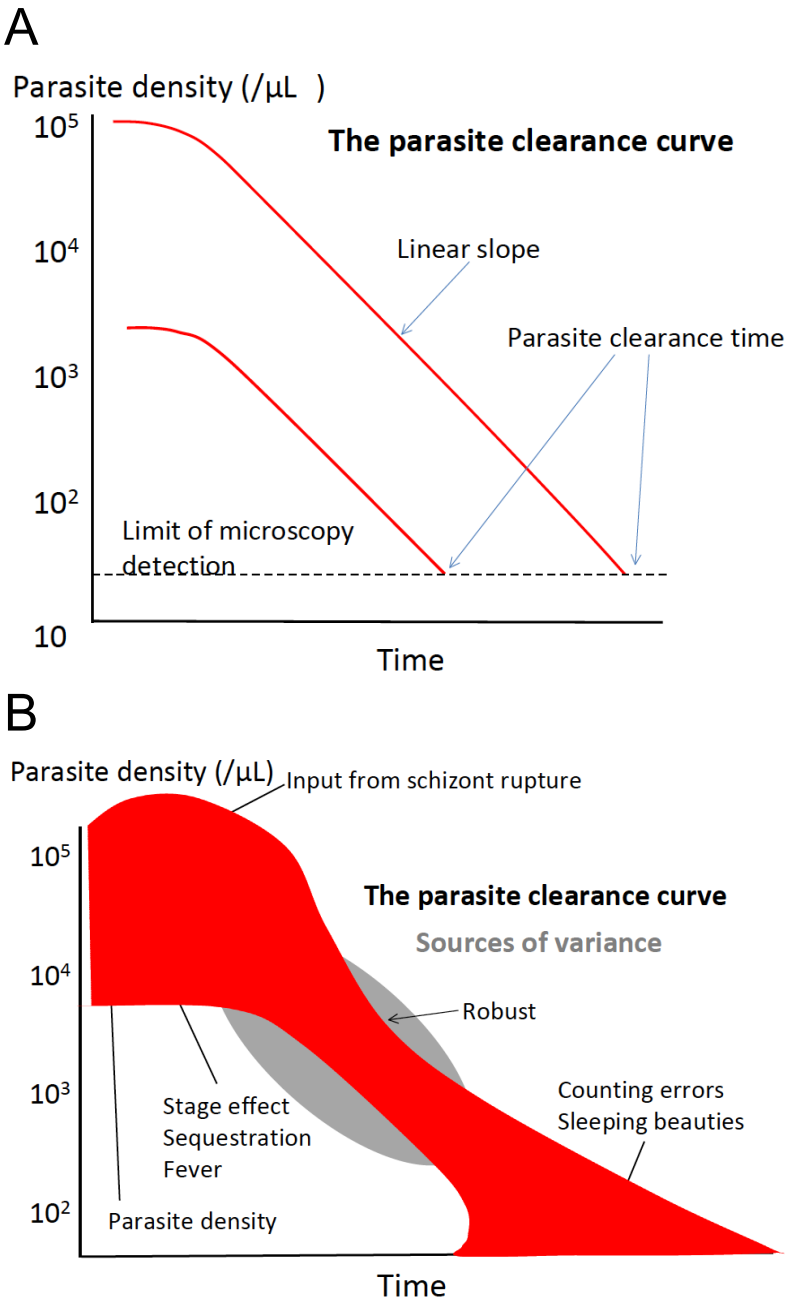


Figure 1.9 The parasite clearance curve showing (A) identical therapeutic responses with dependence of initial parasite count (B) sources of variance in *P. faciparum* (100) (© Malaria Journal 2011, 10:278)

1.11.4 Detection of artemisinin resistance by *in-vitro* and *ex-vivo* methods

In vitro studies are needed for assessing the sensitivity of *Plasmodium falciparum*, and an *ex vivo* method has been developed to identify resistance *P. falciparum* infections in the field. Witkowski and colleagues have described the *in vitro* ring-stage survival assay (RSA 0-3hr), which can differentiate slow from fast clearing parasites in Cambodia. After the first three hours of the ring-stage survival assay, in which parasites are exposed to an 700 nM concentration of dihydroartemisinin, the proportion of viable parasites from slow clearing infections remaining was higher than from fast clearing infections. The RSA 0-3hrs was used to detect and then validate the kelch13 molecular marker (104). The authors have also described an *ex vivo* survival assay which can be done on parasites directly collected from patients in the field.

1.11.5 Monitoring by artemisinin resistance molecular markers

Surveillance of antimalarial drug resistance by using molecular markers in combination with *in vivo* and *in vitro* studies can provide timely and relevant information to policy makers for malaria control and elimination. Improvement in our knowledge of the genetic mechanisms underlying drug resistance can potentially allow early detection of the emergence of resistance. The first genome sequence of *P. falciparum* was reported in 2002, consisting of 14 chromosomes and 5,300 genes (11). Understanding of population genetics of *P. falciparum* can help to identify potential genes related to resistance compared to reference genomes (105-107).

To date, the mechanism of artemisinin resistance is not fully understood. Mok *et al.* (108) carried out transcriptomic analysis of clinical isolates from Southeast Asia and Africa. Clinical isolates were classified into three groups according to parasite clearance half-life, and it was found that increased expression of unfolded protein response pathways is associated with artemisinin resistance. The major PROSC (Plasmodium reactive oxidative stress complex) and TRiC (TCP-1 ring complex) were involved. This experiment suggested that artemisinin resistant parasites remain as young ring-stages for longer ('decelerated development') and are therefore able to survive killing by artemisinin (108). At the same time, Straimer and colleagues demonstrated by transfection using zinc finger nucleases that kelch13 mutations mediated artemisinin resistance. The authors identified that the insertion of a single mutation into kelch13 gene of artemisinin sensitive clone increased RSA survival rates (84). These data also suggested that different types of kelch13 mutations confer different levels of artemisinin resistance. The evidence presented in these two studies supported the hypothesis that kelch13 mutations confer resistance to artemisinin in both laboratory parasite lines and clinical isolates. However additional studies are needed to explore the molecular mechanism of this resistance. A recent study by Mbengue *et al.* (109) reported that *Plasmodium falciparum* phosphatidylinositol-3-kinase (PfPI3K) plays a major role in artemisinin resistance (109). Their studies suggest that PI3K and wild type kelch13 combine together and through the process of polyubiquitination and PI3K degradation result in a low basal level of PI3P (Phosphatidylinositol-3-phosphate)(Figure 1.10). On the other hand, mutations in the propeller region of kelch13 gene decreases polyubiquitination and increases levels of PI3P, resulting in an artemisinin resistant phenotype

through as yet unknown pathways. However, Burrows J (110) argued that this is not a complete picture of the artemisinin resistance puzzle. Mutations in PfPI3K and DHA resistance are not correlated, and further study is needed to answer the problem. Bozdech and colleagues have pointed out that it is crucial to examine PfPI3K-linked mechanisms in the context of other experimental data and to understand the role the PI3K cycle plays in *P. falciparum* blood stage development (111). Future work is warranted to integrate this finding and those of transcroptomic profiling studies.

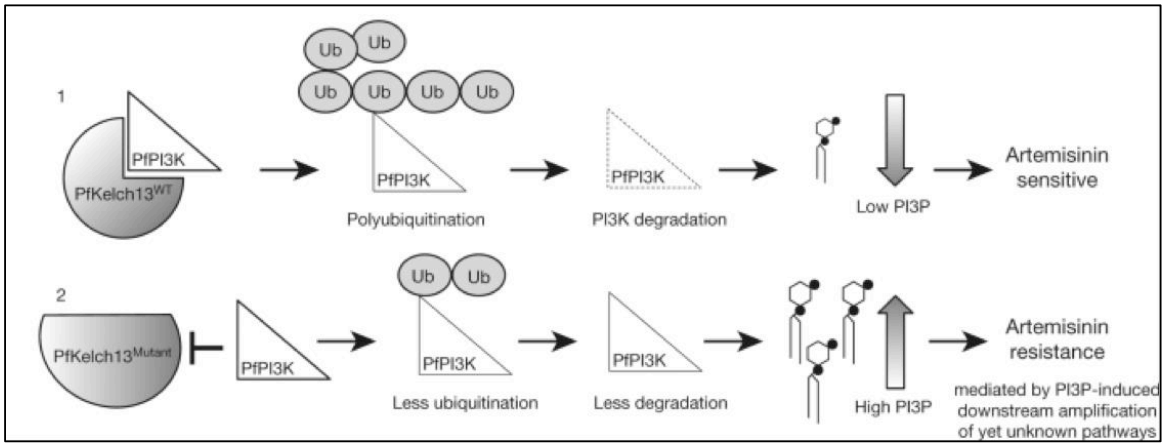


Figure 1.10 A hypothetical model of PI3P-induced artemisinin resistance (109)

1.11.6 Surveillance of *Plasmodium falciparum* kelch13 gene

Table 1.4 The location of *Plasmodium falciparum* kelch13 non-synonymous polymorphisms

No	*Plasmodium falciparum* kelch13 mutations	Geographical distribution	Reference
1	N87K, G112E, E130G, L134P, T149S, A175T, K189T, K189N, R223K, E252Q, R255K, D281V, K438N, P441L, F446I, G449A, N458Y, M476I, A481V, Y493H, S522C, N525D, N537I, G538V, R539T, I543T, P553L, R561H, V568G, P574L, A578S, C580Y, Q613E, F614L, A675V, H719N	**Asia**- Vietnam, Cambodia, Thailand, Myanmar, Bangladesh **Africa**- DR Congo, Nigeria	Ashley *et al.*, 2014(93)
2	P441L, G449A, D452E, N458Y, C469F, M476I, K479I, A481V, V496F, R515T, D516Y, R528T, N537I, R539T, R575K, C580Y	Western and Eastern border, Myanmar	Nyunt *et al.*, 2014(112)
3	A578S	Bangladesh	Mohon *et al.*, 2014(113)
4	K189T, F334L, I465T, L619S, V637D	Uganda	Conrad *et al.*, 2014(114)
5	F446I, N458Y, C469F, A481V, F483S, L492S, F495L, N537I, P553L, E556D, R561H, P574L, A578S, C580Y	Yunnan Province China	Huang *et al.*, 2015 (115)
6	F446I, I511M, N537I, P574L, A676D	Yunnan Province, China	Feng *et al.*, 2015 (116)
7	G533A, S549Y, R561H, A578S	India	Mishra *et al.*, 2015 (117)

Several recent studies have investigated kelch13 mutations in Asia and Africa (Table 1.4)(93, 112-117). Although numerous non-synonymous mutations in the kelch13 propeller domain (starting from amino acid position 441) have been shown to be associated with slow parasite clearance, only one non-synonymous mutation from the stem (BTP/POZ) region, E252Q, has also been associated with resistance (118).

A large multicentre GWAS showed that kelch13 polymorphisms play a major role in determining resistance to artemisinin derivatives in Southeast Asia, but also identified *arps10* (apicoplast ribosomal protein S 10), *fd* (ferredoxin), *mdr2* (multidrug resistance protein 2) and *crt* (chloroquine resistance transporter) as polymorphisms strongly linked to artemisinin resistance (85).

One main question is “have these mutations spread from one geographical location to another location?” Takala-Harrison *et al.* performed a haplotype network analysis on the C580Y mutation and found that it had emerged once in Cambodia and Vietnam and then a second time in Myanmar. In a similar fashion P574Lhas originated once in Vietnam and once in Myanmar. This suggests multiple origins of artemisinin resistance in Southeast Asia (119). Although the number of studies is limited, there is no evidence of artemisinin resistance in Bangladesh and India (93, 113, 117). A therapeutic efficacy study of day 3 parasitaemia and kelch13 typing from 2014 carried out along the western border of Myanmar near to Bangladesh showed relatively few kelch13 mutations (112). For Myanmar the main area of focus for artemisinin resistance is the eastern border with Thailand and China. Several studies have reported that artemisinin resistance has emerged in Thai-Myanmar border (80, 93, 112, 118, 120) and the most prevalent SNPs there were C580Y and E252Q (121). Yunnan province is

one of the few remaining malaria endemic areas in China, and is also part of the Greater Mekong Subregion. Slow parasite clearance was observed there in 2012 (122), and the most prevalent SNPs were F446I in 2015 (115, 116). In addition, Chinese researchers conducted clinical trials in Laiza city in Myanmar (within 100 km from Myitkyina, a capital city of Kachin State) based on a combination of day 3 positivity and subsequent molecular studies (122-124). This study confirmed that artemisinin resistance is present in this region.

1.11.7 Associated and validated *Pf* kelch13 molecular markers for artemisinin resistance

The kelch13 protein comprises a total of 726 amino acids, with the propeller region extending from amino acid position 441 to 726. Recently the WHO published a status report on artemisinin and artemisinin-based combination therapy resistance in September 2015 and listed four “confirmed” and nine “associated” kelch13 mutations (Table 1.5). “Confirmed” kelch13 propeller mutations mean those genotypes confirmed by *in vivo* and *in vitro* studies. “Associated” kelch13 propeller mutations mean those genotypes correlated with delayed parasite clearance (60).

Table 1.5 Associated and validated kelch13 mutation for artemisinin resistance (60)

No.	*kelch13 mutation*	*Classification*
1	P441L	Associated
2	F446I	Associated
3	G449A	Associated
4	N458Y	Associated
5	Y493H	Confirmed
6	R539T	Confirmed
7	I543T	Confirmed
8	P553L	Associated
9	R561H	Associated
10	V568G	Associated
11	P574L	Associated
12	C580Y	Confirmed
13	A675V	Associated

1.11.8 Clinical impact of artemisinin resistance

The main clinical impact of artemisinin resistance is treatment failure after treatment with artemisinin-based combination therapy. The reduced killing of ring-stage parasites of artemisinin causes higher number of parasites to be exposed to the long-acting partner drugs, increases selective pressure on them (20). In Cambodia, the first line drug DP is now failing and WHO and the Cambodian National Malaria Control Programme decided to re-introduce artesunate-mefloquine as first line treatment in Pailin in 2014 (60). It is now clear that piperaquine resistance has emerged on the background of artemisinin resistance (97). Work is ongoing to find piperaquine resistance molecular markers which could be used to track piperaquine resistance in this region.

New drugs to treat falciparum malaria are still in the pipeline, and if partner drug resistance on the back of artemisinin resistance becomes widespread the clinical impact of high malaria treatment failure will be enormous, potentially leading to a disastrous situation where malaria becomes effectively untreatable.

1.11.9 Challenges of artemisinin resistance in Greater Mekong Subregion

Between 2000 and 2013 global malaria control has improved and the total number of cases worldwide has decreased significantly (2). However if the problem of artemisinin resistance is not controlled, this achievement will reverse. Resistance to artemisinin derivatives and other antimalarial drugs were detected first in the Greater Mekong Subregion (GMS) (65). In 2009, the World Health Organization (WHO) initiated a containment project in Cambodia and Thailand. One year later Myanmar and Vietnam were also included in the containment plan and WHO defined zones for containment activities (125). It was argued that containing the problem in the region would prevent the spread of the problem to Africa which already suffers from high malaria transmission and 90% of the world's malaria-associate mortality. The final goal must be elimination of resistant parasite pool by any and all means (95, 126). One of the important threats to containment is malaria importation by air travel. Frequent travel between China and Africa for business purposes and also soldiers from Cambodia travelling to Africa for peace keeping activities raises possibility of transfer of resistant malaria parasites between continents (125, 127). In the GMS, malaria burden remains high in border areas, remote and hard-to-reach locations and among migrant and minority ethnic groups (128).

Table 1.6 Summary of artemisinin resistance in GMS countries (updated on September 2015) (60)

Country	ART resistance suspected year of emergence	ART resistance detected	Activities of containment started	First line treatment
Cambodia	2001	2006	2009	AS-MQ
Lao PDR	2013	2013	2014	AL
Myanmar	2001	2008	2011	AL, AS-MQ, DHA-PPQ
Thailand	2001	2008	2009	DHA-PPQ
Viet Nam	2009	2009	2011	DHA-PPQ

ART, artemisinin; AL, artemether-lumefantrine; AS-MQ, artesunate-mefloquine; DHA-PPQ, dihydroartemisinin-piperaquine; PDR, People's Democratic Republic

1.11.10 Status of artemisinin resistance in Myanmar

In the Greater Mekong Subregion, Myanmar is the last battleground from the public health perspective for preventing the spread of artemisinin resistance from Southeast Asia to Africa along the route taken historically by chloroquine and then pyrimethamine resistance (49). According to WHO, artemisinin resistance probably emerged at the Thai-Myanmar border in 2001 but was not recognized until 2008 (60, 62). Artemisinin resistance has steadily worsened along the Myanmar-Thailand border (91, 94) where the kelch13 C580Y mutation now predominates. In southern Myanmar the median parasite clearance half-life of uncomplicated falciparum malaria has been reported to be 4.8 hours, highlighting the emergence of artemisinin resistance as an important problem in Myanmar (129). A multicentre study on tracking artemisinin resistance collaboration (TRAC) was conducted in Shwe Kyin, Bago region between 2011 and 2013

showed that 14% of patients had a parasite clearance half-life of more than 5 hours and 26% of patients had kelch13 propeller mutations (93). In 2014, Takala-Harrison and others reported that independent mutations have emerged in Myanmar based on kelch13 gene haplotype network analysis (119). Therapeutic efficacy studies (TES) on first line antimalarial drugs were conducted between 2005 and 2014 with the help of WHO in different areas of Myanmar. The TES study design emphasises day 3 parasitaemia and treatment failure after 28 or 42 days of follow up depending on the half-life of partner drug (65). Therapeutic efficacy studies for artemether-lumefantrine were conducted in Myanmar at four sites between 2009 and 2010 and demonstrated treatment efficacy ranged from 94 to 100% (130). TES for dihydroartemisinin-piperaquine were conducted at six sites between 2009 and 2011 and treatment efficacy ranged from 95 to 100% (130). However, in Kawthaung in the southern part of Myanmar, PCR confirmed treatment failure with AL and DP were approximately six percent and in Ranong (Thailand), located across the border immediately adjacent to Kawthaung, treatment failure with artesunate-mefloquine increased to >10%. Concerns over these findings led to initiation of the Myanmar Artemisinin Resistance Containment Project (MARC) in 2011 (130, 131). As shown in figure 1.11, WHO divided areas of the country into three tiers. However the actual situation on the ground regarding artemisinin resistance is largely unknown because of a lack of funding for malaria research. The MARC project aims to delay the artemisinin-resistant parasites within Myanmar and the policy makers need to know current situation of therapeutic efficacy of first line drugs for uncomplicated falciparum malaria and also molecular epidemiology of artemisinin resistance in Myanmar. It

was in response to this situation that I planned the two clinical trials on AL and DP and countrywide molecular survey reported in this thesis.

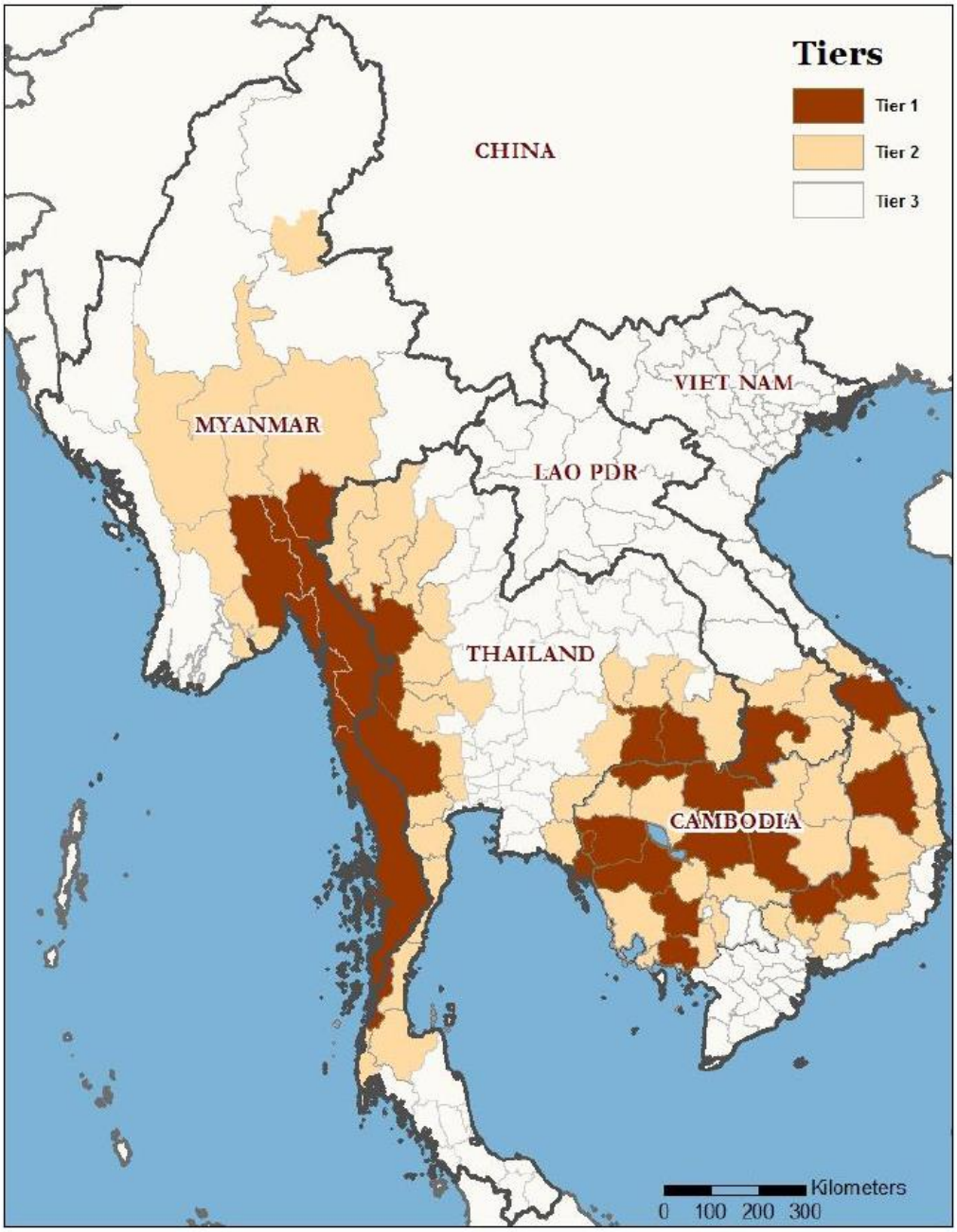


Figure 1.11 Artemisinin resistance containment tier map of Greater Mekong Subregion.

© World Health Organization, Global Malaria Programme Status report on artemisinin resistance September 2014 (page 8) (132)

2 Patients and Methods

2.1 Expansion of artemisinin-resistant *Plasmodium falciparum* in Myanmar

2.1.1 Study sites for molecular survey

Malaria is a major public health problem in Myanmar, but as in most developing countries malaria is under-reported and the available epidemiological data are incomplete. Myanmar consists administratively of fourteen states and regions and one Union Territory (the capital, Naypyitaw). Very few molecular studies related to artemisinin resistance malaria have been conducted in Myanmar to date. To assess the extent of artemisinin resistant malaria in Myanmar I carried out a survey molecular markers of resistance in the ten of these 15 administrative areas, focusing on the areas where malaria is most endemic (omitting only Ayeyarwaddy, Yangon, Magwe and Tanintharyi regions). In particular the survey included the regions bordering Thailand and Bangladesh (Figure 2.1).

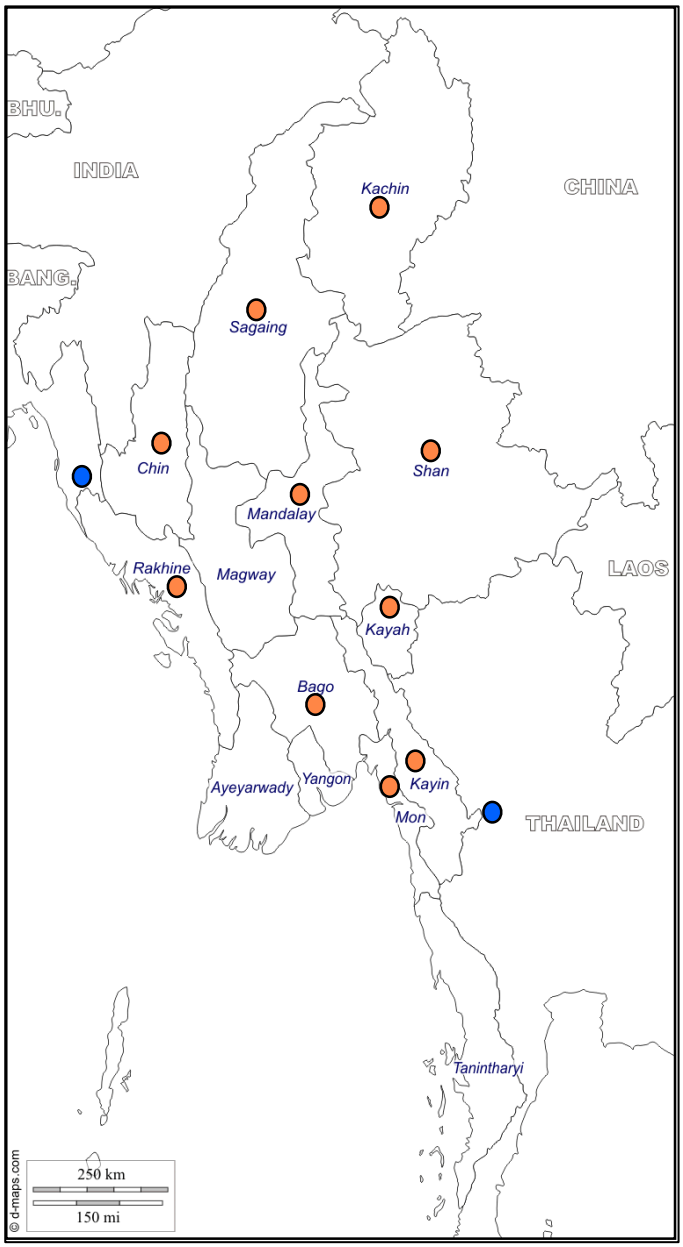


Figure 2.1 Map showing study sites in the molecular survey. Orange circles signify the Myanmar administrative regions where the study was conducted, and the blue circles represent study sites in neighbouring countries (Thailand and Bangladesh).

2.1.2 Study design and patients

Samples for *P. falciparum* kelch13 genotyping were obtained from patients presenting to malaria treatment centres with fever who had falciparum malaria confirmed by blood smear or rapid diagnostic test (RDT) between August 2013 and September 2014.

For patients being managed by routine care, used RDTs were retained anonymously for later *Plasmodium* DNA extraction. Each RDT was stored in separate plastic zip log bag with desiccant at site and then sent to the molecular malariology laboratory at MORU in Bangkok, Thailand.

For patients enrolled in prospective clinical studies, either whole blood samples or filter paper dried blood spots (DBS) were collected after obtaining fully informed consent for the relevant study. In addition we tested whole blood samples being collected as part of ongoing epidemiological studies in villages and camps on either side of the Thai-Myanmar border (in Tak province and Kayin State) over the period January - December 2013 and hospital-based studies in Ramu, Bangladesh undertaken between May and September 2014. In a small subset of these patients, both the screening rapid diagnostic test and the whole blood sample were processed to check for concordance. This collection methodology led to unequal distributions of sample types and patient characteristics (e.g. clinical severity, age, sex and pregnant state) across the 55 sites in ten administrative regions in Myanmar, and the two cross-border areas in Thailand and Bangladesh. However these factors were not considered likely to significantly affect the overall prevalence of kelch13 mutations at each site.

2.1.2.1 *Sample size justification*

The expected population proportion of treatment failure rate of artemether-lumefantrine is less than 0.15; the precision assumed 0.10 and the confidence interval of 0.95 and the minimum sample size must be 50 in each administrative region. In line with power calculations made for WHO Therapeutic Efficacy Studies (133), we planned to obtain at least 50 sequences in each administrative region, although we anticipated that this might not be achieved for operational reasons and possible failure to obtain the necessary kelch13 sequence in a specific sample.

2.1.3 Molecular methods

DNA was extracted from dried blood spots (DBS), used rapid diagnostic test strips (RDT) (both stored desiccated at room temperature) and frozen whole blood samples by standard methods (93).

2.1.3.1 *DNA extraction procedure from used RDTs*

DNA was extracted successfully from used RDTs (134, 135) using the protocol produced by the WWARN Molecular module (136). DNA extraction was only attempted from RDTs which read as positive by the manufacturers' instructions.

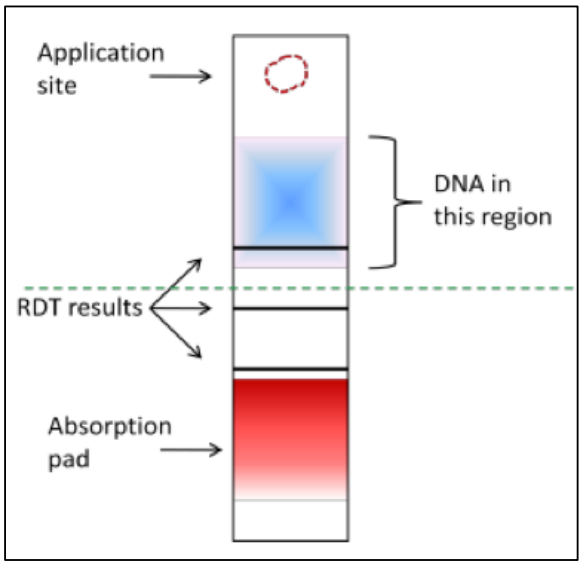


Figure 2.2 Migration of DNA and proteins from rapid diagnostic tests

(Image source: wwarn.org)

The cassette was opened using sterile scissors and the nitrocellose strip was removed. The area of the strip indicated in Figure 2.2 was cut into small pieces and placed in a 1.5 ml microcentrifuge tube. 100 µl of nuclease free water was added and mixed by vortex for 10 seconds. Then the tube was centrifuged and heated at 95°C for 10 minutes. After incubation, the tube was centrifuged for 5 minutes at 14,000 rpm (20000 x g) and the supernatant was transferred to a 1.5 ml DNA LoBind tube. The sample was kept at minus 20°C until used for the next step.

2.1.3.2 *DNA extraction procedure from dry blood spot (DBS)*

At the study sites blood samples were collected on Whatman® 3 MM filter paper, stored at room temperature in separate ziplock bags with dessicant, and transported to the molecular malariology laboratory in Bangkok. For DNA extraction we used the QIAamp DNA Mini kit in accordance with the WWARN molecular testing for malaria standard operating procedure (137). The dried

blood spots were cut into 3mm x 3mm pieces of and placed in a 1.5 microcentrifuge tube. 180 µl of buffer ATL solution (a tissue lysis buffer for use in purification of nucleic acids from QIAGEN) was added to the microcentrifuge tube containing the sample and incubated at 85°C for 10 minutes. After incubation, the tube was centrifuged to remove drops from inside the lid.

Then 20 µl of proteinase K stock solution was added and mixed by vortexing for 15 seconds and incubated at 56°C for one hour. 200 µl of buffer AL (a commercial buffer solution from QIAGEN containing chaotropic salt) was added to each sample and mixed by vortex and incubated at 70°C for 10 minutes. The tube was centrifuged to remove drops from inside the lid.

Next 200 µl of absolute ethanol was added and the tube centrifuged again. Then QIAamp Mini spin columns were labelled with appropriate sample numbers and placed each spin column was placed on a 2 mL collection tube. Each sample mixture was put into the appropriately labelled QIAamp mini spin column using a 1000µl pipette and the spin tube/collection combination centrifuged at 6000 x g (8000 rpm) for 1 min. The tube containing the eluate was discarded. The spin column was placed into a clean 2 ml collection tube and 500µl buffer AW1 (wash buffer) was added and the spin tube/collection combination centrifuged at 6000 x g (8000 rpm) for one minute. The tube containing the eluate was discarded. The spin column was placed into a clean 2 ml collection tube and 500 µl of buffer AW2 (wash buffer) was added and the spin tube/collection combination centrifuged at 20,000 x g (14,000 rpm) for three minutes. The tube containing the eluate was discarded.

The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 150 µl of buffer AE (elution buffer) was added. The spin tube/microcentrifuge

tube combination was centrifuged again at 6000 x g (8000 rpm) for one minute and the spin column was discarded. The microcentrifuge tube containing the extracted DNA was then used for the purification/sequencing process outlined in 1.1.3.4 below.

2.1.3.3 *DNA extraction procedure from whole blood*

We collected 3 ml whole blood samples into EDTA tubes at the study sites. The blood samples were centrifuged and packed red blood cells kept and stored at minus 20°C. At the molecular malariology laboratory, DNA was extracted by using QIAamp Blood Mini kit. 20 µl of QIAGEN protease and 200 µl of sample were added to a 1.5 ml microcentrifuge tube. Then 200 µl of Buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds and incubated at 56°C for 10 minutes. The tube was placed in and briefly spun in the microcentrifuge to remove drops from the inside of the lid. Next 200 µl ethanol was added and mixed again by pulse-vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was centrifuged briefly and the mixture transferred to a QIAamp mini spin column and spun at 6000x g (8000 rpm) for one minute. The QIAamp Mini spin column was placed into a clean 2 ml collection tube and the tube containing the filtrate discarded. Then 500µl Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for one minute. After that 500 µl of Buffer AW2 was added and centrifuged at 20,000 x g (14,000 rpm) for three minutes. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl of Buffer AE was added. The sample was centrifuged again at 6000 x g (8000 rpm) for one minute. The microcentrifuge tube containing the extracted DNA was then used for the purification/sequencing process outlined in 1.1.3.4 below.

2.1.3.4 *Sequencing of the Plasmodium falciparum kelch13 gene (PF3D7_1343700)*

Eluted genomic DNA samples were quantified by PicoGreen analysis and quantitative real-time PCR using the Applied Biosystems StepOne RT-PCR system and frozen at minus 80°C. Samples with more than 50ng DNA and less than 80% human DNA contamination were submitted to the Wellcome Trust Sanger Institute's malaria Illumina sequencing (Illumina Genome Analyzer II) pipeline. Definition of single nucleotide polymorphisms (SNPs) was based on analytical approaches described elsewhere (107).

Filtering of raw sequence file was adapted to the heteroallelic nature of relevant polymorphisms in the kelch13 gene (83), with calls at SNPs requiring one sequence read for the reference allele and two reads for the alternative allele (three if there were 50 or more total reads covering that position). In addition, potential SNPs where the alternative allele was not supported by at least 5 reads in at least one sample, SNPs that were not biallelic, and polymorphisms in the kelch13 low-complexity region (corresponding to amino acid positions 133-143) were not included. Because of the complex effect on phenotype, samples with heterozygosity at one or more positions were also excluded from genotype-phenotype analyses, as were samples with missing data at any of the kelch13 SNP positions. Samples not meeting the Illumina sequencing entry criteria were sequenced by standard dideoxy sequencing of PCR products.

These were obtained by nested PCR, the initial PCR (nest 1) amplifying the whole gene (spanning 2,283 bases) and three nested PCR reactions (nest 2,

fragments a-c) each amplifying a fragment (approximately 840 – 940 bases) of the kelch13 gene. PCR reaction conditions consisted of a final reaction volume of 100 µl containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 2mM MgCl2 (3mM for fragment c), 250µM 4-deoxynucleotide triphosphate (dNTPs), 250nM oligonucleotide primers (see Table 2.1), 2µl for whole blood sample and 5 µl for dried blood spot sample (of each genomic DNA template), and 0.4 units Platinum®Taq DNA polymerase (Invitrogen®, USA). The cycling parameters were pre-denaturation 95°C for 5 min, followed by 25 (nest 1) or 35 (nest 2) PCR cycles involving denaturation at 94°C for 1 min, annealing at 58°C for 2 minutes and extension at 72°C for 2 minutes, with post-extension at 72°C for 7 min, using a MyCyclerTM thermal cycler (Bio-Rad Laboratories, U.S.A.).

After this step, PCR products were purified. 100 µl of PCR product was transferred to a microcentrifuge tube and 5 volumes of FADF Buffer added and mixed by vortexing. The FADF column was placed into a collection tube and the sample mixture was transferred to the FADF column and centrifuged for 30 seconds. 750 µl of wash Buffer was added to the FADF column and centrifuged for 30 seconds and then the flow-through was discarded. The mixture was centrifuged again for an additional 3 minutes and the FADF column placed in a new microcentrifuge tube. A total of 35 µl of elution Buffer was added to the FADF column and after standing for 2 minutes the DNA was eluted by centrifugation for 2 minutes..

Purified PCR products were sequenced at Macrogen, Republic of Korea and analysed by ImageLab (BioRad Gel doc XR) using the 3D7 kelch13 sequence as reference (Accession: XM_001350122.1).

Relevant primers derived from the kelch13 gene sequence were used to amplify the full kelch13 open-reading frame using a nested PCR protocol (93) with the aim of describing the complete sequence from amino acid 210 onwards (the conserved part of the protein). Heterozygous results were considered as mutations.

Table 2.1 PCR primers for kelch13 genotyping

Reaction	*Fragment*	*Primer name*	*Sequence (5'>3')*	*Product (bp)*
Nest 1	Whole gene	K13_c.1F	TGGAAGGAGAAAAAGTAAAAACAAAA	2283
		K13_c.2283R	TGTGCATGAAAATAAATATTAAAGAAG	
Nest 2	Fragment a	K13_c.1F	TGGAAGGAGAAAAAGTAAAAACAAAA	840
		K13_c.840R	TTGTACAATCGTACTCTTTCCATTTC	
Nest 2	Fragment b	K13_c.614F	TTGAAACGGAATTAAGTGATGC	851
		K13_c.1464R	CAATACAGCACTTCCAAAATAAGC	
Nest 2	Fragment c	K13_c.1344F	AGGTGGATTTGATGGTGTAGAA	940
		K13_c.2283R	TGTGCATGAAAATAAATATTAAAGAAG	

2.1.4 Geospatial mapping

See details in appendix A.

2.2 *In vivo* artemisinin resistance in patients with uncomplicated falciparum malaria treated with Dihydroartemisinin-piperaquine in Myanmar (DP Trial)

2.2.1 Study sites

With the spread of the artemisinin resistance phenotype in the Greater Mekong Subregion, it was crucial to determine the extent and nature of resistance within Myanmar, as well as the efficacy in terms of cure rate of existing ACTs. The TRAC 1 study was carried out in Bago and Pyin Oo Lwin districts of Lower and Central Myanmar (93), along with a number of WHO therapeutic efficacy studies carried out in some border areas, but few assessments have been made recently in Upper Myanmar (138). Therefore I planned and coordinated a clinical trial on uncomplicated falciparum malaria with dihydroartemisinin-piperaquine in Myitkyina, Kachin state (100 km from China-Myanmar border) and in Thabeikkyin, Mandalay region (Figure 2.4). The two sites are situated in areas of low entomological inoculation rate (EIR<1) (139).

2.2.2 Study design

We performed an open-label clinical trial in two hospitals located in Upper Myanmar. The recruitment phase of the study started in August 2013 and ended in December 2014.

2.2.2.1 *Sample size justification*

The sample size was calculated following WHO 2009 guidelines for single-arm efficacy studies (140). A target sample size of 73 enables detection of a 5% failure rate of dihydroartemisinin-piperaquine with 95% confidence and 5% precision. An additional 10% will be included to allow for losses to follow up making a total of 80 patients. This number will also enable the distribution of parasite clearance half-lives to be described adequately. Assuming that the geometric mean of the clearance rates in a "sensitive" parasite population is 0.2 from historical data, with 50 (or 100) patients we will be able to show that the clearance rate is significantly reduced if the true value of the clearance rate was 0.157, 0.164, 0.171 (0.169, 0.174, 0.179) for SDs equal to 0.55, 0.5, 0.4 of the log-transformed values, respectively (PASS 2005).

Figure 2.3 Study sites of the DP trial in Upper Myanmar

2.2.3 Study participants

Male and non-pregnant female patients aged between 6 months and 65 years with acute uncomplicated falciparum malaria were the target study population. Inclusion criteria were acute uncomplicated *P. falciparum* malaria, confirmed by positive blood smear with asexual forms of *P. falciparum* (including mixed infection with non-falciparum species), parasitaemia with a parasite count between 5,000 and 200,000 per cubic millimeter determined on a thin or thick blood film and fever defined as ≥ 37.5°C tympanic temperature or a history of fever. The exclusion criteria were signs of severe and/or complicated malaria according to WHO criteria (141), haematocrit less than 25% or haemoglobin (Hb) less than 8 g/dL at enrolment, acute illness other than malaria requiring treatment, patients who received artemisinin derivatives within the previous seven days, history of allergy to artemisinins or to dihydroartemisinin-piperaquine, and previous splenectomy.

2.2.4 Drug Therapy

Dihydroartemisinin-piperaquine (Duo-Cotecxin®) was purchased from Beijing Holley-Cotec Pharmaceuticals, and primaquine from Remedica Pharmaceuticals was provided by the 3MDG Fund. Patients received dihydroartemisinin-piperaquine (DP) administered orally with a target dose of 2 mg/kg of dihydroartemisinin per day and 16 mg/kg of piperaquine per day, for three days. All patients received Primaquine 0.25 mg/kg single dose on Day 0 after the first dose of dihydroartemisinin-piperaquine. One tablet of Duo-cotecxin® contains 40 mg dihydroartemisinin and 320 mg piperaquine. One tablet of primaquine contains 7.5 mg. Dosing was adjusted by weight categories (Appendix C). The

treatment was administered by study medical or nursing staff while the patients were hospitalised. If the patient vomited within half an hour after taking the antimalarial drugs, the dose was repeated. If vomiting occurred between half and one hour, half of the dose was repeated. Repeat doses were recorded on the Case Report Form (CRF).

2.2.5 Rescue treatment

The indications for rescue treatment were:

- The development of any danger signs or signs of severe malaria at any point.
- A parasitaemia rise after 12 hours, e.g. the parasite count at 18 hours is greater than the 12 hour count.

Rescue treatment consisted of parenteral artesunate, 2.4 mg/kg STAT, followed by parenteral artesunate 2.4 milligram per kilogram at 12 hours and 24 hours and then daily until able to take oral medication. Patients with persistent asexual parasitaemia on day 7 or who developed a recurrent parasitaemia on day 14 or beyond with no signs of severity were treated with either another ACT or a different combination e.g. quinine and either doxycycline or clindamycin. A patient who developed a non-falciparum parasitaemia during follow up was treated according to national guidelines.

2.2.6 Study endpoints

2.2.6.1 *Primary endpoint*

The parasite clearance rate as defined by the slope of the linear portion of the natural logarithm parasite clearance curve and expressed as the parasite clearance half-life.

2.2.6.2 *Secondary endpoints*

- Fever clearance time (i.e. the time taken for the tympanic temperature to fall below 37.5˚C and remain there for at least 24 hours)
- Proportion of patients with gametocytaemia before, during and after treatment, assessed at admission, on days 3, 7 and then once weekly, stratified by presence of gametocytes at enrolment
- Parasite clearance below the microscopic level of detection using molecular methods
- PfKelch13 molecular markers of drug resistance
- Efficacy at day 42 (corrected by PCR genotyping)
- Proportion of patients with a positive malaria smear 72 hours after treatment
- Day 7 piperaquine concentration

2.2.7 Study Procedures

2.2.7.1 *Informed Consent*

The patient information sheet was written in Myanmar and local languages, was presented to all participants, and detailed not less than: the exact nature of the study; the implications and constraints of the protocol; and the known side effects and any risks involved in taking part. It clearly stated that participation is voluntary and that the participant or guardian is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to provide a reason for withdrawal.

Patients or parents or guardians of minors who were unable to read or write provided informed consent with a witness present. The patient or guardian had the opportunity to talk with the investigator to decide whether they would (or allow their relative to) participate in the study. If the patients were older than 10 years and under 18 years of age assent was also obtained. The rest of the patients provided written informed consent to enroll in the study. A copy of the signed informed consent/assent document(s) was given to the participants. The informed consent process was as simple and quick as possible because the patients were ill. It was clearly stated and verbally explained that the participant was free to withdraw from the study at any time, for any reason, without interfering with their medical care.

2.2.7.2 *Screening, eligibility and assessments*

Patients presenting to Myitkyina and Thabeikkyin hospitals were screened to assess eligibility. Full consent was obtained before any enrolment procedures were conducted. It was explained that refusal to participate in the study would not jeopardize subsequent antimalarial treatment. A screening log was kept. A full medical history was obtained and recorded in standard CRFs by the research physicians.

2.2.7.3 *Screening physical examination and laboratory tests*

A research physician conducted physical examination at enrolment and recorded all findings in the CRF. Screening blood smears, haematocrit and urine pregnancy tests for females of child bearing potential were performed.

2.2.7.4 *Randomisation and blinding*

This study was an open label, non-randomised, single arm study so blinding of investigators or participants was not applicable.

2.2.7.5 *On Admission*

Patients were admitted to hospital for at least three days for supervised treatment and six-hourly blood smears. Numbers of parasites were counted by blood smear obtained at hour 0 and then every 6 hours up to 48 hours and daily thereafter until two negative consecutive slides were observed. A venous blood sample (5 mL) was collected in a heparinized tube at hour 0 for uqPCR and kelch13 gene sequencing. A venous blood sample (0.5 µL) was collected on filter paper (Whatman® FTA cards) for PCR genotyping (MSP1, MSP2, GLURP in case of recurrence).

2.2.7.6 *Follow up assessments*

Tympanic temperature and haematocrit were measured at the time of malaria smears every 6 hours up to 48 hours and then every 12 hours until discharge from hospital. After patients were discharged from hospital, follow up assessments were performed at days 5, 7, 9, 14, 21 for malaria blood smear, hematocrit, and uqPCR. A dry blood spot was collected on day 7 for piperaquine drug level with Whatman® 3MM cellulose chromatography paper. Thereafter, patients were assessed weekly until day 42 when samples were taken for malaria blood smear and haematocrit at the clinic. If a patient could not come to the clinic, then research staff followed him/her up at his/her home.

The time window for the visit on day 7 was plus 3 days and for the visits on days 14 to 42 was minus 1 to plus 3 days.

The trial was monitored by the Mahidol-Oxford Tropical Medicine Research Unit Clinical Trial Support Group in collaboration with the Myanmar Oxford Clinical Research Unit. Data were evaluated for compliance with the protocol and accuracy in relation to source documents.

2.2.7.7 *Amendments submitted to Ethical Review Committees*

After the first malaria season, the study team analysed uqPCR samples for preliminary analysis. It was found that 75 per cent of the patients showed positive parasite DNA on day 9 and therefore we extended our sample collection for uqPCR from day 5 and day 9 to day 14 and day 21. The changes had no negative consequences for the patients and the Ethics Committees approved this amendment.

2.2.8 Microscopy quality control measures and preparation

Thick and thin Giemsa-stained blood smears were prepared at study sites and counted against white blood cells or red blood cells. All blood smears were checked by a trained laboratory technician at each site and all slides at hour 0 and hour 72 were rechecked by experienced microscopists in the Malaria Laboratory of the Mahidol-Oxford Tropical Medicine Research Unit (MORU), Bangkok. The parasite clearance half-life of each patient was calculated by using the WWARN parasite clearance estimator (PCE) online tool (142).

2.2.8.1 *Preparation of thin and thick blood film*

Thin and thick blood films were prepared on the same glass slide. Blood smears were made from a finger prick or from EDTA blood collected from the patients. A micropipette was used if using EDTA blood from a vacutainer tube. An identifying sticker was put on one edge of the slide containing the patient ID number, and

date and hour of sampling. The slide was cleaned with alcohol-soaked cotton and the fingertip side was pricked using a blood lancet. The first drop of blood was removed with a clean piece of gauze and two to three small drops of blood were collected on the slide for the thick blood film. The 2-3 small drops were joined together with the corner of spreader to form a round thick blood film. Only one drop of blood was collected for the thin film. Then it was spread across the slide with a spreader to make the film. After that, the slide was placed on slide folder on a flat surface and left to dry. Once the thick blood film was completely dried, the thin blood film was quickly immersed into methanol for fixation. Then, the slide was placed face upward on a rack above the sink and stained with Giemsa for 15-20 minutes. After staining the slide was gently immersed in clean water for washing. The slide was placed on a slide rack for drying.

2.2.8.2 *Calculation of parasite count*

Calculation of the parasite count was done automatically within the database. The parasite count per µL of blood was derived as follows: thin film - number of parasitized red cell seen per 1,000 RBC x haematocrit x 125.6; thick film -number of parasites seen per 500 WBC x 16.

2.2.9 Dry blood spot sampling

For PCR genotyping, treated filter paper (Whatman® FTA card) was used to reduce DNA degradation and inhibit microbial growth. The FTA card was labeled before sample collection, using an indelible marker, with the patient ID number, initials, day or hour of follow up, and date and time visit.

EDTA-anticoagulated blood from finger prick or venipuncture was used. Approximately 100 µl of blood was applied to each sample area on the

Whatman® FTA cards by applying the blood in a spiral within the circle printed on the card. The FTA card was dried in the open air away from flies, direct sunlight and dust, and then put into separate zip log plastic bag with silica gel desiccant. The FTA cards were stored in a dry, cool environment in a locked cabinet before shipment.

2.2.10 Sample transport

Blood samples were shipped from the study sites to the Myanmar Oxford Clinical Research Unit (MOCRU) using the commercial company World Courier. Sample packing and transport were performed in compliance with the United Nation committee of Experts/Technical instruction, IATA infectious substances and diagnostic specimens shipping guidelines. Samples were shipped from Yangon to Bangkok (in 2 tranches) by World Courier (classified as biological substances, Division 6.2, Category B) with sufficient dry ice and temperature control in accordance with guidelines.

2.2.11 Source Data

Source documents were the original documents, data reports, and records from which participants' CRF data were obtained. These include, but were not limited to, hospital records (from which medical history and previous and concurrent medication summarized into the CRF), clinical and office charts, laboratory and pharmacy records, diaries, microfiches, radiographs, and CRFs. CRF entries were considered source data if the CRF was the site of the original recording. In this study the CRFs were used as the source document for most of the data points.

All documents were stored safely in confidential conditions in on site lockers at the research clinics at Myitkyina and Thabeikkyin hospitals. On all study-specific documents, other than the signed consent form, the participant were referred to by the patient ID number and initials, not by name.

2.2.12 Safety reporting

This trial involved the administration of drugs licensed for use in Myanmar that have been evaluated extensively in the past. Therefore, the safety aspects of this trial were limited to close observation while patients were in hospital, treating any inter current illnesses or drug-related side effects and recording and reporting only serious adverse events.

2.2.12.1 *Definitions*

2.2.12.1.1 Adverse event (AE)

An adverse event is any untoward medical outcome in a patient undergoing a study related procedure or intervention that is believed to be caused by that study related procedure or intervention. Medical conditions or abnormal laboratory values present at the time of enrolment were considered as baseline and not reported as AEs.

2.2.12.1.2 Serious adverse event (SAE)

A serious adverse event is an AE that:

- results in death
- is life-threatening i.e. the patient was at risk of death at the time of the AE
- requires inpatient hospitalisation or prolongation of existing hospitalisation
- results in persistent or significant disability/incapacity

- is a congenital anomaly/birth defect
- requires acute medical or surgical care to prevent one of the outcomes listed above

2.2.12.2 *Reporting of Serious Adverse Events*

All SAEs were recorded and reported by site physician to the medical monitor, Dr Frank Smithuis, within one day of his or her awareness to the SAE. The medical monitor then reported the SAE to the data safety monitoring board and the Defence Services Medical Ethics Committee (Myanmar) in accordance with local requirements.

2.2.13 Ethics

This study was conducted in compliance with the current revision of the Declaration of Helsinki (Seoul 2008) and according to National Regulations, following the principles of the ICH guidelines for Good Clinical Practice 1996.

2.2.13.1 *Declaration of Helsinki*

As the investigator I ensured that this study was conducted in compliance with the current revision of the Declaration of Helsinki (Seoul 2008).

2.2.13.2 *ICH guidelines for Good Clinical Practice*

I ensured that this study was conducted in accordance with National Regulations and followed the principles of the ICH Guidelines for Good Clinical Practice 1996.

2.2.13.3 *Approvals*

The study protocol and its associated documents were submitted to the Oxford Tropical Research Ethics Committee (OXTREC) (Reference: 06-11) the Defence Services Medical Ethics Committee (Myanmar) (Reference: 03/Ethics/13). We

submitted and, where necessary, obtained approval from the above parties for all substantial amendments to the original approved protocol and documents. This trial was registered at ClinicalTrial.gov (Identifier NCT01350856).

2.2.14 DNA sample preparation

2.2.14.1 *WBC depleted and whole blood samples*

DNA samples were collected for genomic profile studies of the parasite, so host genetic material was removed by using cellulose filtration (CF11)(143). The plunger was removed from a 10 mL syringe and two discs of lens tissue were placed at the bottom of the syringe barrel by using forceps. A total of 2.0 gram loosely packed cellulose powder was added to the syringe barrel and at the same time the lens paper was checked to fully cover the syringe opening. The plunger was inserted and used to pack the cellulose powder to the 5.7 mL mark. Three mL of whole blood was collected from the patient and 3 mL of PBS solution (pH 7.3, without MG^{2+} and Ca^{2+}) was added to this. The cellulose column was suspended over an uncapped 50 mL tube using a clamp and 6 mL of PBS solution was added to the top of the cellulose column. After the PBS was no longer visible at the top of the column, the blood-PBS mixture was applied on the top of the column and allowed to pass through by gravity. When no liquid was visible at the top of the column, 6 mL of PBS was added to the top of the column to wash through the remaining red blood cells and the fluid allowed to run through by gravity. The filtered sample was centrifuged at 1,000 x g at room temperature for 10 minutes to pellet the cells. The supernatant was removed and discarded and the pellet was stored in 3 mL cryovial tube at minus 20°C.

DNA samples were also collected for detection parasites by highly sensitive quantitative polymerase chain reaction method. Three mL of whole blood was collected from the patient and centrifuged at 1,000 x g at room temperature for 10 minutes to pellet the cells. The supernatant was removed and discarded and the pellet stored in 3 mL cryovial tube at minus 20°C.

2.2.15 Molecular studies

2.2.15.1 *DNA extraction*

(same as previous section 2.1.3.3)

2.2.15.2 *Sequencing kelch13 gene*

(same as previous section 2.1.3.4)

2.2.15.3 *Highly sensitive qPCR (uqPCR)*

DNA extraction was done using either QIAgen mini or medi kits (mini kit if sample volume ≤ 400 µL, midi kit if 400-4000 µL). Purified DNA was then concentrated by speed vacuum to 10-40 times the original whole blood concentration (average 66 times for 1,095 samples). The parasite density was assessed by an absolute quantitative real-time PCR (standard curve) method on a Corbett Rotor-Gene Q (Corbett Research, Australia). The 18sRNA *Plasmodium* genus primers, hydrolysis probe, and Quanti-Tect Multiplex PCR (without ROX dye) (Qiagen, Germany; modified from previous protocol) (144) were used. Two µL of concentrated purified DNA was used as the DNA template. This is equivalent to 50-150 µL of whole blood per reaction.

The reference samples used were cultured highly synchronized ring stage 3D7 *P. falciparum* parasites. The parasite density was measured by microscopy and by flow cytometry. Ten fold serial dilutions from 20,000,000 to 20 parasites per mL

(i.e. 7 points) were made with double distilled water. These gave linear standard curves R^2> 0.98, with a slope value of -3.322 and amplification efficiency of 90 to 105%. Using highly concentrated DNA template allowed limits of detection down to 20 parasites in 1000 µL of whole blood. The specificity of uqPCR was tested against uninfected healthy volunteer blood (100 samples). In addition definitely negative blood samples (~5% of total) were added to survey batches, to which those conducting the uqPCR assays were blinded.

Genome parasite density analysis was derived from the sample Ct value against the standard curve using the Rotor-Gene Q series software v 2.0.2. The cut off Ct value was 40. The parasite density results were then derived from the parasite DNA concentration.

If the uqPCR was positive then species detection was attempted using nested PCR methods specific for both *P. falciparum* and *P. vivax* microsatellite markers as described previously (145, 146). Samples for which there was insufficient DNA were reported as of indeterminate species. I calculated the uqPCR result fluorescence-activated cell sorting (FACS) standard curve. If the microscopy result was positive (above detection limit of microscopy) I used parasite count per µL using microscopy at day 0 and day 3, and if the microscopy result was negative I used parasite count per µL using the uqPCR method up to day 21.

2.2.16 Piperaquine drug level

Piperaquine concentrations will be measured in the filter paper blood spots. These assays will be performed by the MORU department of pharmacology using previously validated methods (147, 148), but the results will not form part of this thesis.

2.2.17 Urine tests

Pregnancy tests were carried out on women of child-bearing age to confirm their eligibility for the study.

2.3 The effectiveness and safety of a 3 day versus 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar (3 vs. 5 AL Trial)

2.3.1 Study sites

Kayin state is situated in Southeastern Myanmar and a number of studies along the Myanmar-Thailand border have indicated a high prevalence of kelch13 mutations and a recent decrease in treatment efficacy of uncomplicated falciparum malaria (92, 118, 149). To assess the efficacies of the standard 3 day artemether-lumefantrine regimen and of an extended 5 day regimen we conducted a randomised clinical trial in village health centres in Kyainseikgyi Township, Kayin state, namely, Kyeikdon, Thanpayar and Hpayarthonesu, as shown in Figure 2.5.

2.3.2 Study design

This clinical trial was a randomized, 2 arm, open-label, clinical and parasitological study comparing artemether-lumefantrine (Coartem®) 3 days and 5 days treatment.

2.3.2.1 *Sample size justification*

It was anticipated that there would be a 20% difference in *P. falciparum* positivity at day 5 after 3 day and 5 day ACT treatment (i.e. from 10% to 30% detected by uqPCR method). To detect this difference at 5% significance and 80% statistical power 75 patients needed to be enrolled in each group.

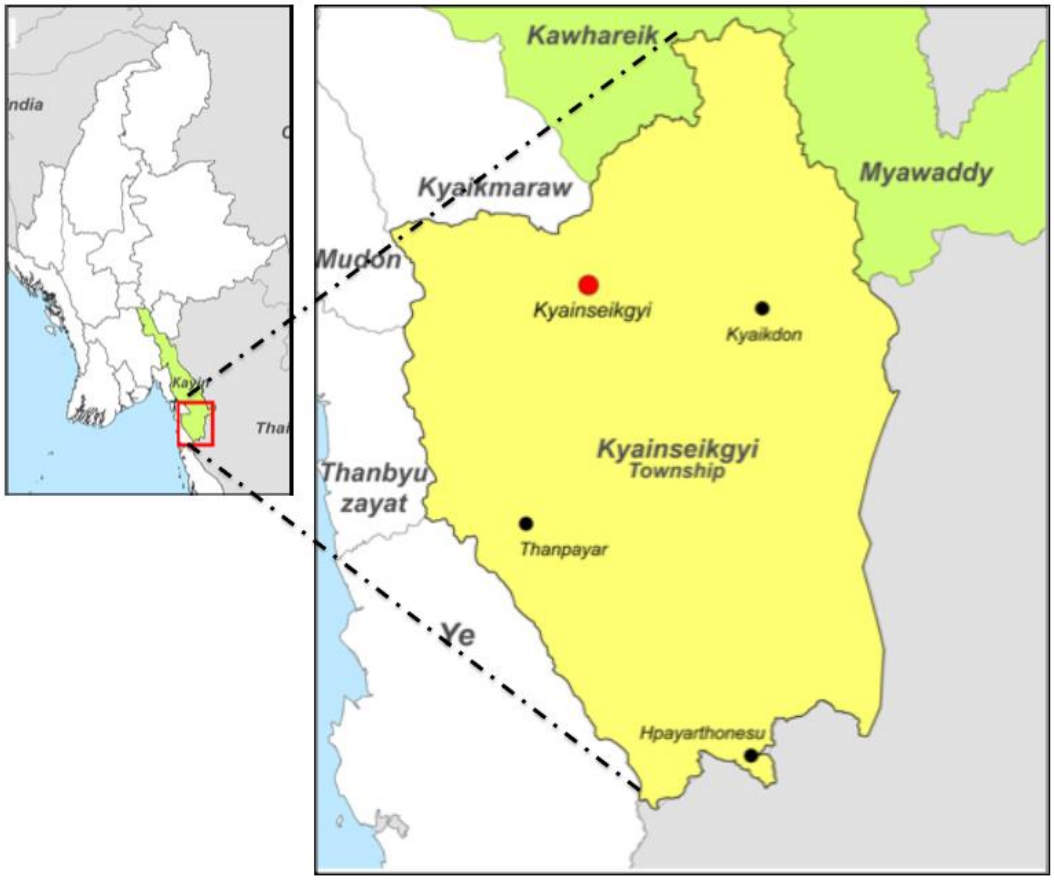


Figure 2.4 Study sites of the 3 vs. 5 AL trial in Kayin state, Myanmar

2.3.3 Study participants

The target study population were males and non-pregnant females aged between 6 and 65 years with acute uncomplicated falciparum or mixed malaria with parasite count between 80 (≥5/500 WBC) and 175,000 per cubic millimeter determined on thick or thin blood film and fever defined as >37.5°C tympanic temperature or a history of fever. Patients with signs of severe and/or complicated malaria according to WHO criteria (20), haemoglobin less than 5 mg/dL at enrolment, patients who had received full course of ACT in the preceding 28 days, known hypersensitivity to artemisinins defined as history of erythroderma/other severe cutaneous reaction, angioedema or anaphylaxis, or previous splenectomy. Females aged between 12 and 18 years were excluded because of cultural sensitivities around pregnancy questioning/testing.

Written informed consent was obtained from the patient or parent/guardian (with impartial witness for illiterate individuals).

2.3.4 Drug Therapy

Artemether-lumefantrine (Coartem®, Novartis, Switzerland) was used in this trial. One tablet contains 20mg artemether and 120mg lumefantrine. The standard regimen is twice daily for 3 days with a delay of at least 8 hours between the first and second dose. It is dosed by weight categories (Appendix C). Patients receiving the 5 day course continued to take the same daily dose for 2 additional days.

A single dose of primaquine (0.25 mg/kg) (Primaquine Phosphate, Remedica, Cyprus) was given to all patients on the first day of treatment for its gametocytocidal activity.

The initial treatment was given under supervision and all other subsequent doses were given to the patient to be taken at home. If the patient vomited within 30 min of the initial treatment, the full dose was repeated. If vomiting occurred after 30 min but within the hour, half the dose was repeated. Patients with persistent vomiting more than twice were withdrawn from the study and given rescue treatment. (Dosing chart - Appendix C)

2.3.5 Rescue Treatment

Rescue treatment consisted of parenteral artesunate at a dose of 2.4 mg/kg STAT, followed by parenteral 2.4 mg/kg at 12 hours and 24 hours and then daily until able to take oral medication. Patients with persistent asexual parasitaemia on day 7 or who developed a recurrent parasitaemia on day 14 or beyond with no signs of severity were treated with either another ACTs or a different combination e.g. quinine and either doxycycline or clindamycin. A patient who developed a non-falciparum parasitaemia during follow up was treated according to national guidelines.

2.3.6 Study Endpoints

The primary endpoint of the study was the proportion of patients with detectable parasitaemia by ultrasensitive PCR on days 5 and 7 after treatment in each treatment arm.

Secondary endpoints were:

- Parasitaemia by ultrasensitive PCR on day 3 in the 3 day arm and day 5 in the 5 day arm, and on days 14 and 21 in both arms.
- Comparison of effectiveness on day 42 uncorrected and corrected by PCR genotyping

- Haematological recovery by day 28 (assessed by haemoglobin %)
- Gametocyte carriage on day 7
- Tolerability of 5 days artemether-lumefantrine

2.3.7 Study Procedures

2.3.7.1 *Informed Consent*

The patient information sheet presented to the participants was written in Myanmar and local Kayin languages. This detailed: the exact nature of the study; the implications and constraints of the protocol; the known side effects and any risks involved in taking part. It was clearly stated that participation is voluntary and that the participant or guardian is free to withdraw the patient from the study at any time for any reason without prejudice to future care, and with no obligation to provide the reason for withdrawal.

Patients or parents/guardians of minors who were unable to read or write provided informed consent with a witness present. The patient or guardian had the opportunity to talk with investigator to decide whether they would (or allow their relative to) participate in the study. If the patient was older than 10 years and under 18 years of age, assent was also obtained. The rest of the patients provided written informed consent to enroll in the study. A copy of the signed informed consent/assent document(s) was given to the participants. The informed consent process was as simple and quick as possible because the patients were ill. It was clearly stated and verbally explained that the participant was free to withdraw from the study at any time, for any reason without interfere to their medical care.

2.3.7.2 *Screening, eligibility and assessments*

Patients presenting to Kyaikdon or Thanpaya research clinics were screened to access eligibility. Full consent was obtained before any enrolment procedures were conducted. It was explained that refusal to participate in the study would not jeopardize subsequent antimalarial treatment. A screening log was kept. A full medical history was obtained and recorded in standard CRFs by the research physicians.

2.3.7.3 *Screening physical examination and laboratory tests*

A research physician conducted physical examination at enrolment and recorded all findings in the CRF. Screening blood smears, haematocrit and urine pregnancy test for women of child bearing potential were performed.

2.3.7.4 *Randomisation and blinding*

From 2013 to 2015, the 3 vs. 5 AL trial was conducted at Kyaikdon and Thanpaya research clinics in Kyainseikgyi township, Kayin state. All enrolled patients were randomised into two groups using a computer-generated sequence in blocks of 12. Study arms were as follows: (Arm 1) 3 day AL, or (Arm 2) 5 day AL. The allocation sequences were computer generated using QuickCalcs online (Graphpad prism software). The allocations were placed in sequentially numbered sealed envelopes which were sent in batches to the study sites. This trial was open label and the investigator and patients were not blinded to the treatment randomisation. However, laboratory staff performing highly sensitive qPCR and molecular markers identification were blind to the treatment allocation.

2.3.7.5 *On admission*

All patients enrolled in the study were given a unique patient number. A case record form was completed for each patient documenting symptoms prior to clinic attendance, concomitant illness, and drug history. Height, weight, vital signs and physical examination findings were recorded. After enrollment, parasite asexual stages and gametocyte counts were determined at day 0, 3, 5, 6 and 7 until blood slides were free of asexual parasites. Thick and thin blood films stained with Giemsa were read and counts expressed as the number of parasites per 500 white blood cells (parasitaemia per µl= count x WBC/500) or as the number of parasitized red blood cells per µl (no. of parasitized red cells seen per 1,000 RBC x HCT x 125.6).

Filter paper blood blots (3 dots on Whatman® 3MM filter paper approx. 180-300 µL blood) for parasite genotyping (MSP1, MSP2, GLURP in case of recurrence during follow-up) were taken on admission. Haemoglobin level was measured on admission and day 28 with a Hemocue® device (HemoCue Hb 301+ System, AB Leo Diagnostics, Helsingborg, Sweden).

2.3.7.6 *Follow up assessments*

All patients were called back for follow up on days 3, 5, 7, 14 and 21. On each visit 3 mL of venous blood was taken into an EDTA tube for parasite DNA quantitation. Samples for lumefantrine concentration measurement were taken on day 7 (3 mL of blood was taken into an EDTA tube). Weekly follow up was done on day 7, 14, 21, 28, 35 and 42 for temperature measurement, physical examination and blood smear. A symptom questionnaire was completed at each visit. Any additional medications taken during the trial period for whatever reason

was documented. Patients were advised to visit the clinic at any time they felt unwell.

Paired samples of filter paper blood blots were collected from patients with recurrent parasitaemia during follow up for the purpose of genotyping, which was based on MSP-1, MSP-2 and GLURP polymorphisms (150), to determine whether the recurrence was caused by reinfection or recrudescence.

Recurrent *P. falciparum* infections were treated with dihydroartemisinin-piperaquine at a dose of 2 mg/kg of dihydroartemisinin per day and 16 mg/kg of piperaquine per day, for three days. Recurrent *P. vivax* infections were treated with chloroquine in accordance with national guidelines.

2.3.7.7 *Amendment to Ethical Review Committee*

After the first few patients had been enrolled, the study team decided to change uqPCR collection time points based upon the preliminary results on uqPCR positivity from the DP clinical trial. During the DP trial it was found that 75 per cent of the patients showed positive parasite DNA on day 9 and therefore we extended our sample collection for uqPCR from day 5 and day 6 to day 14 and day 21. This amendment was approved by the Ethics Committees.

The trial was monitored by the Mahidol Oxford Tropical Medicine Research Unit (MORU) Clinical Trial Support Group in collaboration with the Myanmar Oxford Clinical Research Unit. Data were evaluated for compliance with the protocol and accuracy in relation to source documents. The monitors checked whether the clinical trial was conducted and data generated, documented and reported in compliance with the protocol, GCP and the applicable regulatory requirements.

2.3.8 Microscopy quality control measures and preparation

Giemsa-stained blood smears were prepared at study sites and counted against white blood cells or red blood cells. All blood smears were checked by a trained laboratory technician in site clinic and all slides were rechecked by experienced microscopists in the Malaria Laboratory of Myanmar Oxford Clinical Research Unit (MOCRU), Yangon, Myanmar.

2.3.8.1 *Preparation of thin and thick blood film*

(Same as section 2.2.8)

2.3.8.2 *Calculation of parasite count*

(Same as section 2.2.8)

2.3.9 Dry blood spot sampling and PCR genotyping

(Same as section 2.2.9)

2.3.10 Sample transport

Blood samples were shipped from study sites (Kyaikdon and Thanpaya) to Myanmar Oxford Clinical Research Unit (MOCRU) by using the commercial World Courier service. Sample packaging for transportation was carried out in compliance with the United Nation committee of Experts/Technical instructions, IATA infectious substances and diagnostic specimens shipping guidelines. Samples were shipped from Yangon to Bangkok (in 3 shipments by World Courier, classified as biological substances, Division 6.2, Category B) with sufficient dry ice and temperature control in accordance with UN guidelines.

2.3.11 Source Data

Source documents were original documents, data reports, and records from which participants' CRF data were obtained. These included, but were not limited to, hospital records (from which medical history and previous and concurrent medication summarized into the CRF), clinical and office charts, laboratory and pharmacy records, diaries, microfiches, radiographs, and CRFs. CRF entries were considered source data if the CRF is the site of the original recording. In this study the CRF were used as the source document for most of the data points.

All documents were stored safely and confidentially in on-site lockers at the Kyaikdon and Thanpaya research clinics. On all study-specific documents, other than the signed consent form, the participants were referred to by the patient ID and initials, not by name.

2.3.12 Safety reporting

This trial involved the administration of drugs that have either been registered or evaluated extensively. However, in this study, we extended the standard three days treatment of artemther-lumfantrine to five days treatment. The safety aspects of this trial were limited to close observation while patients were in the research clinic during follow-up, treating any inter current illnesses or drug-related side effects and recording and reporting only serious adverse events.

2.3.12.1 ***Definitions (AE and SAE)***

(Same as section 2.2.12.1)

2.3.12.2 *Reporting of Serious Adverse Events*

All SAEs were recorded and reported by site physician to the medical monitor, Dr. Frank Smithuis, within one day of his or her awareness to the SAE. The medical monitor then reported the SAE to the data safety monitoring board and the Department of Medical Research Ethics Committee (Myanmar) in accordance with local requirements.

2.3.13 Ethics

This study was conducted in compliance with the current revision of the Declaration of Helsinki (Seoul 2008) and according to National Regulations, following the principles of the ICH guidelines for Good Clinical Practice 1996.

2.3.13.1 *Declaration of Helsinki*

As the Principal Investigator I ensured that this study was conducted in compliance with the current revision of the Declaration of Helsinki (Seoul 2008).

2.3.13.2 *ICH guidelines for Good Clinical Practice*

I ensured that this study is conducted according to any National Regulations and that it will follow the principles of the ICH Guidelines for Good Clinical Practice 1996.

2.3.13.3 *Approvals*

The study protocol and its associated documents were submitted to the Oxford Tropical Research Ethics Committee (OXTREC) (Reference: 1046-13) the Department of Medical Research Ethics Committee (Myanmar) (Reference: 50/Ethics2013). The investigator submitted and, where necessary, obtained approval from the above parties for all substantial amendments to the original

approved protocol and documents. This trial was registered at ClinicalTrial.gov (Identifier: NCT02020330).

2.3.14 DNA sample preparation

Blood samples were obtained consisting of 3 mL venous blood from individuals aged ≥6 years.

For highly sensitive polymerase chain reaction (uqPCR) 3 mL of whole blood sample was collected from the patient and centrifuged at 1,000 x g at room temperature for 10 minutes to pellet the cells. The supernatant was removed and discarded and the pellet stored in 3 mL cryovial tube at minus 20°C.

For lumefantrine level at day 7, 3 mL of whole blood was collected from the patient and centrifuged at 1,000 x g at room temperature for 10 minutes to pellet the cells. The supernatant was removed and discarded and the pellet stored in 3 mL cryovial tube at minus 20°C.

2.3.15 Molecular studies

The frozen packed red cells were transported to the molecular laboratory at MORU, Bangkok, Thailand, for sample processing.

2.3.15.1 *DNA extraction*

(same as previous section 2.1.3.3)

2.3.15.2 *Sequencing kelch13 gene*

Purified PCR products were sequenced at Macrogen, Republic of Korea and analysed by ImageLab (BioRad Gel doc XR) using the 3D7 kelch13 sequence as reference (Accession: XM_001350122.1) (same as previous section 2.1.3.4).

2.3.15.3 *Highly sensitive qPCR (uqPCR)*

For qPCR, DNA extraction was done using either QIAgen mini or medi kit (≤ 400 µL for mini kit or 400-4000 µL for midi kit). Use of highly concentrated DNA template allowed limits of detection down to 20 parasites in 1000 µL of whole blood. The parasite density results were then derived from the parasite DNA concentration (same as previous section 2.2.15.3).

2.3.16 Lumefantrine drug level

Lumefantrine concentrations will be measured by the MORU department of pharmacology using a published validated method (151), but the results will not form part of this thesis.

2.3.17 Urine test

Pregnancy tests were done on women of child-bearing age to confirm their eligibility in the study.

3 Expansion of artemisinin-resistant *Plasmodium falciparum* infections in Myanmar

3.1 Chapter 3 Abstract

Background: Emergence of artemisinin resistance in Southeast Asia poses a serious threat to the global control of *Plasmodium falciparum* malaria. Discovery of the kelch13 marker has transformed approaches to the monitoring of artemisinin resistance, allowing introduction of molecular surveillance in remote areas through analysis of DNA. The aim of the study was to assess the spread of artemisinin-resistant *P. falciparum* in Myanmar by determining the relative prevalence of *P. falciparum* parasites carrying kelch13 propeller mutations.

Methods: This cross-sectional survey was conducted at malaria treatment centres at 55 sites in ten administrative regions in Myanmar, and in relevant border regions in Thailand and Bangladesh, between January 2013 and September 2014. Kelch13 sequences from *P. falciparum* infections were obtained mainly by passive case detection. Data were entered into two geostatistical models to produce predictive maps of the estimated prevalence of mutations of the kelch13 propeller region across Myanmar.

Results: Overall, 371 (39%) of 940 samples carried a kelch13 propeller mutation. We recorded 26 different mutations, including nine mutations not described previously. In seven (70%) of the ten administrative regions of Myanmar, the combined kelch13 mutation prevalence was more than 20%. Geospatial mapping showed that the overall prevalence of kelch13 mutations exceeded 10% in much of the east and north of the country. In Homalin, Sagaing Region, 25 km from the Indian border, 21 (47%) of 45 parasite samples carried kelch13 propeller mutations.

Conclusion: Artemisinin resistance extends across much of Myanmar. We recorded *P. falciparum* parasites carrying kelch13 propeller mutations at high prevalence next to the northwestern border with India. Appropriate therapeutic regimens should be tested urgently and implemented comprehensively if spread of artemisinin resistance to other regions is to be avoided.

3.2 Background

Artemisinin-based combination treatments (ACTs) are the mainstay of treatment for *Plasmodium falciparum* malaria globally, but resistance to artemisinin is now prevalent across an expanding area of Southeast Asia (62, 77, 83, 91, 93, 99, 132). Artemisinin resistance is characterized by reduced susceptibility of the ring stage of parasite development (152, 153) and is now accompanied by increasing ACT failure rates in Cambodia (75, 83, 96, 154) and Thailand (94). Mutations in the "propeller" region of the kelch-motif containing gene, designated *PF3D7_1343700* and known as kelch13, have been identified recently as a key determinant in Southeast Asia of delayed parasite clearance following artemisinin treatment (83) and reduced *in vitro* responses (84, 155). Resistance conferred by PfK13 mutations are thought to act through upregulation of the unfolded protein response pathway (108). Various kelch13 propeller mutations have been documented in population surveys in the region, with the most prevalent mutations are associated with prolonged parasite clearance after artemisinin treatment (83, 93, 99, 119) and reduced *in vitro* response (156, 157). Frequently occurring non-synonymous propeller mutations associated with normal rates of parasite clearance have not so far been identified (83, 93, 119).

Increasing evidence shows that away from areas of artemisinin resistance, mutations in the kelch13 propeller are not present at significant frequencies (93,

113, 114, 158-161), and the total prevalence of the kelch13 propeller mutation is less than 5% in surveys from a range of transmission settings. Monitoring of the prevalence of mutations in the kelch13 gene can provide a unique opportunity for real-time surveillance regarding spread of artemisinin resistance to support containment and elimination strategies (162). WHO has incorporated results of kelch13 marker surveillance into a revised definition of artemisinin resistance (132).

Myanmar stretches from the Bay of Bengal and Andaman Sea in the south to the Himalayas in the north (Figure 3.1), and therefore provides the only route for drug-resistant falciparum malaria to spread contiguously from Southeast Asia to the Indian subcontinent (139), a path followed by chloroquine and probably pyrimethamine resistance nearly half a century ago (163). Artemisinin resistance has been present at the Thai-Myanmar border for several years (91, 164) and evidence of slow parasite clearance following ACTs has also been reported in south-eastern Myanmar (129, 130). In 2011-2012 in Shwe Kyin, Bago Region, approximately 14% of patients showed delayed clearance (parasite clearance half-life more than 5 hours) and a quarter of all patients (19/73) had mutations in the propeller region of the kelch13 protein (93).

The geographic extent of artemisinin resistance in Myanmar has not been defined. I report in this chapter a detailed molecular survey of kelch13 based on *P. falciparum* field isolates obtained from symptomatic patients presenting to clinics at more than 50 sites extending to 10 states and regions in Myanmar as well as relevant border areas in Thailand and Bangladesh.

3.3 Objective

- To assess the spread of artemisinin-resistant *P. falciparum* in Myanmar by determining the relative prevalence of *P. falciparum* parasites carrying kelch13 propeller mutations.

3.4 Methods

A cross-sectional survey in symptomatic patients presenting to malaria treatment centres was conducted at 55 sites in ten administrative regions in Myanmar and in relevant border regions in Thailand and Bangladesh between August 2013 and September 2014. DNA was extracted from rapid diagnostic tests, dry blood spots and from venous blood samples. Kelch13 gene sequences from *P. falciparum* infections were obtained mainly by passive case detection (please see chapter 2, section 2.1.3.4 for details).

In a small subset of these patients, both the screening rapid diagnostic test and the whole blood sample were processed to check for concordance. In line with power calculations made for WHO Therapeutic Efficacy Studies (133), I planned to obtain at least 50 sequences in each administrative region.

Geospatial modelling techniques using molecular markers have been applied to estimate the prevalence of drug resistance (85). These models can be used to identify geographical areas where data are currently insufficient for policy makers to determine whether drug resistance is present or not. In this study, for predictive geospatial mapping, the total proportion of samples in each location with a non-synonymous mutation after amino acid position 440 of kelch13 gene was calculated. These data and geographical information system co-ordinates for each sampling site were entered into two geostatistical models (for details procedures please see Appendix A).

3.5 Results

Of the 2,378 samples tested, obtained from 55 townships across 12 administrative regions in Myanmar and neighbouring areas (Figure 3.1), 940 samples produced clear DNA sequence covering amino acids 210 to 726 of the kelch13 gene. The overall extraction and sequencing success rates were 97% for whole blood, 84% for dried blood spots, and 26% for rapid diagnostic tests (Figure 3.2). We did not screen samples with PCR before attempting to sequence the kelch13 gene. Eleven of 12 samples sequenced from both rapid diagnostic tests and whole blood showed concordant sequences, with the remaining sample failing to produce a sequence from the diagnostic test.

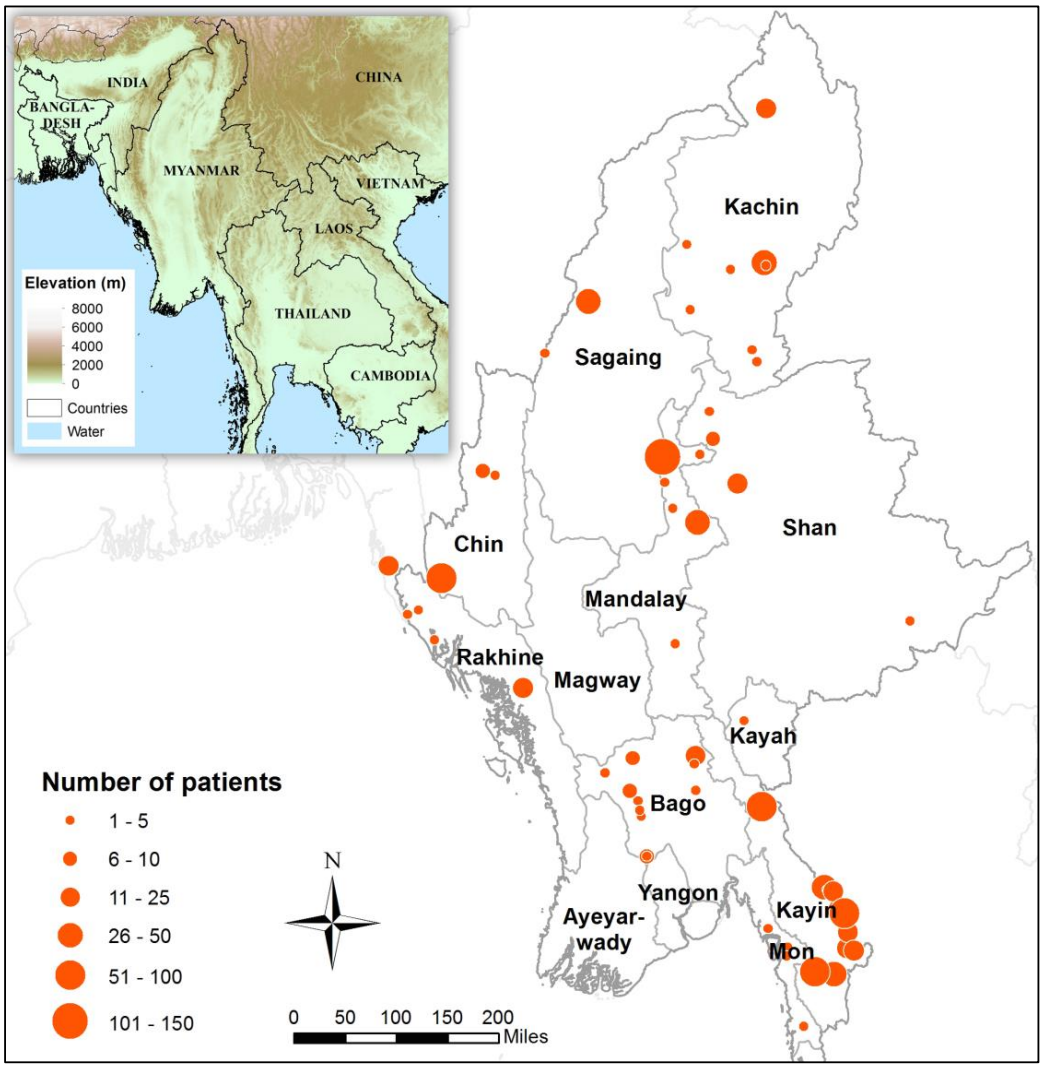


Figure 3.1 Location of sampling sites, sample sizes, and administrative states and regions of Myanmar, and a relief map of Southeast Asia. Red circles show numbers of patients in each region.

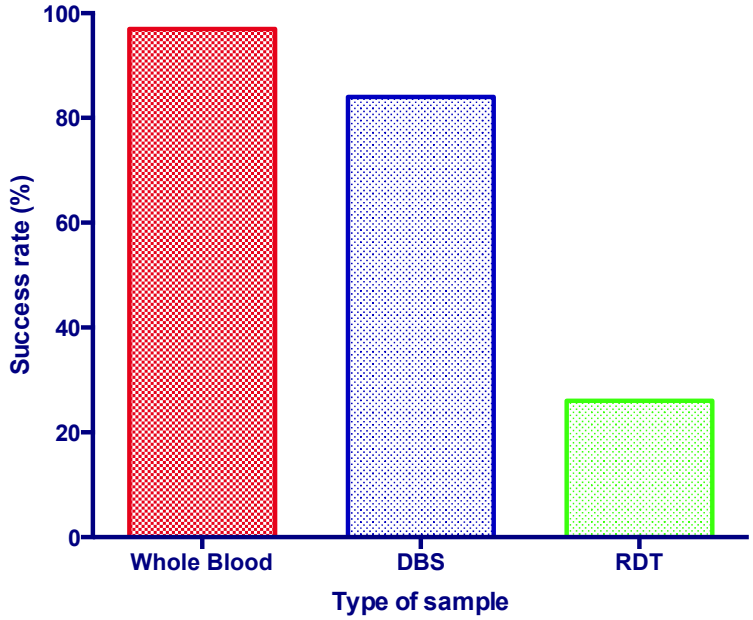


Figure 3.2 Extraction-sequencing success rates by different sample type (n=940) (DBS, dried blood spot; RDT, rapid diagnostic test)

Twenty-nine different mutations after amino acid 210 were identified, of which 26 (90%) were after amino acid 440 (Table 3.1). Three hundred and seventy one (39%) isolates had a kelch13 propeller domain mutation (Table 3.2). In addition 17 isolates had an E252Q mutation in the so-called 'stem' section of the kelch13 protein; one (2%) from Bago Region, 11 (4%) from Kayin State, and five (3%) from Tak Province. No single sample had more than one mutation in these conserved domains of the protein.

Consistent with previous reports(83, 93), mutations in the propeller region were concentrated within blades 1-4 (Figure 3.2).

Table 3.1 List of mutations found in 940 samples

Mutation	*No. of samples with mutation*	*No. of States/Regions where mutation was found*	*Notes and references*
Unique to this survey before its publication			
N371I	1	1	
P443S	4	2	
D452E	1	1	
N458I	9	1	
C469F	1	1	
K479I	8	1	
S485N	1	1	
N490T	1	1	
P527H	1	1	
G533A	3	2	
R575K	6	2	
A675V	5	2	
A676D	6	3	
Previously reported in Myanmar or Myanmar-Thailand border (93, 165)			
E252Q	17	3	
K438N	1	1	
P441L	8	2	
F446I	80	6	
G449A	5	4	Also reported in Cambodia (83)
N458Y	2	1	Also reported in Cambodia (83)
M476I	18	2	
A481V	1	1	Also reported in Cambodia (83, 93)
N537I	15	2	Also reported in Cambodia (83)
G538V	30	3	
R561H	25	5	Also reported in Cambodia (83)
P574L	41	7	Also reported in Cambodia (83)
C580Y	97	2	Also reported in Cambodia (83, 93)
F614L	1	1	
Previously reported in Cambodia only			
H719N	2	1	
Previously reported in Africa only (93)			
R255K	2	2	

Table 3.2 Number of samples per region and proportion with mutations in the propeller domain of kelch13

Country	*Administrative State / Region*	*Total samples*	*Propeller mutants*	*Proportion (%)*
Myanmar	Bago	52	0	0 (0 to 6.9)
	Chin	62	2	3.2 (0.9 to 11)
	Kachin	70	26	37.1 (26.8 to 48.9)
	Kayah	2	2	100 (34.2 to 100)
	Kayin	288	137	47.6 (41.9 to 53.3)
	Mandalay	181	43	23.8 (18.1 to 30.5)
	Mon	8	3	37.5 (13.7 to 69.4)
	Rakhine	29	2	6.9 (1.9 to 22)
	Sagaing	46	21	45.7 (32.2 to 59.8)
	Shan	21	14	66.7 (45.4 to 82.8)
Bangladesh	Chittagong	25	0	0 (0 to 13.3)
Thailand	Tak	156	121	77.6 (70.4 to 83.4)
	Total	940	371	39.5 (36.4 to 42.6)

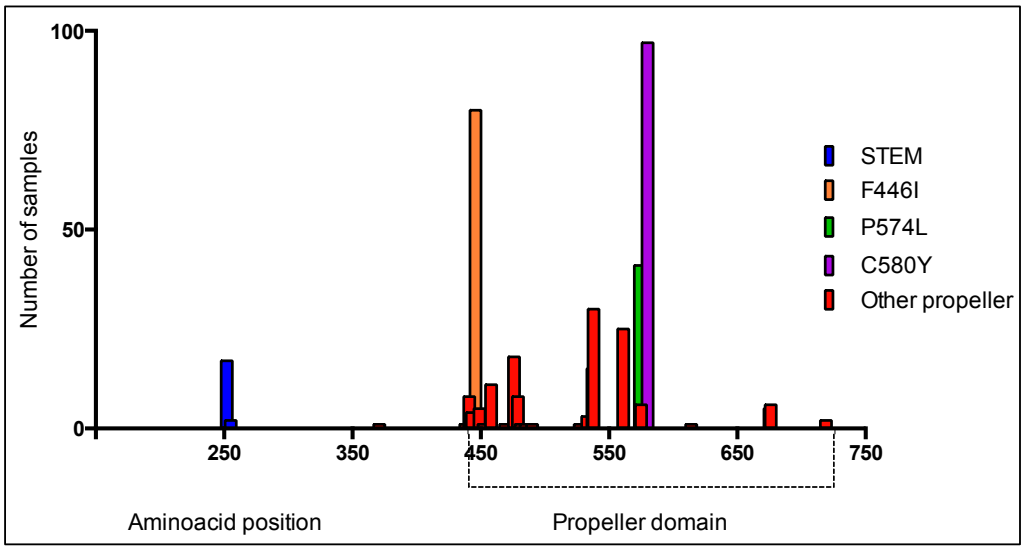


Figure 3.3 Primary aminoacid postions of kelch13 mutations identified by the survey in Myanmar and border areas

Several mutations appeared to be concentrated in particular areas of Myanmar (Figure 3.3). F446I was identified in 80 samples across six states or regions with several areas in Upper Myanmar showing prevalences in excess of 10%; 21 (47%) of 45 samples obtained in Homalin, Sagaing Region (25 km from the India border) carried kelch13 propeller mutations (mostly the F446I mutation). The P574L mutation was also wide ranging, being found in 41 samples across seven states or regions while the A676D mutation was seen in three northern states or regions only. The C580Y mutation found at high prevalence in western Cambodia (83) was confined to Kayin state, and was also present at high prevalence across the adjacent western part of Tak province in Thailand (Figure 3.3). Notably, the M476I mutation shown to develop after prolonged in vitro artemisinin selection (83) was seen in 18 isolates.

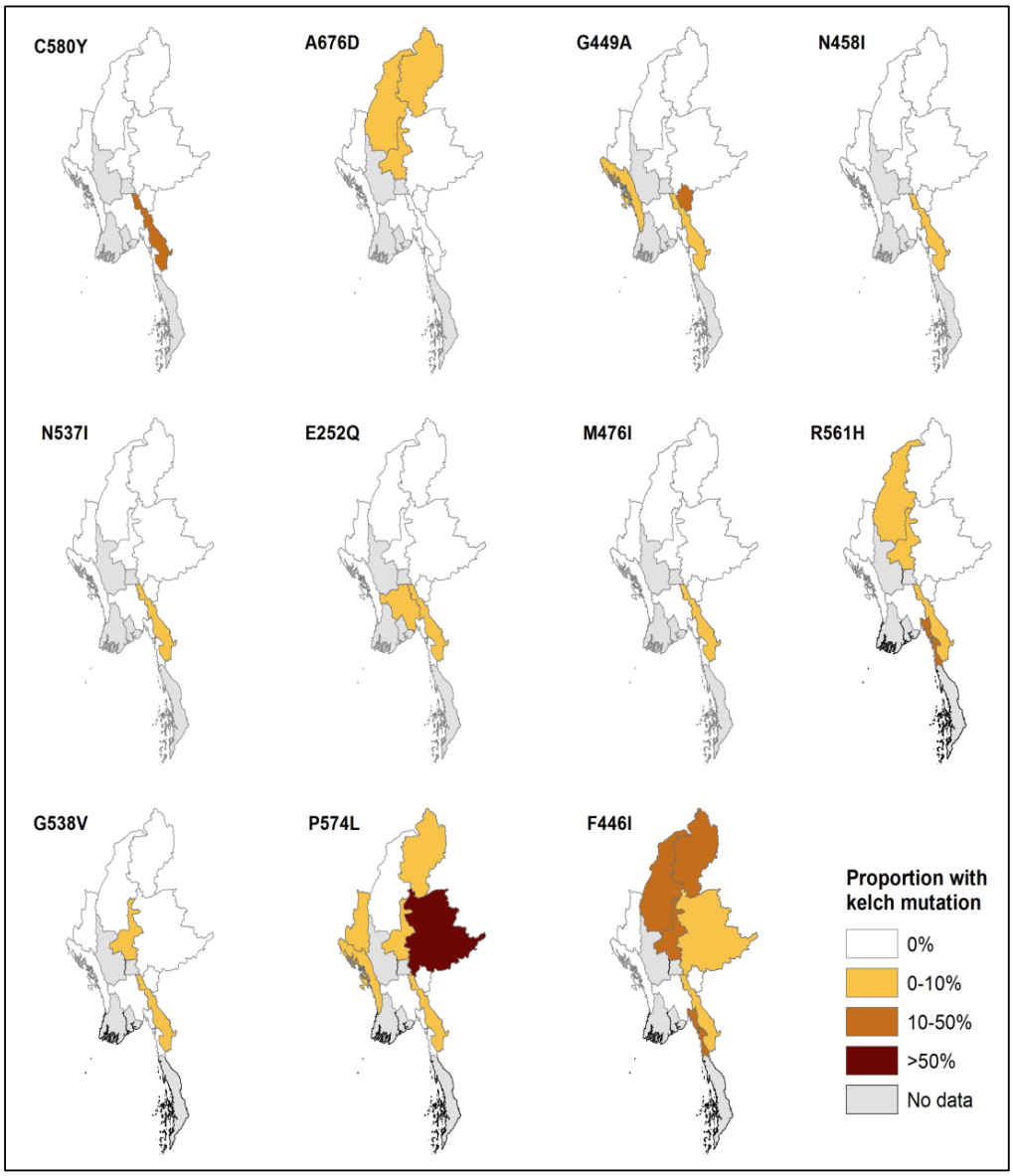


Figure 3.4 Local prevalence of individual kelch13 mutations by administrative state or region in Myanmar

(Only mutations found in at least nine isolates, or at least three states or regions, are shown.)

Around two-thirds of the observed kelch13 mutations had been described previously in Myanmar or at the Myanmar-Thailand border (93, 112, 119), and a further subset of these had also been observed in Cambodia (83, 93). These previously observed mutations tended to have higher prevalence than the mutations that were unique to Myanmar, none of which was found in more than 10 isolates (Table 3.1). We identified three, rare (three samples or less) synonymous mutations in the sequence after aminoacid in the set of 759 samples from within Myanmar. Calculation of intraspecific evolutionary coefficients (with Jukes-Cantor correction) produced values of 0.0123 for dS and of 0.0136 for dN. Inference of selective forces from the resulting intraspecific dN to dS ratio of 1.10 is challenging, but the findings are compatible with strong positive selection (166).

The total prevalence of kelch13 propeller mutations for each site was calculated as the proportion of samples with any mutation after amino acid 440. These point metrics were entered into two independent geospatial models to obtain continuous prevalence maps for Myanmar (Figure 3.5).

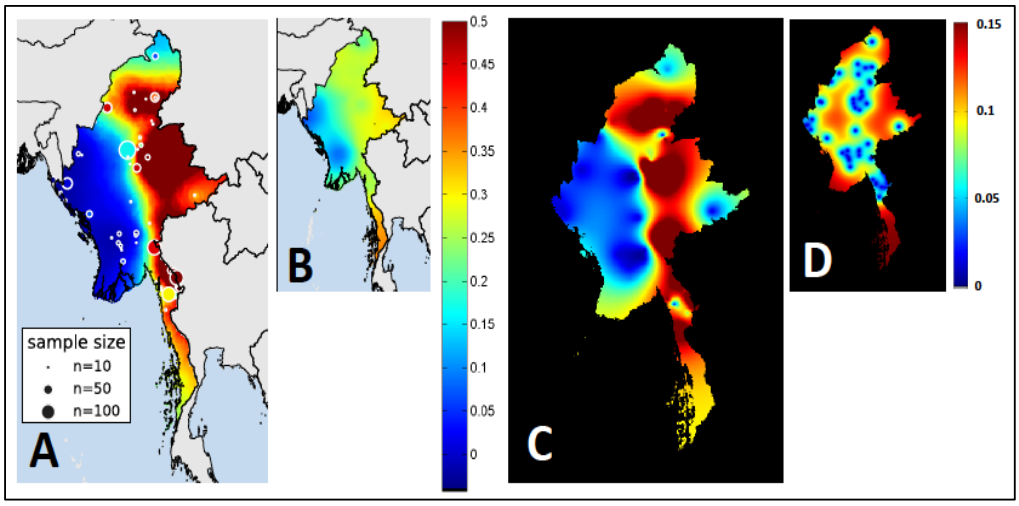


Figure 3.5 Geographical extent of predicted artemisinin resistance as determined by the prevalence of kelch13 propeller mutations (>440 aminoacids) visualized by approaches using Bayesian model (A, with uncertainty shown in B) and kriging interpolation (C, with uncertainty shown in D).

(In the uncertainty maps colour represents the standard deviation of the distribution with orange/red areas indicating greatest uncertainty in Shan state (in the east) and Thanintaryi region (south)).

We calculated the total prevalence of kelch13 propeller mutations for each administrative region and site as the proportion of samples with any mutation after aminoacid position 440. These point metrics were entered into two independent geospatial models to obtain continuous prevalence maps for Myanmar (Figure 3.5). In spite of differing in their approach, both maps indicated a large area of relatively high mutation prevalence (substantially more than 10%) extending from the south-east to the north of the country. Much of Lower Myanmar, and Chin and Rakhine states in the west, had a very low prevalence of kelch13 mutations, a finding consistent with the absence of kelch13 mutant parasites in adjacent Bangladesh (Figure 3.5).

3.6 Discussion

Artemisinin resistant falciparum malaria is spreading westwards. Previous studies have already indicated high kelch13 mutation prevalence along the mountain ranges in the south-east near the Thai border (80, 93, 165). This work provides strong evidence that kelch13 propeller mutations also extend across much of Upper Myanmar, and including the regions close to the Indian border in the north-west.

By comparison, Lower Myanmar and the western states (Rakhine and Chin) presently have a relatively low prevalence of kelch13 mutations, and there is no evidence of spread into south-eastern Bangladesh. There is also no evidence yet that artemisinin resistance has reached India, but there are few available data (117, 167). An independent study examining samples from 91 patients in Kayin state in east Myanmar and Chin state in the west likewise reported a range of kelch13 propeller mutations, with a higher prevalence in the eastern region and

relatively few kelch13 mutant parasites at the western border with Bangladesh (112).

Artemisinin resistance became established in western Cambodia more than a decade ago (83). Population studies show evidence of several founder populations that have risen to relatively high levels and can be linked to specific kelch13 mutations (85, 168) and high levels of artemisinin resistance both *in vivo* and *in vitro* (169). Seven individual mutations appear to have arisen independently on more than one occasion on different genetic backgrounds and in different locations; these mutations include C580Y, which is reaching fixation in a number of regions (83, 92, 170).

In this survey, C580Y parasites were only found along the Thai-Myanmar border, although this appears to be a separate lineage to that found in Cambodia (119). Few of the kelch13 mutants currently found in Myanmar have been encountered at high prevalence in Cambodia; furthermore the Myanmar mutations appear to be clustered towards the first kelch domain (amino acids 441-475) with one mutant in the conserved stem of the protein (E252Q) also reaching significant frequencies levels in south-east Myanmar. In the north of Myanmar, including sites very close to the Indian border, the F446I mutation prevalence is at high levels. Why there are different sets of mutations in different locations is not yet clear, but one possible explanation is that Myanmar is at a relatively earlier stage of an evolutionary process than Cambodia. The prevalent mutations in Myanmar may provide relatively low levels of artemisinin resistance (although the widespread P574L mutation appears to be associated with clearance that is at least as slow as C580Y (93, 119) or might bring fitness costs, so that they are outcompeted by ‘fitter’ alternatives such as C580Y over time. Alternatively there

might be distinctive selective forces resulting from different antimalarial use, host genetics or mosquito biology that promote differential sets of mutations in the two regions. Further studies on parasite fitness and *in vitro* drug sensitivity are likely to shed light on these questions.

This study shows that valuable “real-time” molecular epidemiological surveillance and monitoring can be performed with used rapid diagnostic tests (as well as dried blood spots), if there is a system for obtaining these and sending them to a reference laboratory. The two geospatial models described provide consistent and informative up to date knowledge of the extent of resistance to artemisinins in Myanmar and could be used to guide and prioritise interventions. Additional data are needed to reduce the uncertainty of the current estimates of resistance in certain locations, potentially guided by surveillance modelling methods.

The earlier global spread of chloroquine resistance along this route resulted in the loss of millions of lives in Africa, and clearly, Myanmar can be considered the “frontline” in the battle to contain artemisinin resistance. These data highlight the concern that artemisinin resistance could follow historical paths of antimalarial drug resistance spread from Southeast Asia, via Myanmar, through India to Africa (171). Substantial increases in international travel and migration could promote direct spread of artemisinin resistance (so-called jumping) (172). Local emergence of resistant parasites is an alternative scenario (so-called popping).

Myanmar also has by some distance more malaria than any other country in Southeast Asia (173), so aside from the wider implications the spread of drug resistance holds the potential to reverse recent downward trends in morbidity and mortality from malaria in the country. Knowledge of the level of kelch13 propeller mutations provides a snapshot of the extent of artemisinin resistance, but does

not in itself provide direct information about the effectiveness of Myanmar's present first-line artemisinin-based combination treatment, artemether–lumefantrine.

If, for example, the artemisinin resistance reported here results from early wide-scale availability of artemisinin monotherapy, the lumefantrine component could be sufficiently effective for the combination to retain high effectiveness in at least some regions of Myanmar. However, declining effectiveness of artemisinin-based combination treatments, representing a combination of artemisinin resistance and failing partner drug (mefloquine), is already a substantial problem on the eastern Myanmar border (94) and, in view of the cross-resistance between mefloquine and lumefantrine, effectiveness in that region is probably poor. In other regions where kelch13 mutant parasites are prevalent, prediction of effectiveness is difficult, and therapeutic efficacy studies—the definitive method for identification of whether a combination is beginning to fail—are urgently needed, with a focus on the areas of emerging artemisinin resistance evident in this study (see Chapters 4 and 5 for therapeutic efficacy studies carried out as part of the research work for this thesis). Measurement of *pfmdr1* copy number in these samples would also be useful in this respect.

Artemisinin resistance has not been contained. Present artemisinin-based combination treatments are failing in areas affected by artemisinin resistance and there is a real threat that the incidence of *P. falciparum* will begin to rise again, thus confounding regional aspirations to eliminate malaria.

Even low numbers of recrudescence fuel the emergence and spread of resistance to the partner drug (which is exposed to a higher parasite burden

because of reduced parasite killing by the artemisinin derivative), substantially shortening the lifetime of any artemisinin-based combination treatment (78).

Switching to an alternative partner (such as piperaquine) successfully, but only temporarily, improved the effectiveness of artemisinin-based combination treatments in western Cambodia (154). These considerations suggest that local malaria treatment guidelines need to be constantly assessed and if necessary revised. Use of regimens of more than 3 days duration, or containing more than one partner drug, will become necessary across an expanding area of Southeast Asia.

3.7 Conclusion

As well as molecular surveillance, *in vivo* therapeutic efficacy studies based on Myanmar's currently recommended first line therapy for *P. falciparum*, artemether-lumefantrine, are also critical (see Chapters 4 and 5). In addition, studies for antimalarial drug regimens with longer courses or different combinations are need to be considered in the areas of emerging artemisinin resistance now evident in Myanmar. The pace at which the geographical extent of artemisinin resistance is spreading is faster than the rate at which control and elimination measures are being developed and instituted, or new drugs being introduced. A vigorous international effort to contain this enormous threat is needed.

3.8 Summary of findings

The salient findings of chapter 3 are as follows:

- Twenty-nine different mutations after amino acid 210 were identified, of which 26 (90%) were after amino acid 440.
- Three hundred and seventy one (39%) isolates had a kelch13 propeller domain mutation.
- In seven of ten administrative regions of Myanmar, the combined kelch13 mutations prevalence was more than 20%.
- The F446I was identified in 80 samples across six states/regions and mostly in Upper Myanmar.
- Geospatial mapping showed that the overall prevalence of kelch13 mutations exceeded 10% in much of east and north of Myanmar however in the west there was a very low prevalence of kelch13 mutations.
- This study shows that artemisinin resistance extends over more of Southeast Asia than had previously been known, and is now present close to the border with India.
- This finding expands the area in which containment and elimination are needed to prevent the possibility of global spread of artemisinin resistance.

4 *In vivo* artemisinin resistance in patients with uncomplicated falciparum malaria treated with dihydroartemisinin-piperaquine in Upper Myanmar (DP trial)

4.1 Chapter 4 abstract

Background: Artemisinin resistance in *Plasmodium falciparum* extends across Southeast Asia where it is associated with delayed parasite clearance, worsening partner drug resistance and a decline in the efficacy of frontline artemisinin-based combination treatments. Dihydroartemisinin-piperaquine (DP) is an essential component of preventive and curative treatment in the region, but its therapeutic efficacy has recently fallen in Cambodia.

Methods: To determine the prevalence of clinical artemisinin resistance and to assess the efficacy of DP in northern and central Myanmar we enrolled 116 patients with acute uncomplicated falciparum malaria in a prospective clinical and parasitological evaluation of DP conducted at two sites in Upper Myanmar between August 2013 and December 2014. Patients received DP orally for three days together with primaquine 0.25 mg/kg on admission. Parasite clearance half-lives based on six hourly blood smears, and day 42 therapeutic responses were assessed as well as parasite kelch13 genotypes. We determined parasite clearance dynamics from day 0 to day 21 using highly sensitive PCR (uqPCR) .

Results: Median parasite clearance half-life was prolonged, and clearance half life was greater than 5 hours in 21% of patients. Delayed parasite clearance was significantly associated with mutations in the propeller region of the parasite kelch13 gene. The kelch13 F446I mutation was found in 25.4% of infections and was associated with a median clearance half-life of 4.7 hours compared with 2.7 hours for infections without kelch13 mutations ($p< 0.001$). There were no failures after 42 days of follow-up, although 18% of patients had persistent parasitaemia on blood smear on day 3. The Kaplan-Meier curves showed that kelch13

mutations has higher positivity rate than wild type at day 21 using uqPCR but this was not statistically significant.

Conclusion: The dominant kelch13 mutation observed in Upper Myanmar, F446I, appears to be associated with an intermediate rate of parasite clearance compared to other common mutations described elsewhere in the Greater Mekong Subregion. Discerning this phenotype requires relatively detailed clearance measurements, highlighting the importance of methodology in assessing artemisinin resistance.

4.2 Background

Artemisinin resistance in *Plasmodium falciparum* has emerged in Cambodia, along the Thai-Myanmar border, and in Vietnam and Myanmar. Artemisinin resistance is characterised by the clinical phenotype of delayed parasite clearance (62). A large prospective trial conducted on the Thai-Myanmar border (91) showed that the geometric mean parasite clearance half-life had increased from 2.6 hours to 3.7 hours within ten years. In southern Myanmar the median parasite clearance half life of uncomplicated falciparum malaria has been reported to be 4.8 hours, highlighting the emergence of artemisinin resistance as an important problem in Myanmar (129).

The main ACT currently in use in Myanmar is artemether-lumefantrine, one of three different ACTs permitted to be used as first line treatment for uncomplicated falciparum malaria. When the current study was planned a molecular marker for artemisinin resistance had not been reported, but during the course of patient recruitment Ariey *et al.* (83) described the strong association of mutations in the propeller region of the parasite kelch13 gene with slow parasite

clearance. This marker has since been validated in clinical and transfection studies (84, 93).

A large number of kelch13 propeller mutations have been identified and C580Y, R539T, Y493H and F446I have been associated with delayed clearance half-life in recent studies (83, 93, 115). But questions remain about the evolution of resistance and whether other mutations are associated with resistant phenotypes or not.

For these reasons detailed *in vivo* assessment is important to detect the presence of artemisinin resistance. The clinical study outlined here had the primary objective of defining the extent of the spread of resistance in northern and central Myanmar by *in vivo* assessment, describing the clinical relevance of the prevalent kelch13 mutations, so that containment efforts can be planned sensibly. The study also assessed the efficacy of dihydroartemisinin-piperaquine, important in the light of recent reports of reduced clinical efficacy in Cambodia probably related to the development of piperaquine resistance on the background of artemisinin resistance (154, 174).

In 2013 and 2014, two studies were carried out to describe artemisinin resistance in Myanmar using artesunate monotherapy for 7 days (93, 129). Although this purist approach assesses the parasite clearance rate of artesunate alone, a seven day monitored treatment regimen substantially limits recruitment and is operationally difficult. Our aim in the current study was to develop a protocol for assessing efficacy which can be used easily and widely. Using standard 3 day ACT treatment regimens has the additional advantage of enabling monitoring of both the initial parasite clearance of the combination of artemisinin derivative plus

partner drug and the cure rate of treatments recommended and implemented by malaria control programmes across the region.

During the course of treated falciparum malaria, the peak of asexual parasitaemia is followed by gametocytaemia after approximately one week (175). Although artemisinin derivatives have a broader spectrum of anti-gametocyte activity than other antimalarial drugs, the mature stage 5 *P. falciparum* gametocytes are relatively insensitive to artemisinins. To prevent ongoing infectiousness after the treatment of asexual parasitaemia the World Health Organization has recommended the addition of a single dose of primaquine 0.25 mg/kg to the ACT treatment of uncomplicated falciparum malaria (141).

4.3 Objectives

The primary objectives of this study were to determine the *P. falciparum* parasite clearance rate in two different geographical regions of Myanmar and to identify parasite genetic determinants of the slow *P. falciparum* clearance phenotype in Myanmar.

The secondary objectives were to measure the efficacy of dihydroartmisinin-piperaquine 42 days after treatment, to measure the incidence and duration of gametocyte carriage after treatment, to describe the parasite clearance below the microscopic level of detection using highly sensitive qPCR method, to identify molecular markers of the slow *P. falciparum* clearance phenotype and to measure day 7 piperaquine drug level.

4.4 Methods

4.4.1 Study design

I conducted an open-label clinical trial in two hospitals, one located in Myitkyina, Kachin state in northern Myanmar and the other in Thabeikkyin, Mandalay Region in central Myanmar. The two sites are situated in areas of low entomological inoculation rate (EIR<1) (139). This trial was registered at ClinicalTrial.gov (NCT01350856). The recruitment phase of the study started in August 2013 and ended in December 2014. The Oxford Tropical Research Ethics Committee (UK) and the Defence Services Medical Ethics Committee (Myanmar) approved the study protocol.

4.4.2 Primary and secondary endpoints

4.4.2.1 *Primary Endpoint*

- Parasite clearance rate as defined by the slope of the linear portion of the natural logarithm parasite clearance curve.

4.4.2.2 *Secondary Endpoints*

- Fever clearance time (i.e. the time taken for the tympanic temperature to fall below 37.5°C and remain there for at least 24 hours)
- Proportion of patients with gametocytemia before, during and after treatment, assessed at admission, on days 3, 7 and then once weekly, stratified by presence of gametocytes at enrolment
- Parasite clearance below the microscopic level of detection using molecular methods
- *Pf* kelch13 molecular markers of artemisinin resistance

- Efficacy at day 42
- Proportion of patients with a positive malaria smear 72 hours after treatment
- Day 7 piperaquine concentration (not reported in this thesis)

4.4.3 Study participants

Please see Chapter 2 (section 2.2.3) for more details.

4.4.4 Drug Therapy

Dihydroartemisinin-piperaquine (Duo-Cotecxin®) was purchased from Beijing Holley-Cotec Pharmaceuticals, and primaquine was provided by 3MDG from Remedica Pharmaceuticals. Patients received dihydroartemisinin-piperaquine (DP) administered orally with a target dose of 2.4 (2 to 10) mg of dihydroartemisinin per day and 18 (16 to 27) mg/kg of piperaquine per day, for three days (20). All patients received primaquine 0.25 mg/kg single dose on day 0 after the first dose of DP. One tablet of Duo-cotecxin® contains 40 mg dihydroartemisinin and 320 mg piperaquine, and 7.5mg primaquine tablets were used. Dosing was by weight categories (Appendix C).

4.4.5 Study Procedures

Please see Chapter 2 (section 2.2.7) for more details.

4.4.5.1 *Molecular studies*

Please see Chapter 2(section 2.2.15) for more details.

4.4.5.2 *Drug assays*

Piperaquine concentrations will be measured in filter paper blood spots taken during the study. This assay will be performed by the MORU pharmacology department, but the results will not form part of this thesis.

4.4.5.3 *Statistical Analysis*

Sample size was based on WHO guidelines for single-arm efficacy studies (140). A target sample size of 73 enables detection of a 5% failure rate with 95% confidence and 5% precision. An additional 10% was included to allow for losses to follow up. Based on previous studies, this number would also enable distribution of parasite clearance half-lives to be described adequately. Data were entered into a web-based database, OpenClinica, version 3.0. Data cleaning and analysis were done by using Stata statistical software, version 13 (StataCorp, College Station, Tx, USA) and GraphPad Prism software 6.0 (Graphpad Software Inc., La Jolla, CA, USA). Parasite clearance half-lives were calculated by using the online Parasite Clearance Estimator (http://www.wwarn.org/tools-resources/toolkit/analyse/parasite-clearance-estimator-pce) tool from the Worldwide Antimalarial Resistance Network (102). Parasite clearance time (PCT) were calculated as the time to the first of two negative malaria blood smears and reported as median and interquartile range.

Fever clearance was defined as the time to the start of the first 24-hour period during which the temperature remained below 37.5°C. The gametocyte clearance time (GCT) was the interval from first detection to last detection of gametocytes in a peripheral blood smear. Person-gametocyte weeks (PGW) were calculated for each case as the number of weeks in which gametocytaemia was patent

(excluding admission) divided by duration of follow-up, expressed per 1,000 person-weeks.

In accordance with WHO criteria (176), anaemia was defined as haemoglobin less than 11 g/dL or less than 10 g/dL in children under five years of age. Estimated haemoglobin values were obtained from original haematocrit measurements using a published conversion method (haematocrit = 5.62 + 2.60 x haemoglobin) (177).

Categorical baseline data were presented by site, as number (%) and included all patients who were recruited. Continuous baseline characteristics were presented, by site, as median and interquartile range (IQR), means (95% confidence interval), or geometric mean (95% confidence interval) as appropriate. Tests of association between two categorical variables were performed using the chi-squared test, and comparison of non-normally distributed continuous variables by Mann-Whitney U test. Wild type was defined as no mutation present at any position before amino acid 210 for synonymous SNPs. Effect of F446I mutation on parasite clearance half-life compared to wild type was evaluated using Mann-Whitney U test. Survival time data were analysed by the use of Kaplan-Meier method and Cox regression and hazard ratio were calculated. Survival rates are expressed as the percentage of uqPCR positivity for 21 days.

Simple logistic regression was conducted for association between each potential risk factor and prolonged parasite clearance half-life, with multiple logistic regression to analyse resulting risk factors. In general, a test of association between two categorical variables was performed using the chi-squared test, and comparison of non-normally distributed continuous variables was compared

using the Mann-Whitney U test. All hypothesis testing was two tailed and a p value of <0.05 was considered to be statistically significant.

4.5 Results

The study was conducted between August 2013 and December 2014. A total of 3,211 patients were screened and two-thirds of the *P. falciparum* cases were excluded because the parasite count was lower than 5,000 per µL (Figure 4.1). A total of 116 patients were enrolled, 44 in Myitkyina (Kachin state) and 72 in Thabeikkyin (Mandalay region). Early discontinuation occurred in two patients and therefore a total of 114 patients were analysed (Figure 4.2). In accordance with an Ethics Committee recommendation, children less than 5 years of age were not included in the Thabeikkyin site because of the absence of a paediatrician at the hospital. We aimed to recruit 80 patients per site but conflict in the Myitkyina area restricted recruitment.

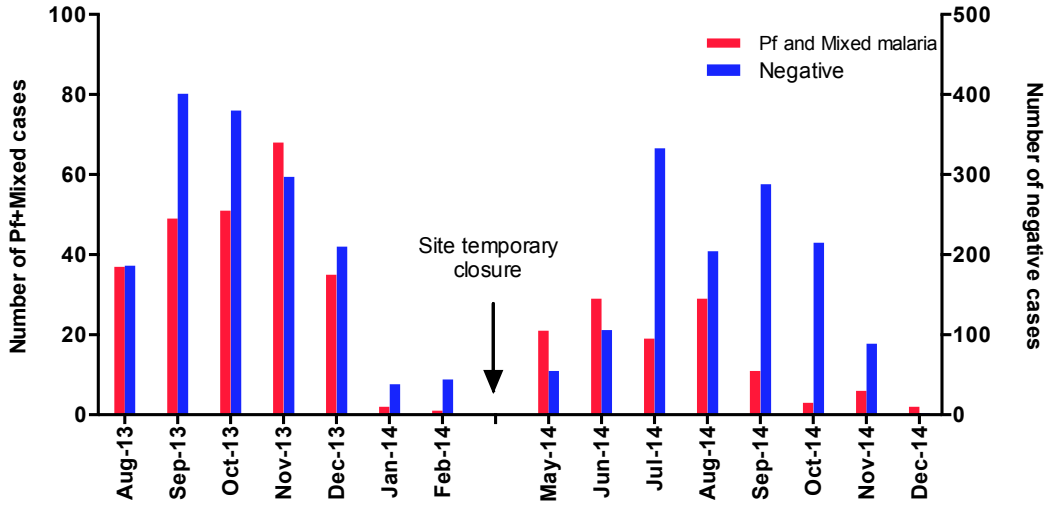


Figure 4.1 Study enrollment screening from Aug 2013 to Dec 2014

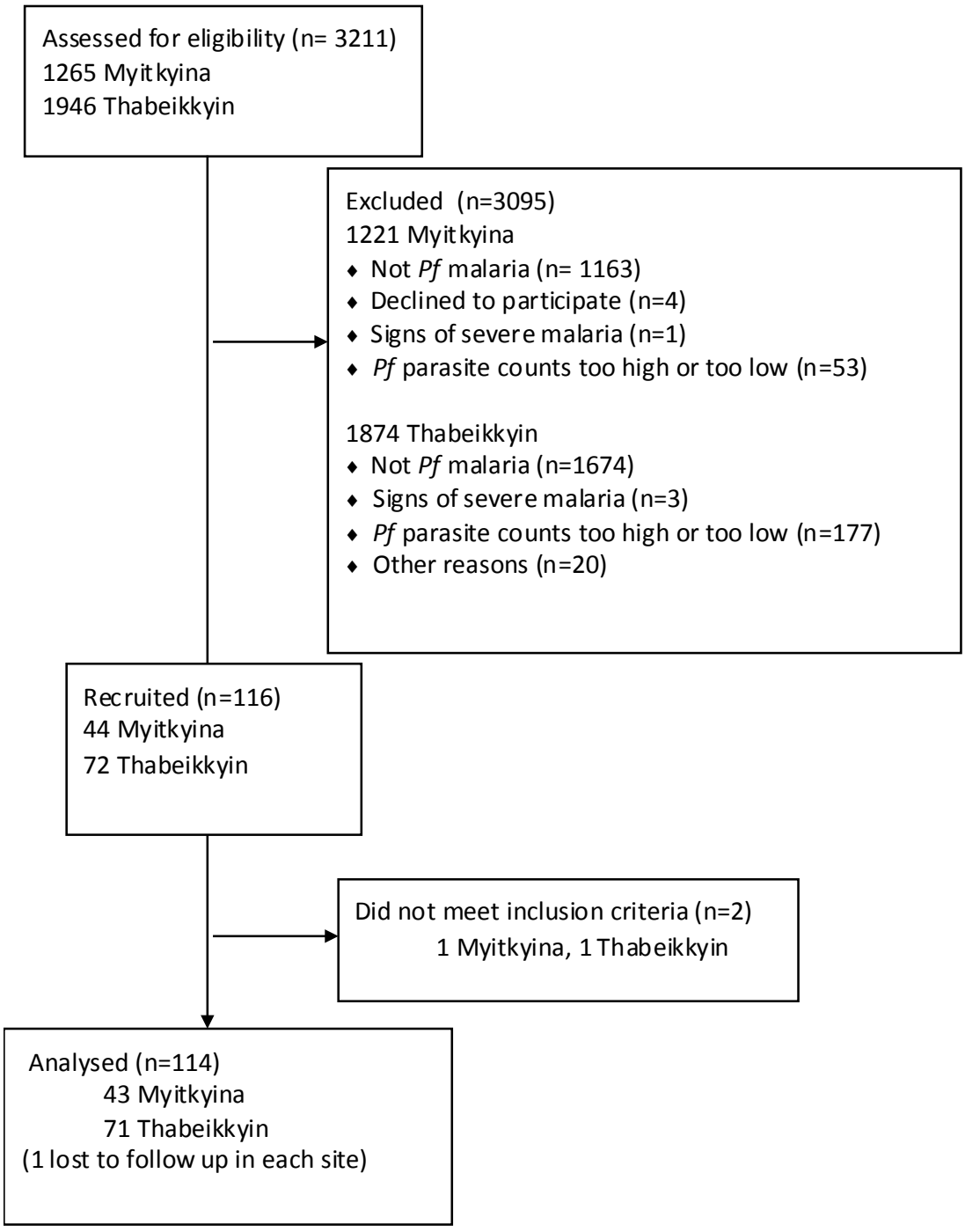


Figure 4.2 Participant flow diagram

4.5.1 Patient characteristics

The median patient age was 19 years (IQR, 12 to 25), and males accounted for 75% of all participants. The median body mass index was 18.3 (IQR, 15.2 to 20.5). The geometric mean (95%CI) parasite count on admission was 35,011 and ranged from 28,613 to 42,839 parasites per μL.

Twelve percent (14/114) of patients presented with mixed infections on admission. Thirty three percent of patients had an axillary temperature >37.5°C before treatment and the median of haematocrit was 37 (IQR, 32 to 42) (Table 4.1). The distributions of symptoms, drug and medical history are shown in Table 4.2. The most frequently reported symptoms were headache (67%), dizziness (49%), muscular pain (36%) and joint pain (35%) in all patients. Only 3.5% of patients reported past history of malaria. The median times to resolution of fever in Myitkyina and Thabeikkyin were 6 and 12 hours respectively. There was a significant difference in fever resolution time between the two sites (Mann Whitney U Test, p=0.008)(Table 4.3)

4.5.2 Clinical and early parasitological responses

Median (IQR) parasite clearance half-life at Myitkyina was 4.4 hour (3.4 to 6.7), significantly longer than Thabeikkyin (2.6 hour (2.0 to 3.8, p<0.001))(Figure 4.3A). In Myitkyina 39% of patients had a parasite clearance half-life more than a previously used cutoff (5 hours) compared with 13% in Thabeikkyin (p=0.001); using a 4h cutoff these proportions were 63% and 23% respectively (p<0.001). Twenty of 114 participants (18%) remained parasitaemic 3 days after treatment (30.2% of patients in Myitkyina and 9.9% in Thabeikkyin (p=0.01)). Fever

resolution was rapid (median resolution time was 6 hours in Myitkyina, 12 hours in Thabeikkyin, p=0.008) (Table 4.3).

Table 4.1 Baseline characteristics of the patients by study site

Study site		**Total**	**Myitkyina**	**Thabeikkyin**
Number of patients		114	43	71
Gender (Male sex)	n (%)	86 (75.4%)	40(93.0%)	46(64.8%)
Age	yr	19 (12 to 25)	23 (19 to 29)	13 (11 to 21)
Weight	kg	45 (30 to 54)	54 (45 to 56)	34 (23 to 50)
Height	m	1.57 (1.36 to 1.63)	1.63 (1.6 to 1.65)	1.47 (1.3 to 1.58)
BMI	kg/m2	18.3 (15.2 to 20.5)	20.5 (18.4 to 21.6)	16.3 (14.3 to 19.5)
Parasite count (geometric mean, 95%CI)	No./μL	35,011 (28,613 to 42,839)	36,779 (26,809 to 50,446)	33,982 (26,016 to 44,287)
Gametocytaemia on Day 0	n/N (%)	10/114 (8.8)	9/43 (20.9)	1/71 (1.4)
Mixed infection	n/N (%)	13/114 (11.4)	8/43 (18.6)	5/71 (7.1)
Haematocrit	%	37 (32 to 42)	38 (33 to 42)	37 (32 to 42)
Fever on admission	n/N (%)	37/114 (32.4)	12/43 (27.9)	25/71 (35.2)
Heart rate	bpm	98 (87 to 111)	92 (82 to 102)	104 (92 to 121)
Respiratory rate	bpm	24 (24 to 27)	24 (24 to 25)	26 (23 to 29)
Systolic Blood Pressure	mmHg	108.5 (100 to 115)	110 (100 to 115)	108 (100 to 118)
Diastolic Blood Pressure	mmHg	63 (60 to 70)	60 (60 to 70)	65 (59 to 71)

Data are presented as median (IQR) unless otherwise indicated.

Table 4.2 Symptoms on admission, and drug and past medical history

History	*No (%)*
Symptoms	
Dizziness	56/114 (49.1)
Headache	76/114 (66.7)
Nausea	37/114 (32.5)
Anorexia	31/114 (27.2)
Vomiting	29/114 (25.4)
Diarrhoea	8/114 (7.0)
Abdominal pain	3/114 (2.6)
Joint pain	40/114 (35.1)
Muscular pain	41/114 (36.0)
Palpitations	5/114 (4.4)
Difficulty breathing	3/114 (2.6)
Tiredness	4/114 (3.5)
Drug history	
Paracetamol	19/114 (16.7)
Amoxicillin	2/114 (1.8)
Chlopheniramine maleate	3/114 (2.6)
Vitamins	3/114 (2.6)
Milk of magnesia	1/114 (0.9)
Traditional medicine	3/114 (2.6)
Past Medical history	
Malaria	4/114 (3.5)
Febrile convulsion	2/114 (1.8)
Pulmonary tuberculosis	1/114 (0.9)

Table 4.3 Fever clearance time, parasite clearance time and clinical presentation

Study site		**Total**	**Myitkyina**	**Thabeikkyin**	**P value**
Median time of fever clearance (IQR)	hours	6 (6 to 12)	6 (6 to 6)	12 (6 to 18)	0.008**
Median parasite clearance Half-life (IQR)		3.3 (2.1 to 4.8)	4.4 (3.4 to 6.7)	2.6 (2.0 to 3.8)	<0.0001**
Parasite clearance time (Median, IQR)		42 (30 to 72)	72 (42 to 96)	36 (24 to 72)	<0.0001**
Parasite clearance Half-life >4 hours	n/N (%)	42/114 (37.7)	27/43 (62.8)	16/71 (22.5)	P<0.001*
Parasite clearance Half-life >5 hours		26/114 (22.8)	17/43 (39.5)	9/71 (12.7)	P<0.01*
Day 3 parasitaemia		20/114 (17.5)	13/43 (30.2)	7/71 (9.9%)	P<0.01*
Gametocyte carriage time (Median, IQR)	hours	18 (9 to 57)	57 (6 to 81)	16.5 (12 to 24)	0.31
ACPR on day 42	% (95%CI)	100 (97 to 100)	100 (92 to 100)	100 (95 to 100)	

* Chi square statistics, ** Mann-Whitney test, ACPR- Adequate Clinical and Parasitological Response

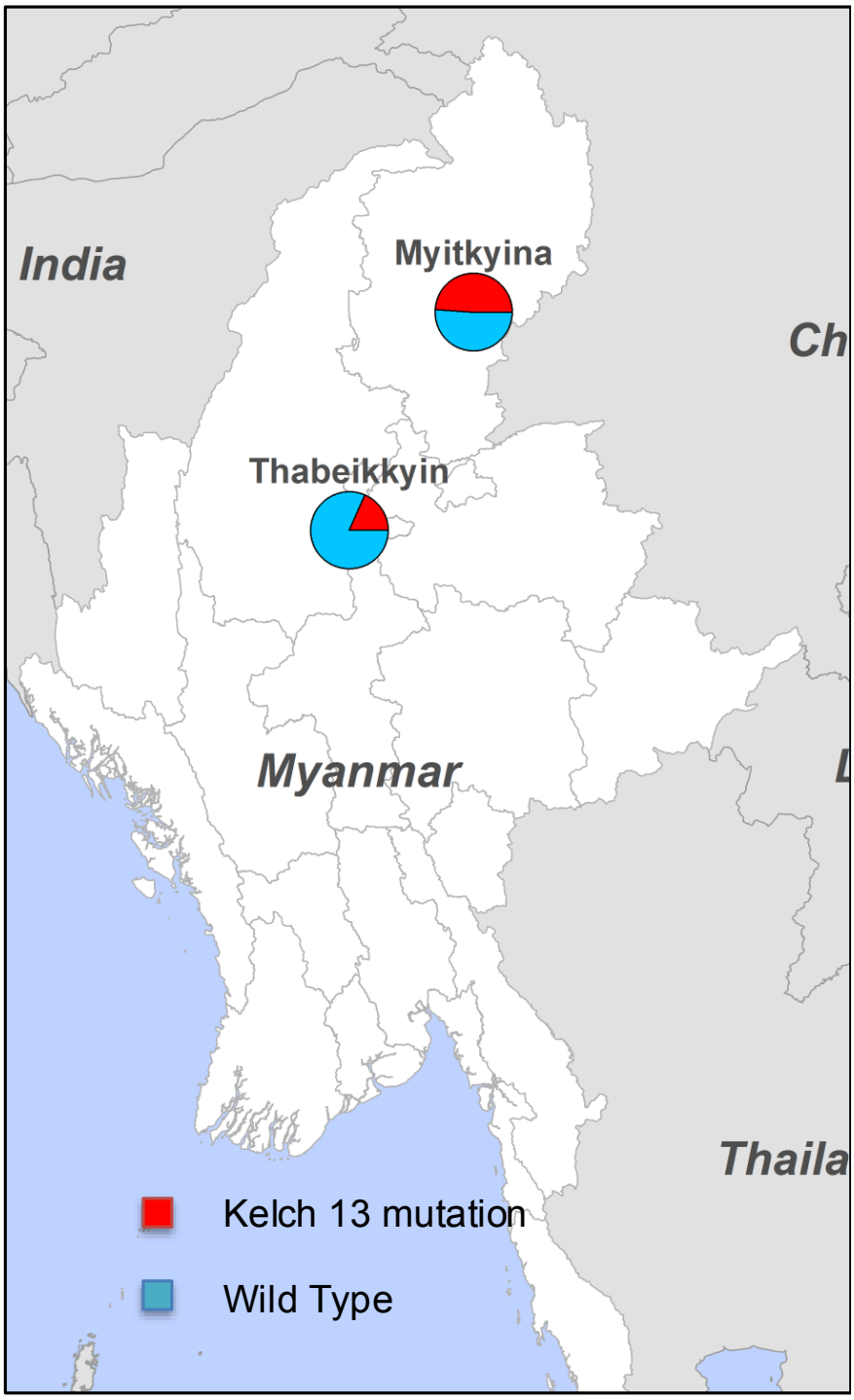


Figure 4.3 Geographic distribution of *Pf* kelch13 mutations in two study sites of Myanmar

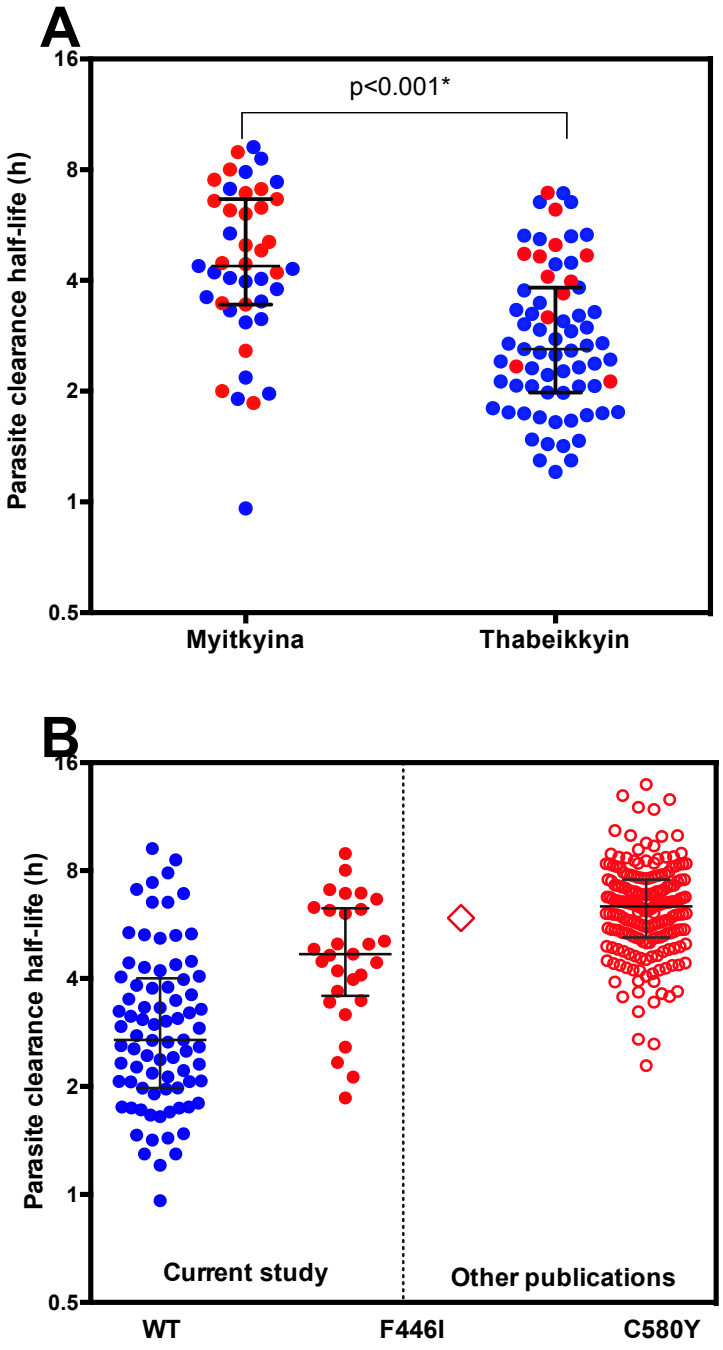


Figure 4.4 Distribution of parasite clearance half-lives by (A) geographical region (B) *Pf* kelch13 sequence. Solid circles are from the current study, the diamond represents summary data from Huang *et al.* (115) and hollow circles are from Ashley *et al.* (93). Red symbols represent parasites with kelch13 propeller mutations, blue symbols represent wild type. Median and IQR are shown.

Table 4.4 Prevalence of *Pf* kelch13 mutations

Kelch13	Total No (%)	Myitkyina No (%)	Thabeikkyin No (%)
Wild type	81 (71.1)	22 (51.2)	59 (83.1)
P443S	1	1	-
F446I	29	18	11
G538V	1	-	1
P574L	1	1	-
A676D	1	1	-
All kelch13 propeller	33 (28.9)	21 (48.8)	12 (16.9)

4.5.3 Curative efficacy

After initial parasite clearance all patients remained parasite free until 42 days (Table 4.3); two patients were lost to follow-up before 42 days with negative blood films at their last follow-up visit.

4.5.4 Kelch13 sequence and parasitological response

The kelch13 gene was successfully sequenced in all 114 samples. The prevalence of propeller (>440) mutations was 28.9% (33/114) (Table 4.4); among these the F446I mutation predominated (29/33, 87.8%). In Myitkyina 21 of 43 (49%) of patients had kelch13 mutations compared with 12 of 71 (16.9%) in Thabeikkyin (Figure 4.3). All kelch13 mutations observed had been reported in previous studies. No mutations were found outside the propeller region (amino acids 210-440).

The parasite clearance half-life was associated with kelch13 sequence. Median parasite clearance half-lives associated with wild type and the F446I mutation was 2.7 and 4.7 hours respectively ($p<0.001$)(Figure 4.4B). The single cases of propeller mutations P443S, G538V, P574L and A676D were associated with half-lives of 6.6, 4.7, 7.5, and 2.0 hours respectively.

The proportion of patients still parasite positive at day 3 was higher in infections with parasites with kelch13 mutations (10/33, 30.3%) compared to those with wild type alleles (10/81, 12.3%) ($p=0.02$).

4.5.5 Factor associated with prolonged parasite clearance

For multivariable analyses parasite clearance half-life cut-off values of both 4 and 5 hours were used. With the 4h cutoff, presence of a kelch13 propeller mutation had an odds ratio of 5.0 (95% CI 1.9 to 13.0, $p=0.001$) for predicting slow clearance while study site ('recruitment in Myitkyina') had an odds ratio of 4.0 (1.7 to 10.1, $p=0.002$). Using a cutoff of 5h, these ratios were 2.3 (95%CI 0.9 to 6.1, $p=0.1$) and 3.5 (95%CI 1.3 to 9.4, $p=0.01$) respectively (Table 4.5).

Persistent parasitaemia at day 3 was significantly associated with site and parasitaemia on admission (using a cutoff of 1%, close to the median) (Table 4.5); in this multivariable analysis kelch13 sequence was not a significant factor.

Table 4.5 Multivariate analysis examines relationship between clearance half-life or day 3 positivity and covariates kelch13 propeller mutations, study site and admission parasitaemia

Dependent variable	*Odds ratio*	*95% CI*	*P value*
Half-life cutoff 4 hours			
Site (Myitkyina)	4.1	1.7 to 10.1	0.002*
Kelch 13 (>440)	5.0	1.9 to 12.9	0.001*
Half-life cutoff 5 hours			
Site (Myitkyina)	3.5	1.3 to 9.4	0.01*
Kelch 13 (>440)	2.3	0.9 to 6.1	0.1
Day 3 positivity			
Parasitaemia on admission (>1% parasitaemia)	4.0	1.2 to 11.4	0.02*
Site (Myitkyina)	3.2	1.1 to 9.6	0.04*
Kelch 13 (>440)	2.0	0.7 to 4.0	0.68

4.5.6 Gametocytaemia

Ten patients (8.8%) had patent gametocytaemia at the time of enrollment, with most of these recruited in Myitkyina (Table 4.1). The geometric mean (95%CI) gametocyte density on admission was 83/μL (34 to 201). Fourteen patients (12%) had patent gametocytaemia after admission.

The median (IQR) durations of gametocyte carriage in Myitkyina and Thabeikkyin were 57 (6 to 81) and 16.5 (12 to 24) hours respectively. Overall gametocyte carriage rate was 26 PGW per 1,000 weeks of follow up. There was no association between gametocyte carriage duration and geographical location (p=0.31), or parasite clearance half-life (p=0.25). All patients with gametocytaemia cleared gametocytes within 96 hours from the time gametocytes were first seen (Figure 4.5).

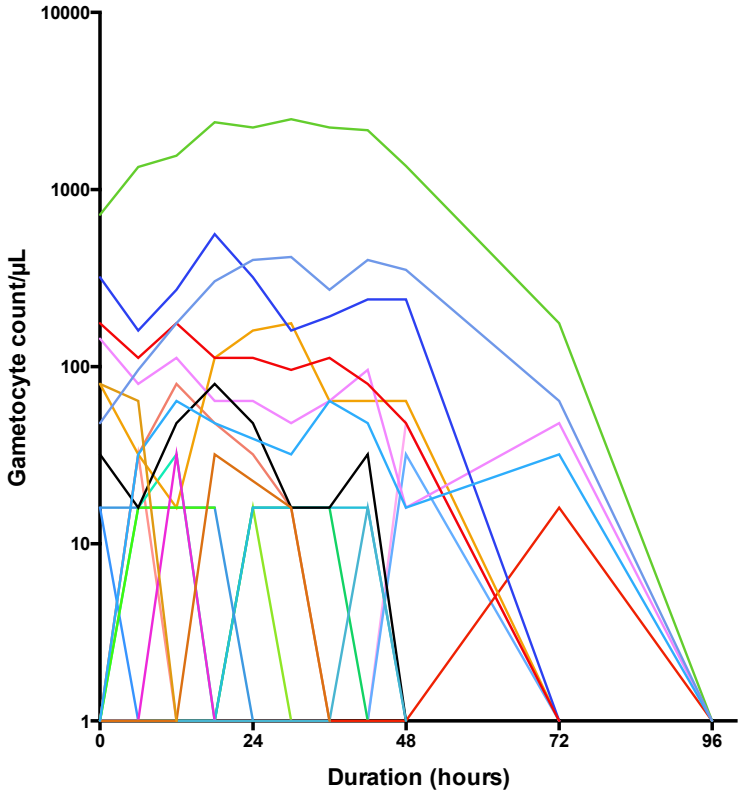


Figure 4.5 Gametocyte carriage time – counts over time from individual patients

4.5.7 Haematological changes

One third of participants (35%) were anaemic on enrolment (Table 4.6). The nadir in mean haematocrit occurred on day 3, after which haematocrit values gradually increased (Figure 4.6). There were no clinically significant falls in haemoglobin, or haemoglobinuria. By day 28, most of the participants' haematocrit values exceeded baseline measurements (Figure 4.6).

Table 4.6 Prevalence of anaemia during study period

Site	*Day 0* *n/N(%)*	*Day 7* *n/N(%)*	*Day 14* *n/N(%)*	*Day 21* *n/N(%)*
Myitkyina	14/43 (32.5)	12/43(27.9)	10/43(23.2)	11/43(25.5)
Thabeikkyin	26/71(36.6)	38/71(53.5)	35/71(49.3)	23/71(32.3)
Total	40/114(35)	50/114(43.8)	45/114(39.4)	34/114(29.8)

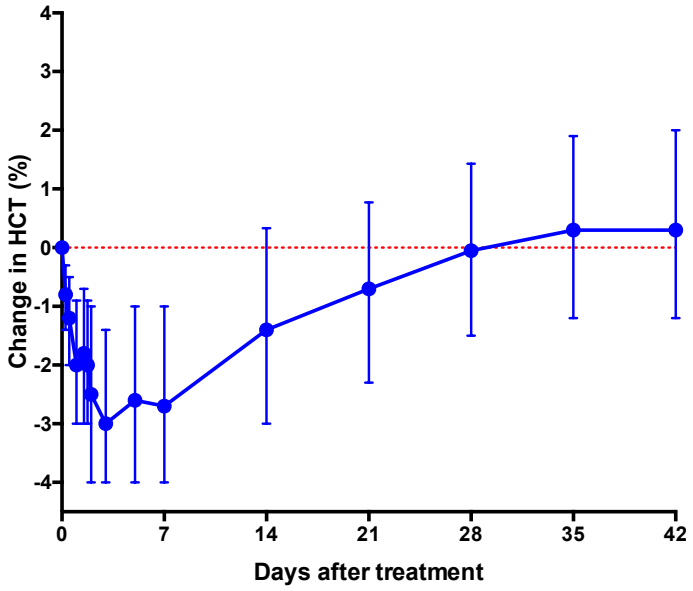


Figure 4.6 Mean (95%CI) change in haematocrit compared with admission value during 42 day follow up (both study sites)

4.5.8 Highly sensitive PCR (uqPCR) findings

The total number of patients with parasitaemia assessments by uqPCR on days 0, 3, 5, 7, 9, 14 and 21 were 109, 109, 108, 109, 105, 40 and 42, respectively (collection of day 14 and 21 samples began after analyses of the first batch of samples showed longer than expected uqPCR parasite clearance times). On admission every patient had positive uqPCR parasitaemia and at day 21 40% were still positive using uqPCR (Figure 4.7) even though all clinical parameters were normal and microscopy negative.

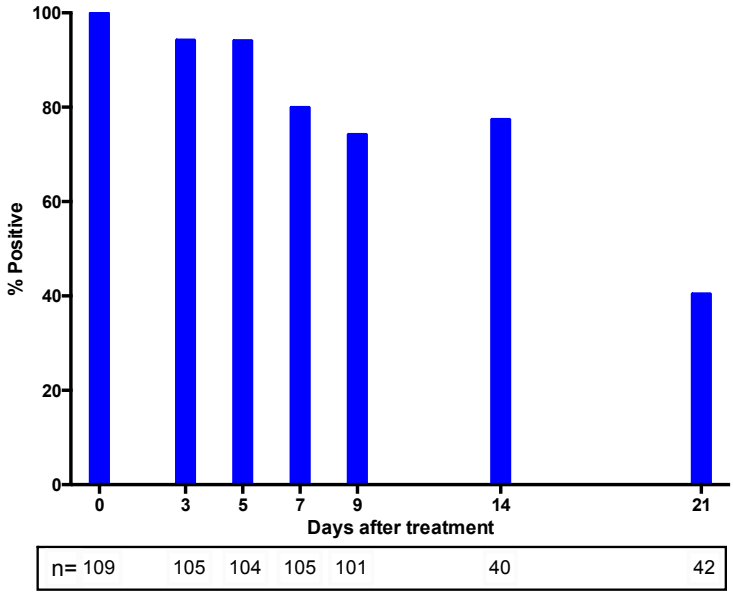


Figure 4.7 Prevalence of parasitaemia determined by ultrasensitive PCR (uqPCR) from day 0 to day 21. (n=number of sample size at each time point)

Figure 4.8 shows individual patients' parasite density by kelch13 mutations using uqPCR method from day 0 to day 21 after ACT treatment. Patients with kelch13 propeller mutations on admission showed 50% (8/16) positive parasitaemia and those with wild type 35% (9/26) at day 21 using uqPCR.

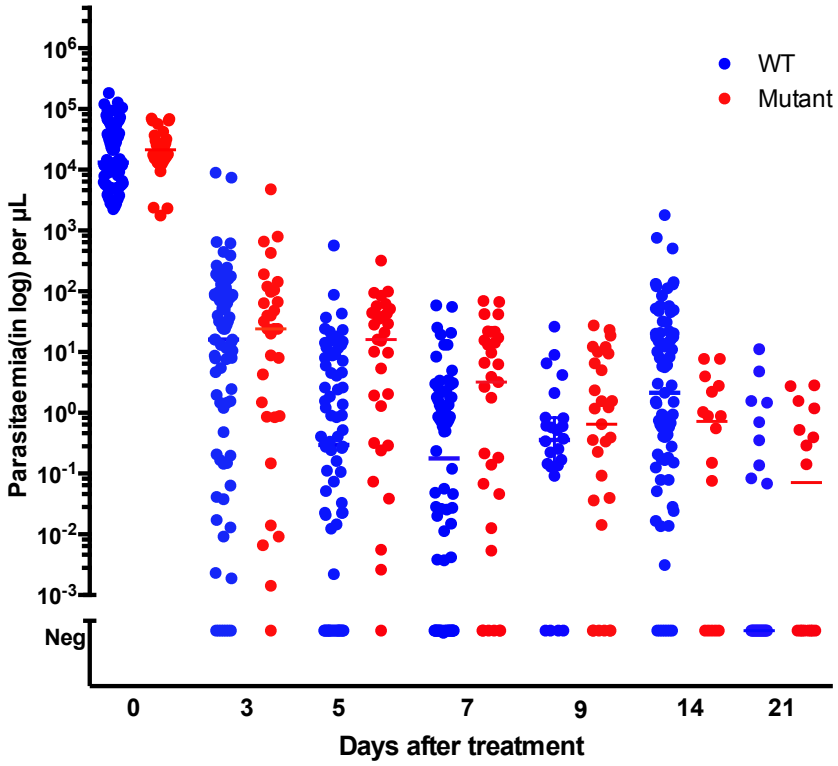


Figure 4.8 Kelch13 propeller mutations and days after ACT treatment. One circle represents one patient, red circles are mutation after amino acid position 440, blue circles represent wild type alleles.

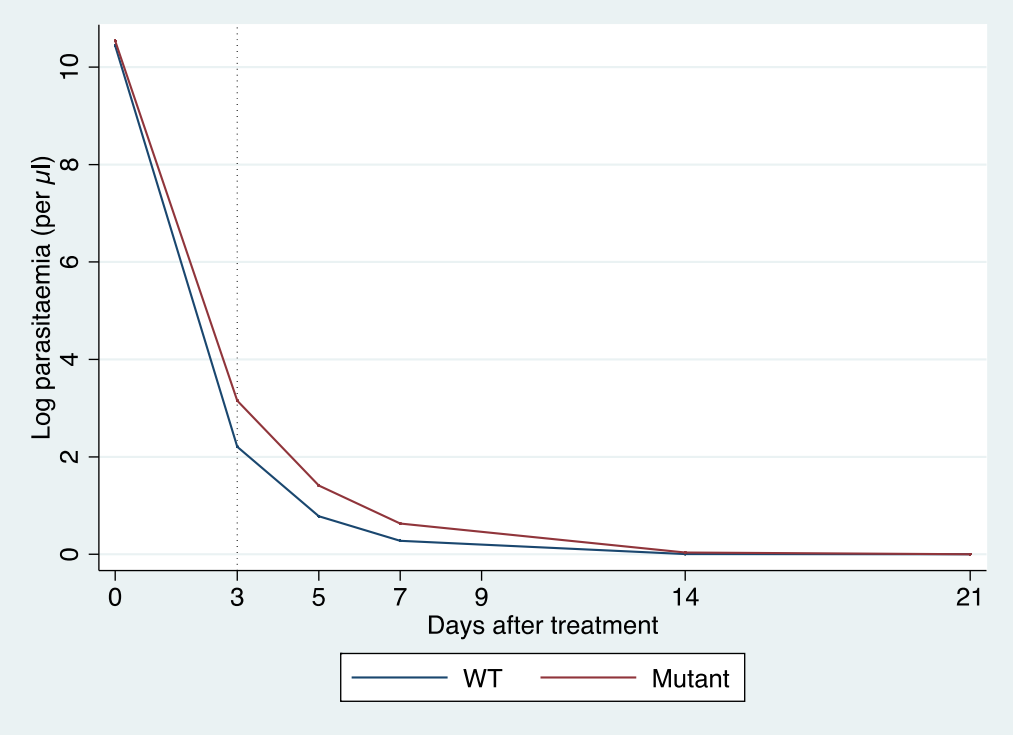


Figure 4.9 Parasitaemia and days after treatment determined using uqPCR by wild type and kelch13 mutations

Nonlinear exponential regression was used to fit the model for the reduction in parasitaemia over time after the treatment as shown in Figure 4.9. The first compartment shows rapid reduction of residual parasites due to dihydroartemisinin and the slow reduction of residual parasites in the second compartment is probably due to partner durg action. After adjusting admission parasitaemia, no significant reduction of parasite density in patients with wild type was found compared with kelch13 propeller mutations at day 3 using analysis of covariance (p=0.2).

A total of 114 patients were included in the survival analysis and total time at risk was 1,286 days. The percentage uqPCR positive between wild type and kelch13 mutations were 27 (13 to 42) and 42 (21 to 62) respectively and the hazard ratio is 0. 82 (0.4 to 1.6)(Table 4.7).

The chance of becoming uqPCR negative by day 21 after ACT treatment in patients with kelch13 mutations is 18% lower than those with wild type, however statistically this difference was not significant (p=0.6).

The Kaplan-Meier curves showed that infections with kelch13 mutation-carrying parasites had a higher positivity rate than wild type at day 21 (Figure 4.10) but this was not statistically significant.

Table 4.7 Positivity rates and hazard ratio for risk factor of uqPCR positivity at 21 days after treatment

Risk factor		*Percentage of uqPCR positive*	*Hazard Ratio (95% CI)*
Kelch13	WT	27 (13 to 42)	0.82 (0.4 to 1.6)
	Mutations (>440)	42 (21 to 62)	

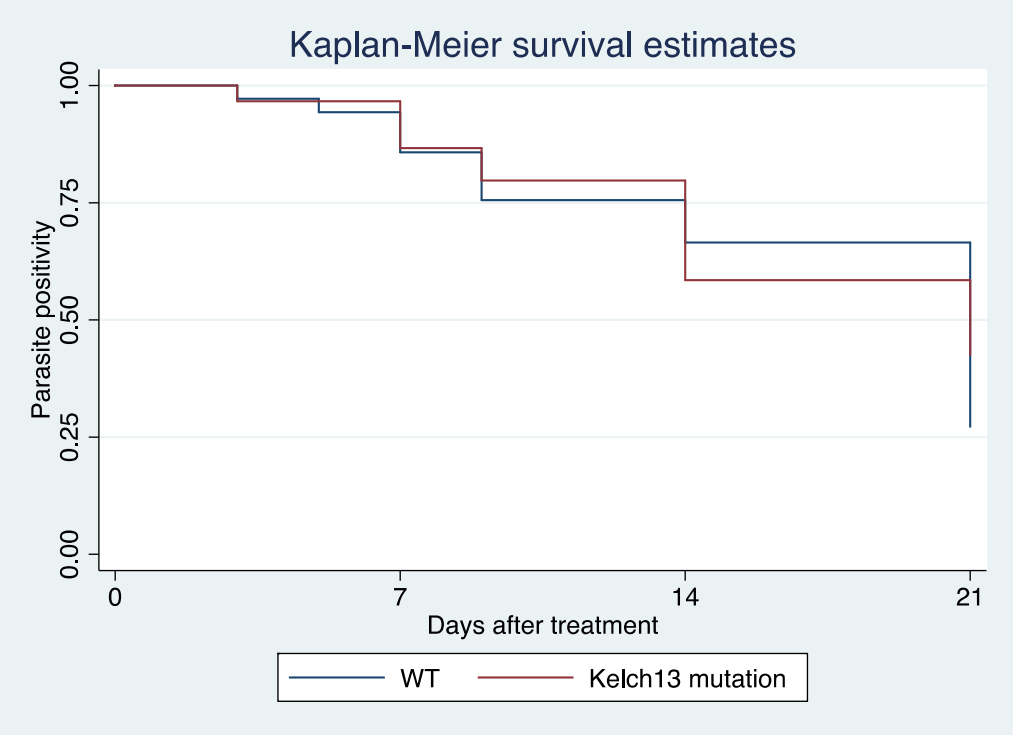


Figure 4.10 Kaplan-Meier survival curves of kelch13 mutations and wild type.

4.5.9 Safety

The actual doses (median and IQR) received by patients for dihydroartemisinin, piperaquine and primaquine were 2.2 (2.1 to 2.3), 17.5 (16.6 to 18.5) and 0.26 (0.24 to 0.28) mg per kilogram respectively. Study medications were generally well tolerated although six patients vomited the day 0 medication, and one vomited on day 2; all were retreated according to the protocol (Table 4.8). One serious adverse event was reported at the Myitkyina site; one patient who had already missed follow-up visits was killed in a car accident approximately two weeks after the last visit.

Table 4.8 Patients who vomited study drugs during first three days of treatment

Subject ID	Site	Day of Treatment	Retreatment
MM004-028	Thabeikkyin	Day 0	Yes
MM004-035	Thabeikkyin	Day 0	Yes
MM004-040	Thabeikkyin	Day 0	Yes
MM004-041	Thabeikkyin	Day 0	Yes
MM004-044	Thabeikkyin	Day 0	Yes
MM004-054	Thabeikkyin	Day 0	Yes
MM004-001	Thabeikkyin	Day 2	Yes

4.6 Discussion

In this study conducted in northern and central Myanmar dihydroartemisinin-piperaquine was highly efficacious, despite emerging artemisinin resistance. The day 3 positivity rate was 18% overall (30% in Myitkyina) (60).

Our study protocol was designed specifically to assess prolongation of parasite clearance half-lives, the hallmark of artemisinin resistance in *P. falciparum* (60). Parasite clearance half-life is less prone to confounding by baseline parasitaemia than day 3 parasite positivity (102, 103, 178), and remains the gold-standard for determining resistance phenotypes needed for the characterization of artemisinin resistance (80, 82, 85).

The use of clearance half-life to define artemisinin resistance was first validated in western Cambodia, where artemisinin resistance was first documented. For the kelch13 genotypes associated with long clearance times in Cambodia a dichotomous distribution of half-lives is evident and a 5-hour cut-off provides satisfactory discrimination (103). At the Thai-Myanmar border where longitudinal studies have been undertaken for more than two decades, the C580Y mutation only recently replaced earlier kelch13 mutations associated with lesser effects on parasite clearance (93, 170).

Consistent with previous data from the Myanmar-China border and molecular surveys (115, 149, 179), F446I was the predominant kelch13 mutation in our study. This mutation is associated with a significant slowing in parasite clearance, but not to the same extent as that associated with the mutations prevalent further east. The median parasite clearance half-life of F446I infections in our study was 4.7 hours, less than that obtained by Huang *et al*. (115) in patients recruited

nearby at the Myanmar-China border (less than 100km from our Myitkyina site). This difference may reflect the more frequent measurement of parasitaemia in the current study; our protocol used 6-hourly assessment of parasitaemia in order to capture the lag phase of clearance and avoid systematic overestimation of slope half-life (101, 102). Other factors may also contribute to the differences, including stochastic effects due to sample size, baseline parasitaemia (although clearance half-life is less prone to this influence than day 3 positivity (180)), host effects (fever, haematocrit, immune status and genetics (171)), drug factors and accuracy of microscopic assessment.

Measuring parasite counts for every six hours in first three days until two blood slides were negative have been recommended and TRAC and other studies (62, 93) have measured parasite count in accordance with the above recommendation. Artemisinin monotherapy for first 3 to 7 days and then ACTs were used to treat the patients in these two studies. A modelling approach has been used to determine the optimal sampling design for parasite clearance (101), leading to the proposal that in areas of unknown resistance status, a more intense schedule for the first two days and then daily blood smears until two blood slides are negative. Therefore, in this trial a new optimal sampling schedule was used because the recommended 6 hourly for first three days was a difficult schedule for study nurses and research physicians in remote clinical settings. Moreover, in this study we used ACT directly to treat the uncomplicated malaria according to national guidelines and therefore, the rate of parasite clearance half-lives of my study might be faster than these two studies (62, 93).

There has been uncertainty whether there is significant artemisinin resistance in northern Myanmar. The finding that F446I, the prevalent genotype in the region,

is associated with an intermediate artemisinin resistance phenotype helps to interpret clinical and laboratory studies undertaken recently at Myanmar-China border. Several publications have documented relatively low day 3 parasite positivity rates in clinical studies from Yingjiang County, Yunnan Province (115, 123, 124), where frequencies of the F446I mutation are generally 40-60% (115, 179, 181). For example, in the series of therapeutic efficacy studies from Yingjiang county described by Huang *et al.* (115), day 3 positivity rate was approximately 12% while kelch13 propeller mutation prevalence was 56% (mostly F446I mutants). Most infections carrying kelch13 propeller mutants were cleared by microscopy by day 3, suggesting a 'milder' resistance phenotype than observed in the TRAC study (centred on western Cambodia) where most patients carrying kelch13 mutations remained parasite positive at day 3 (93). This indicates that day 3 positivity has limited sensitivity as an indicator of intermediate levels of artemisinin resistance. The F446I mutation prevalence has clearly risen at the Myanmar-China border (115, 116, 179), suggesting preferential survival after treatment compared to wild-type parasites, and thus selection.

In this study, site was also significantly associated with delayed parasite clearance (day 3 positivity and prolonged half-life), independent of kelch13 mutations. This may again reflect host effects although an interesting, but less likely, possibility is that artemisinin resistance might be additionally mediated through an alternative (non-kelch13) mechanism. Kelch13 wild-type infections remaining positive at day 3 have been found in other parts of Myanmar (112). Most probably, this could be a stochastic effect with long half-life infections

simply representing the top end of the log-normal distribution of parasite half lives observed with kelch13 wild-type infections (103).

Genome-wide studies of parasite polymorphism will allow study of the population structure of F446I mutant parasites and determine whether underlying 'backbone' mutations play a role (85).

What is the future for DHA-piperaquine in Myanmar? At present, it is still a highly efficacious and well tolerated treatment for falciparum malaria in Upper Myanmar, but if artemisinin resistance worsens, then increasing reliance will be placed on the piperaquine component. Replacement of the intermediate phenotype with a severe phenotype as has happened further east would place greater pressure on the piperaquine component. Worsening partner drug resistance has rapidly followed emergence of artemisinin resistance at both the Thai-Myanmar border (94) and in Cambodia, where piperaquine resistance has now emerged and DP failure rates have risen alarmingly in the last two years (97, 98, 154, 182). In spite of spread of artemisinin resistance, we found that dihydroartemisinin-piperaquine is still efficacious in Upper Myanmar. The Global Fund distributes artemether-lumefantrine through the Myanmar government health system and international non-governmental organizations free of charge across the entire country. Therefore dihydroartemesinin-piperaquine is not very popular among practitioners because of higher market price compare to artemether-lumefantrine and that is one of the possible explanations for its high treatment efficacy.

ACTs clear gametocytes four fold more efficiently than non-ACT treatments and when combined with PQ gametocyte clearance is four fold faster than ACTs alone (183). The gametocyte clearance time (GCT) after treatment with AL is about 7-10 days and if single dose PQ 7.5mg/kg is added the GCT is <7hours

(184). The active metabolite(s) of PQ remain undetermined, though host factors, particularly polymorphisms in CYP2D6, the enzyme thought to be responsible for their *in vivo* production, appear to play a role in determining PQ efficacy. The half-life of PQ is 4-8 hours (185, 186). Treatment with PQ at 0.065 mg/kg along with a full course of AL resulted in very low infectivity to mosquitoes, and 0.25 mg performed even better (184). In the current study, treatment with DP and low dose primaquine resulted in 26 PGW per 1,000 weeks. In comparison with other studies done in Myanmar or in Africa (63, 187, 188), the PGW is shorter than with DP alone but three times longer than DP plus 0.75 mg primaquine. It is possible that this is because we used a three times lower dose of primaquine than Smithius and collegues' study, which was also conducted in Myanmar (63).

The median (IQR) gametocyte carriage time in this study was 18 (9 to 57) hours, significantly less than in the TRAC study (160 (<6 to 252) hours) where primaquine was not used (93). Hence the addition of a single low dose of primaquine (0.25 mg/kg) resulted in low rates of gametocyte carriage and was well tolerated, with rapid haematological recovery from malarial anaemia. G6PD deficiency gene frequencies in Myanmar typically range from 10-20% so it is likely that G6PD-deficient male patients in this study were exposed to primaquine, but without significant haemolysis.

Studies have looked at the effect of different dosages of PQ on P. falciparum gametocyte clearance. Eziefula *et al.* (64) stated that 0.4 mg/kg was as good as 0.75 mg/kg dose but doses between 0.1 and 0.4 mg/kg needed further evaluation. Our results demonstrate that adding 0.25 mg/kg of PQ appears to be effective at clearing gametocytes, though our study consisted of a single arm

without a control and gametocytes were detected by microscopy only and infectivity was not assessed. Adding primaquine to DP would be easy, safe and practical for both health care providers and patients. Further studies with more focus on gametocyte detection by PCR methods and/or mosquito infectivity after treatment with low dose primaquine plus ACT are therefore indicated.

We used a highly sensitive qPCR method developed to detect very low parasitaemia (down to 20 parasites per mL) (21). Because of the emergence of artemisinin resistance problem in Southeast Asia, health authorities have committed to contain and ultimately eliminate artemisinin resistance malaria in this region. For these reasons it is important to know the epidemiology of malaria, especially that of submicroscopic asymptomatic malaria. There is very little published data on the time course of parasitaemia measured by uqPCR following the treatment of symptomatic malaria (189, 190). Although all patients in our study had cleared parasitaemia measured by microscopy by day 5, using uqPCR we found that 40% of patients still carried parasites on day 21 despite treatment with a supervised full course of an ACT. uqPCR positivity rate at day 21 was higher than found in the Thriemer *et al.* study in central Vietnam, which found on conventional qPCR 25% residual parasitaemia after treatment with the same ACT (190). In this study despite the high percentage of uqPCR positivity at day 21 there were no treatment failures by the standard WHO criteria (i.e. all patients except for the 2 lost to follow up achieved adequate clinical and parasitological response – ACPR). At day 21 after treatment, the chance of becoming uqPCR negative in infections with kelch13 mutation-carrying parasites was 18% lower than those with wild type (p=0.6). Further research is necessary to determine

whether this submicroscopic infection consists of asexual parasites, possibly in a dormant or ‘sleeping’ form, or gametocytes.

4.7 Conclusion

In summary, this study shows that although artemisinin resistance has emerged and is established in Upper Myanmar, treatment efficacy remains high with DP. Clearance half-life remains the gold-standard measure in research studies of artemisinin resistance, particularly those conducted in new geographical contexts. The main kelch13 mutation observed, F446I, is associated with a median clearance half-life of slightly under 5 hours, representing an “intermediate resistance” phenotype. The study also emphasises the value of frequent *in vivo* assessments of parasite clearance in locations where susceptibility to artemisinins is previously unknown (101). The sampling schedule of blood smears (6 hourly for 48 hours then daily, until two negative slides) is more convenient for patients and investigators than the one used in TRAC (93), but performs well (101). Approximately one third of patients treated with DP still carried residual parasite using uqPCR on day 21 despite treatment with a supervised full course of an ACT. As artemisinin resistance is clearly well established in Myanmar, piperaquine and other ACT partner drugs are at risk, more research need to assess therapeutic efficacy of first line antimalarial drugs carefully and possible deployment of triple ACTs or extended ACT courses.

4.8 Summary of findings

The salient findings of chapter 4 are as follows:

- The median (IQR) parasite clearance half-life with DP at Myitkyina was 4.4 hour (3.4 to 6.7), significantly longer than Thabeikkyin (2.6 hour (2.0 to 3.8, $p<0.001$)).
- Parasite clearance half-life was greater than 5 hours in 21% of patients.

- There was no failures after 42 days of follow-up, although 18% of patients had persistent parasitaemia on day 3 blood smear (p=0.6).
- Patients infected with parasites with kelch13 propeller mutations showed 50% positive parasitaemia and those with wild type 35% at day 21 using uqPCR.
- After adjusting for admission parasitaemia, there was no significant difference in parasite density (measured using uqPCR) at day 3 in patients with wild type parasites compared with those with kelch13 propeller mutant parasites (using analysis of covariance, p=0.2).
- The dominant kelch13 propeller mutation observed in Upper Myanmar, F446I, was associated with an intermediate rate of parasite clearance (median clearance half-life of 4.7 hours) compared to other kelch13 propeller mutations described elsewhere in the Greater Mekong Subregion.
- The presence of distinctive phenotypes outside the 'epicentre' of artemisinin resistance has implications for our understanding of artemisinin resistance and how it should be detected and monitored.
- The findings in this chapter are likely to be of relevance to agencies and researchers involved in monitoring artemisinin resistance and deploying artemisinin combination therapies.

5 The effectiveness and safety of a 3 day versus 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar (3 vs. 5 AL trial)

5.1 Chapter 5 Abstract

Background: A therapeutic efficacy study in *P. falciparum* malaria conducted at 7 sites across Myanmar in 2010 under the auspices of WHO showed that the 28 days adequate clinical and parasitological response rate with artemether-lumefantrine (AL) ranged from 94% to 100% (130). More recently, with the spread of artemisinin resistance, there is evidence ACT failure in some areas. In 2014 artesunate-mefloquine was reported as starting to fail in Kayin State along the Thai-Myanmar border, with a day 42-cure rate of only 42% (121). It became clear that new therapies needed to be evaluated to treat artemisinin-resistant malaria in Greater Mekong Subregion. One option is to use an extended 5 day course of ACT, allowing more parasites to be killed by the artemisinin derivatives leaving a smaller residual number for the partner drug to act on and thereby reducing the probability of parasites surviving to recrudesce during follow up. The aim of the study reported in this chapter was to compare a 5 day regimen of AL with the standard 3 day regimen for the treatment of uncomplicated falciparum malaria. A novel aspect of this trial was the use of sensitive molecular detection methods on day 5 and 7 to assess parasitocidal efficacy.

Method: A randomized, 2 arm, open-label, clinical and parasitological study was conducted in village health centres in Kyainseikgyi Township, Kayin state comparing AL 3 days with AL 5 days. Both arms received single low dose primaquine 0.25 mg/kg at day 0. This trial was registered at ClinicalTrial.gov (NCT02020330). We determined parasite clearance dynamics using highly ultra-sensitive PCR (uqPCR) of samples from day 0 to day 21. Parasitaemia on blood

smears was checked from day 0 to day 42 and therapeutic responses were assessed as well as parasite kelch13 genotypes.

Results: 155 patients with falciparum malaria were recruited into this study; one patient on microscopy did not have malaria and was withdrawn, leaving 154 patients in the ITT analysis. Of these patients 78 were randomised to 3 days AL and 76 to 5 days AL. The percentage of parasites remaining was not significantly between the two treatment arms at any of the time points. The prevalence of kelch13 mutations was 47.4%, with F446I at 28% the most prevalent mutation. After adjusting for admission parasitaemia, there was no significant difference in the percentage reduction in parasite density at day 3 between infections with wild type parasites compared with those carrying kelch13 propeller mutations (ANCOVA, p=0.48). The presence of kelch13 propeller mutations and duration of AL treatment had no impact on gametocytaemia. There was no significant difference between the two treatment regimens in terms of tolerability. An adequate clinical and parasitological response on day 42 was observed in 100% (95%CI, 94.9 to 100) and 97.1% (95%CI, 90.1 to 99.7) of patients in the 3 day and 5 day arms respectively.

Conclusion: Despite a nearly 50% prevalence of kelch13 mutations, the treatment efficacy of artemether-lumefantrine remains high in the south-eastern part of Myanmar. The 3 day treatment regimen is still efficacious. The 5 day regimen was well tolerated though its use is not currently indicated.

5.2 Background

Artemisinin resistance in *Plasmodium falciparum* has emerged in Southeast Asia (83, 93, 119, 149). It is characterised by the clinical phenotype of delayed parasite clearance. In 2010, multicentre therapeutic efficacy studies conducted across Myanmar of dihydroartemisinin-piperaquine (DP) and of artemether-lumefantrine (AL) were reported as showing 28-day adequate clinical and parasitological response levels of 94% to 98% and 94% to 100% respectively (130). However, recent data from the Thai-Myanmar border show that artemisinin-based combination treatments (ACTs) have started to fail, with a day 42 cure rate of 42% following treatment with artesunate and mefloquine (121). New therapies need to be evaluated to treat artemisinin-resistant malaria, but there are currently very few alternative drugs with a different mode of action to the ACTs. New compounds such as the spiroindolone KAE609 and artefenomel OZ439 have shown promising results in phase 2 trials but at the time of writing were still in the pipeline (191-193). In the absence of new drugs, strategies to halt the loss of ACT efficacy include newer dosing regimens of existing ACTs to optimise efficacy against resistant parasite strains. The Technical Expert Group (TEG) on drug resistance and containment has recommended that research be conducted to assess prolonged ACT regimens (194). Prolonging the treatment course allows more parasites to be killed by the artemisinin derivatives, leaving a smaller residual number for the partner drug to act on and thereby reducing the probability of parasites surviving to cause recrudescence during follow-up.

The main ACT used in Myanmar is artemether-lumefantrine. This drug is very well-tolerated. The artemether component of AL is absorbed rapidly and

transformed to dihydroartemisinin (20, 195). Dihydroartemisinin is eliminated quickly from the body. Most of the parasite biomass is cleared by the artemether and dihydroartemisinin components (195). Fever and symptoms resolve rapidly after treatment.

The absorption of the lumefantrine is slow and varies widely between individuals. Elimination is also relatively slow (195). The lumefantrine concentrations that remain at the end of the 3 to 5 day treatment course are responsible for eliminating the residual parasites (61).

Detailed dose-finding pharmacokinetic studies in Thailand indicated that the plasma concentration on day 7 was a principal determinant of the probability of cure in acute falciparum malaria (196). The standard dose of AL seemed adequate for most individuals but there is a large individual variability in absorption. Pregnancy has a significant effect on pharmacokinetic properties and levels of artemether, dihydroartemisinin and lumefantrine. Levels of these three drugs are low in pregnancy, contributing to high failure rates (197). The levels of lumefantrine are lower in children than in adults (198, 199). Children and pregnant women are therefore at higher risk of inadequate treatment of malaria and consequent severe morbidity, and it is important to ensure appropriate dosing of ACTs in these groups.

It has been shown that splitting the 6 dose regimen of AL over 5 days without increasing the total dose improves drug exposure of lumefantrine and the efficacy of AL (200). A five-day treatment regimen with AL provides more reliable lumefantrine bioavailability as the treatment is given for a longer period in a recovering clinical state, resulting in better absorption. Predictive modelling suggested that both an increased dose and increased treatment duration (a twice

daily regimen given for 5 days) are needed for adequate drug exposure for pregnant women (197), significantly increasing the area under the lumefantrine plasma concentration curve (AUC), and therefore the probability of cure. No major effect of prolonged treatment on maximum lumefantrine concentrations could be seen, which suggested that no increase in acute toxicity would occur (197). Giving five days of an ACT (versus for example 7 days) is a practical compromise between longer artemether and lumefantrine administration and the risk of poor compliance with longer regimens.

A 5 day course AL has not been used routinely before, and side effects and compliance have to be monitored. The peak concentrations of lumefantrine with a 5 day twice daily regimen were predicted to be no higher than with 3 days (200). There is no evidence that the highest concentrations would be associated with an increase in adverse effects.

Given current levels of artemisinin resistance in Myanmar it is unlikely that standard measures of assessment of parasitological effectiveness by microscopy will show any difference between an extended regimen and the standard treatment at this time. This study evaluated an alternative method of measuring the parasitocidal effect of the 5 day regimen compared to the standard regimen by using ultra-sensitive detection methods (high volume quantitative PCR) once the parasitaemia has fallen below the microscopic level of detection. It also investigated whether a prolonged regimen has a higher gametocytocidal effect than 3 days treatment, with implications for reduction of onward transmission of resistance.

5.3 Hypothesis

This study examined the hypothesis that in uncomplicated falciparum malaria the parasitocidal efficacy of 5 day AL is higher than that of the standard 3 day treatment regimen, as measured using ultra-sensitive molecular detection methods.

5.4 Objectives

The primary objective of this study was to compare a 5 day treatment regimen of AL with the standard 3 day regimen for treating uncomplicated falciparum malaria by comparing the parasitocidal efficacy on day 5 and 7using an ultra-sensitive molecular detection method.

The secondary objectives were to:

- Compare the proportion of patients with detectable parasitaemia by ultra-sensitive PCR facing effective lumefantrine monotherapy (on day 3 after 3 days AL and on day 5 after 5 days AL).
- Compare the number of parasites facing lumefantrine mono-therapy.
- Compare the tolerability of a 5 day course of artemether-lumefantrine compared to the standard 3 day course
- Compare day 7 gametocytemia between the arms as assessed by microscopy
- Compare the effectiveness on day 42, both uncorrected and corrected by PCR parasite genotyping.
- Compare the haematological recovery on day 28 (as assessed by changes in haemoglobin measurements)

5.5 Methods

5.5.1 Study design

This was a randomized, 2 arm, open-label, clinical trial comparing the standard 3 day course of AL (Coartem®) with an extended 5 day treatment course of AL. This trial was registered at ClinicalTrial.gov (NCT02020330). The study was conducted in village health centres in the proximity of Kyeikdon sub-township and Thanpayar and Hpayarthonesu villages, in Kyainseikgyi Township, Kayin state. The Oxford Tropical Research Ethics Committee (UK) and the Department of Medical Research Ethics Committee (Myanmar) approved the study protocol. It was anticipated that 75 patients would be enrolled in each treatment arm during the study period, giving a total sample size of 150.

5.5.2 Primary and secondary endpoints

5.5.2.1 *Primary endpoint*

- Proportion of patients with detectable parasitaemia by ultra-sensitive PCR on days 5 and 7 after treatment

5.5.2.2 *Secondary endpoints*

- Proportion of patients with detectable parasitaemia by ultra-sensitive PCR facing lumefantrine monotherapy (on day 3 after 3 days AL and on day 5 after 5 days AL).
- Comparison of effectiveness on day 42, both uncorrected and corrected by PCR genotyping

- Haematological recovery on day 28 (assessed by percentage change in haemoglobin from day 0)
- Day 7 gametocytemia assessed by microscopy
- Tolerability in terms of adverse events

5.5.3 Study participants

Please see Chapter 2 (section 2.3.3) for more details.

5.5.4 Drug Therapy

Patients were randomised in blocks of 12 to one of the 2 treatment arms. The randomisation was concealed in sealed envelopes labelled with the patient study code at the site. Artemether-lumefantrine (Coartem®, Novartis, Switzerland) was used. One tablet contains 20 mg artemether and 120 mg lumefantrine. The standard regimen is twice daily for 3 days with a delay of at least 8 hours between the first and second dose. Dosing was by weight categories (See more details on dosing chart -Appendix D). Patients receiving the 5 day course continued to take the same daily dose for 2 additional days. A single dose of primaquine (0.25 mg/kg) (Primaquine Phosphate, Remedica, Cyprus) was given to all patients on the first day of treatment for its gametocytocidal activity. The initial treatment dose was given under supervision. All subsequent doses were given to the patient to be taken at home. At initial treatment, if the patient vomited within 30 min, the full dose was repeated. If vomiting occurred after 30 min but within an hour, half the dose was repeated. Patients with persistent vomiting (more than twice) were withdrawn from the study and given rescue treatment (DP).

5.5.5 Study Procedures

Please see Chapter 2 (section 2.3.7) for more details.

After the first few patients had been enrolled, the study team decided to change the uqPCR collection time points based upon the preliminary uqPCR findings results from a previous clinical trial (the DP trial). In the DP study it was found that 75% of the patients showed positive parasite DNA on day 9 and therefore sample collection for uqPCR was delayed from day 5 and day 6 to day 14 and day 21. These changes were assessed as unlikely to have negative consequences for the patients and this amendment was approved by the relevant Ethics Committees.

The trial was monitored by the Mahidol-Oxford Tropical Medicine Research Unit (MORU) Clinical Trial Support Group in collaboration with the Myanmar Oxford Clinical Research Unit. Data were evaluated for compliance with the protocol and accuracy in relation to source documents. The trial monitors checked whether the clinical trial was conducted and data generated, documented and reported in compliance with the protocol, GCP and the applicable regulatory requirements.

5.5.6 Molecular studies

Please see Chapter 2 (section 2.3.15) for more details.

5.5.7 Drug measurements

Lumefantrine concentrations will be measured by using in blood samples. This assay will be performed by the MORU Department of Pharmacology. At the time of thesis write-up drug measurements were not yet available. These results will be published elsewhere in the future.

5.5.8 Statistical Analysis

It was anticipated that there would be a 20% difference in uqPCR *Plasmodium falciparum* positivity after 3 day and 5 day ACT treatment (i.e. from 30 % to 10% as detected by the uqPCR method). To detect this difference at 5% significance with 80% power 75 patients needed to be enrolled in each group. Data were entered into a web-based database, Macro version 4.2.3.3850 (InferMed). Data cleaning and analysis were done using Stata statistical software, version 14 (StataCorp) and GraphPad Prism software 6.0 (Graphpad Software Inc.). Data were analysed by means of t-tests, Wilcoxon-rank sum tests and chi-squared tests as appropriate.

Survival time data were analysed using the Kaplan-Meier method and Cox regression, and attributable hazard ratios were calculated. Survival rates are expressed as the percentage of uqPCR positivity for 21 days.

Percentage of parasites remaining were calculated as 100 minus the percentage remaining from admission parasitaemia.

Random effects maximum likelihood regression analysis was used to examine the relationship between residual parasitaemia and treatment arm. The outcome variable was parasite density (as measured by uqPCR) as a continuous variable with treatment arm as random effects, and kelch13 mutations as covariate.

The gametocyte clearance time (GCT) was the interval from the first detection to the last detection of gametocytes in a peripheral blood smear. Person-gametocyte weeks (PGW) were calculated for each case as the number of weeks in which gametocytaemia was present (excluding at admission) divided by the duration of follow-up, expressed per 1,000 person-weeks. The area under the curve (AUC) of the gametocyte distribution was calculated according to methods

published elsewhere (201, 202). AUC was analysed against two variables, day of treatment and presence of kelch13 mutation (after the 440 amino acid position).

5.6 Results

The study was conducted between November 2013 and February 2015 in Kyainseikgyi Township, in the south-eastern part of Myanmar. The research clinic was initially based at Kyeikdon sub-township and clinic was moved to Thanpayar within Kyainseikgyi Township after one year. A total of 1,311 patients were screened and 155 patients were enrolled as shown in figure 5.1, 5.2 and 5.3. Early discontinuation occurred in 15 patients, 6 patients withdrew consent, 1 patient was withdrawn due to ineligibility, 7 patients were lost to follow up and 1 patient was withdrawn due to treatment failure. Since Kyainseikgyi Township is situated near Kanchanaburi Province of Thailand, most patients who were lost to follow up were found to have temporarily migrated to Thailand for seasonal work. In keeping with the recommendation of the local Ethics Committee, females of 12 to 18 years age were excluded from the recruitment for cultural reasons.

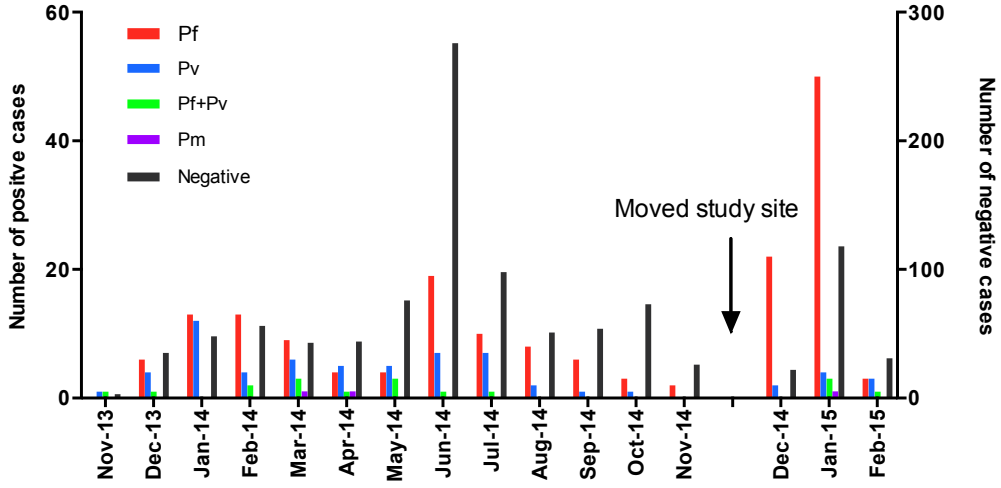


Figure 5.1 Study enrolment screening from November 2013 to February 2015

Figure 5.2 Study sites in Kyainseikgyi Township, Kayin State, Myanmar

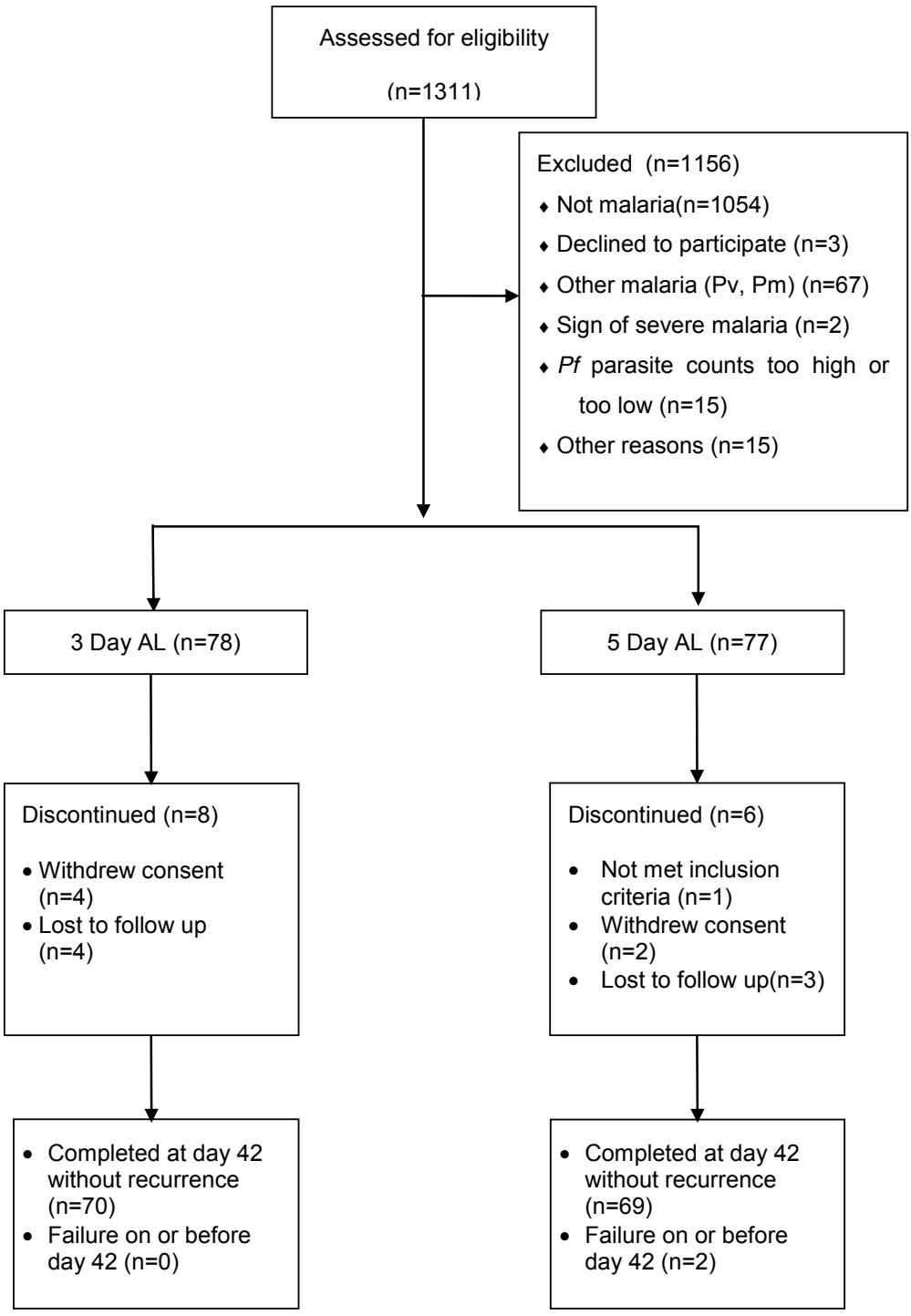


Figure 5.3 Participant flow diagram

5.6.1 Patient characteristics

Males accounted for 62% of all patients. The median age of patients was 19 years (IQR 11-30). The median body mass index was 18.6 (IQR 16.2-20.8) kg/m^2. The geometric mean parasite counts on admission were 3,081 (95%CI 1,751-5,430) in the 3 day treatment arm and 2,140 (95%CI 1,268-3,610) parasites per µL in the 5 day treatment arm. 1.3% (1/78) of patients in the 3 day treatment arm and 2.6% (2/76) in the 5 day treatment arm presented with mixed infections on admission. The median haemoglobin in both treatment arms was 12.2 g/dL. There was no significant difference between the two treatment arms by appropriate statistical test for each variable and the treatment groups were balanced with respect to baseline variables (Table 5.1).

The most frequently reported symptoms were headache (72%), dizziness (31%), muscular pain (23%) and joint pain (20%) in all patients. 32% of patients took paracetamol before coming to clinic, and 33% of patients had a past history of malaria. (Table 5.2)

Table 5.1 Baseline characteristics of the patients by treatment arm

Variables	*Unit*	*Treatment arm*		*Total*
		3 Day AL	5 Day AL	
Gender (Male)	n/N (%)	48/78 (61.5)	48/76 (63.1)	96/154(62.3)
Age	yr	20 (11-31)	18 (10-29.5)	19(11-30)
BMI	Kg/m^2	19.1 (16.8-21.1)	18.4 (15.8-20.2)	18.6(16.2-20.8)
Parasite count (Geometric mean, 95%CI)	No./μL	3,081 (1,751-5,430)	2,140 (1,268-3,610)	2,575(1,756-3,774)
Gametocytaemia on admission	n/N (%)	17/78 (21.8)	16/76 (21.1)	33/154(21.4)
Mixed infection	n/N (%)	1/78 (1.3)	2/76 (2.6)	3/154(1.9)
Haemoglobin	g/dL	12.2 (11.1-13.7)	12.2 (10.8-13.4)	12.2(10.9-13.4)

Data are presented as median (IQR) unless otherwise indicated. There were no statistically significant differences between the treatment arms

Table 5.2 Symptoms on admission, and drug and past medical history

History	*No (%)*
Symptoms	
Dizziness	48/154 (31)
Headache	111/154 (72)
Nausea	18/154 (11.7)
Anorexia	17/154 (11)
Vomiting	9/154 (5.8)
Abdominal pain	15/154 (9.7)
Joint pain	30/154 (19.5)
Muscular pain	35/154 (22.7)
Palpitations	2/154 (1.3)
Blurred vision	1/154 (0.7)
Tiredness	2/154 (1.3)
Drug history	
Paracetamol	49/154 (31.9)
Amoxicillin	9/154 (5.9)
Chlopheniramine maleate	3/154 (2.0)
Vitamins	2/154 (1.3)
Ampicillin	1/154 (0.7)
Past medical history	
Malaria	50/154 (32.5)
Hypertension	1/154 (0.7)
Acute gastritis	1/154 (0.7)

5.6.2 Percentage parasites remaining by uqPCR

The percentage of parasites remaining was not significantly between the two treatment arms at any of the time points (Table 5.3). Most had negative malaria blood smears at 5 days after treatment, however 50% of the 3 day AL treatment arm patients and 44.4% of those in the 5 day AL treatment arm were positive for parasites on uqPCR (Table 5.4). Blood samples for uqPCR were collected on days 0, 3, 5 and 7 from the first 39 patients and on days 0, 3, 7, 14 and 21 for the rest of the patients after amendments were approved by the Ethics Committees. Therefore the findings are separated by sub-group according to day of uqPCR collection and summarised in Table 5.4.

Table 5.3 Percentage parasites remaining, using uqPCR method

		Treatment arm		
		3 Day AL	5 Day AL	
Time point	*n*	(Median, IQR)	(Median, IQR)	*p value*[#]
At 3 days	147	0.39 (0.04 to 2.81)	0.72 (0.06 to 2.27)	0.42
At 5 days	38	0.01 (0 to 0.16)	0 (0 to 0.07)	0.50
At 7 days	144	0.002 (0 to 0.03)	0.003 (0 to 0.05)	0.72
At 14 days	107	0 (0 to 0)	0 (0 to 0)	0.58
At 21 days	105	0 (0 to 0)	0 (0 to 0)	0.33

[#]Wilcoxon rank-sum (Mann-Whitney) test

Table 5.4 uqPCR positivity by treatment arm

	Treatment arm	
	3 Day AL	5 Day AL
Time point	no of positive/total (%)	no of positive/total (%)
All cases (n=154)		
On admission	78/78 (100)	76/76 (100)
3 day after treatment	61/75 (81.3)	61/72 (84.8)
5 day after treatment	10/20 (50.0)	8/18 (44.4)
7 day after treatment	41/74 (55.4)	40/71 (56.3)
14 day after treatment	15/54 (27.8)	16/55 (29.1)
21 day after treatment	5/52 (9.6)	8/53 (15.1)
Sub group analysis (KD-001 to KD-039) (n=37)		
On admission	20/20 (100)	17/17 (100)
3 day after treatment	13/20 (65.0)	12/17 (70.6)
5 day after treatment	10/20 (50.0)	7/17 (41.1)
7 day after treatment	6/20 (30.0)	6/17 (35.3)
Sub group analysis (KD-040 to KD-155) (n=110)		
On admission	55/55 (100)	55/55 (100)
3 day after treatment	48/55 (87.3)	49/55 (89.1)
7 day after treatment	35/54 (64.8)	34/54 (62.9)
14 day after treatment	15/54 (27.8)	16/55 (29.1)
21 day after treatment	5/52 (9.6)	8/53 (15.1)

Two sample tests of proportions were calculated at each time point between treatment arms and statistically no significant difference was found.

5.6.3 Prevalence of *Pf* kelch13 mutations

Of the 154 samples tested, the *Pf* kelch13 gene was successfully sequenced in 152 (98.7%). The prevalence of kelch13 mutations was 47.4% (72/152). The F446I mutation was the most common, accounting for 28% (20/72) of all mutations. There was no difference in kelch13 mutation rates between the two groups. 45% of patients in both the 3 day and 5 day treatment groups had kelch13 mutations after amino acid position 440 (Table 5.5). Most of the mutations were concentrated within propeller blades 1 to 4 in the kelch gene. According to the recent WHO status update on artemisinin resistance (60), one 'confirmed' mutation (C580Y) was detected, along with 6 'associated' mutations (P441L, F446I, G449A, P553L, R561H and P574L) (Figure 5.4).

One non-synonymous mutation identified in our study, P667R, had not to my knowledge been identified previously (Table 5.6).

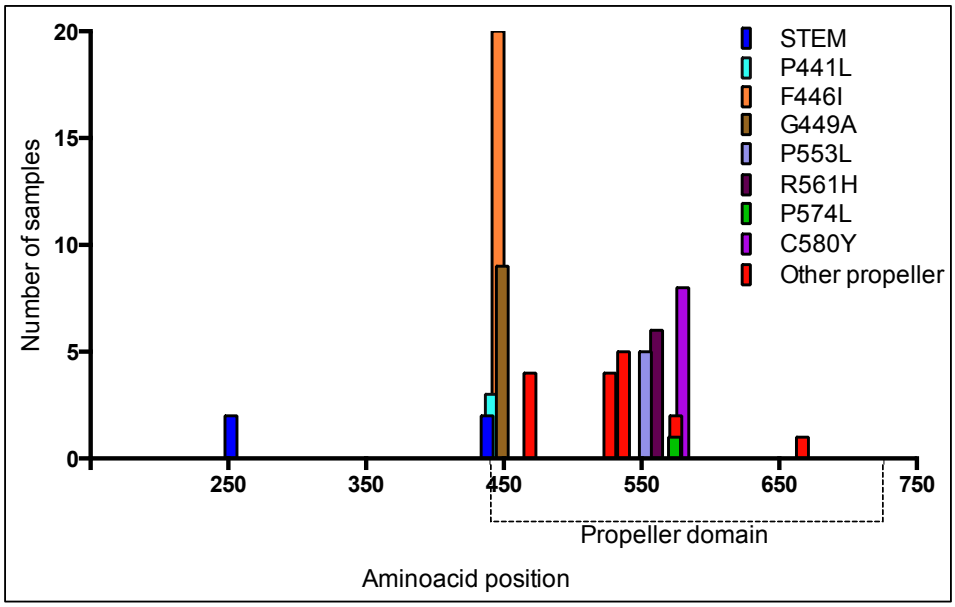


Figure 5.4 Primary amino acid positions of kelch13 mutations in Kyainseikgyi Township, Kayin state, Myanmar

Table 5.5 *Plasmodium falciparum* kelch13 mutation and treatment arm

kelch13	**Treatment arm**	
	3 Day AL (n,%)	5 Day AL (n,%)
Wild Type	40 (52.6)	40 (52.6)
Mutation ≤440 amino acid position	2 (2.7)	2 (2.7)
Mutation >440 amino acid position	34 (44.7)	34 (44.7)
Total	76 (100)	76 (100)

Table 5.6 Prevalence of kelch13 molecular markers (n=152)

kelch13	**3 Day AL (n, %)**	**5 Day AL (n, %)**	**Total (n, %)**
E252Q	1 (1.39)	1 (1.39)	2 (2.78)
K438N	1 (1.39)	1 (1.39)	2 (2.78)
P441L	0 (0)	3 (4.17)	3 (4.17)
F446I	10 (13.89)	10 (13.89)	20 (27.78)
G449A	1 (1.39)	8 (11.11)	9 (12.5)
C469F	3 (4.17)	1 (1.39)	4 (5.56)
P527H	2 (2.78)	2 (2.78)	4 (5.56)
N537I	4 (5.56)	1 (1.39)	5 (6.94)
P553L	3 (4.17)	2 (2.78)	5 (6.94)
R561H	4 (5.56)	2 (2.78)	6 (8.33)
P574L	1 (1.39)	0 (0)	1 (1.39)
R575K	1 (1.39)	1 (1.39)	2 (2.78)
C580Y	5 (6.94)	3 (4.17)	8 (11.11)
P667R	0 (0)	1 (1.39)	1 (1.39)
Total	36 (50.0)	36 (50.0)	72 (100)

5.6.4 Day 3 blood smear positivity

Twenty percent (31/154) of all patients were still parasitaemic on day 3 blood smear after AL treatment: 23% (18/78) of 3D AL treatment arm and 17% (13/76) in the 5D AL treatment arm respectively (unsurprisingly as up to day 3 treatment in the two arms was identical). Out of these 31 patients, 52% (16/31) had kelch13 propeller mutations (p=0.21).

Patients with day 3 parasitaemia on blood smear associated with higher uqPCR positivity on days 5 and 7 and the difference was statistically significant between blood smear day 3 positive and negative patients (Table 5.7).

On multivariable logistic regression >1% parasitaemia on admission was significantly associated with day 3 blood smear positivity, while infection with a kelch13 propeller mutation-carrying parasite was not (Table 5.8).

Table 5.7 Relationship between blood smear day 3 positivity and uqPCR positivity during follow up (n=31)

Time point	*Day 3 Microscopy Positive n (%)*		*Day 3 Microscopy negative n (%)*		*P value*
	uqPCR positive	uqPCR negative	uqPCR positive	uqPCR negative	
Day 3	31 (21.1)	0 (0)	91 (61.9)	25 (17)	0.005*
Day 5	10 (25)	1 (2.5)	10(25)	19(47.5)	0.001*
Day 7	26 (17.9)	4 (2.8)	55(37.9)	60(41.4)	<0.001*
Day 14	9 (8.3)	12 (11)	22(20.2)	66(60.5)	0.10
Day 21	4 (3.8)	14 (15.2)	9(8.6)	76(72.4)	0.25

Table 5.8 Risk factors for a positive blood smear on day 3

Day 3 positivity	**Odds ratio**	**95% confidence interval**	**P value**
>1% admission parasitaemia	6.9	2.6 to 18.4	<0.001*
Kelch13 (>440) mutation	1.7	0.7 to 3.9	0.25

5.6.5 Relationship between residual parasitaemia, treatment arm, and kelch13 propeller mutations

Kelch13 was successfully sequenced in 152 patients, and therefore 152 patients' parasite densities measured using uqPCR methods from day 0 to 21 were included in this analysis. Figure 5.5 shows individual patients' parasite density over time using uqPCR. 21 days after treatment, patients with the kelch13 propeller mutation showed 12.5% (7/56) positive parasitaemia and those with wild type 12.2% (6/49)(p=0.97).

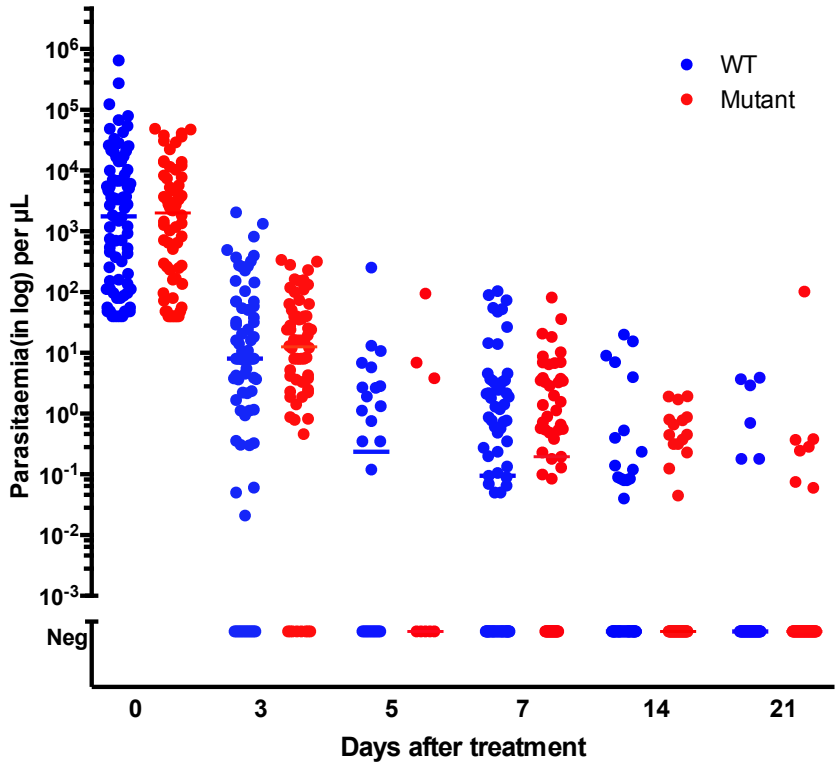


Figure 5.5 Kelch13 propeller mutation and days after treatment. One circle represents one patient, red circles are mutation after position 440, blue circles represent wild types alleles.

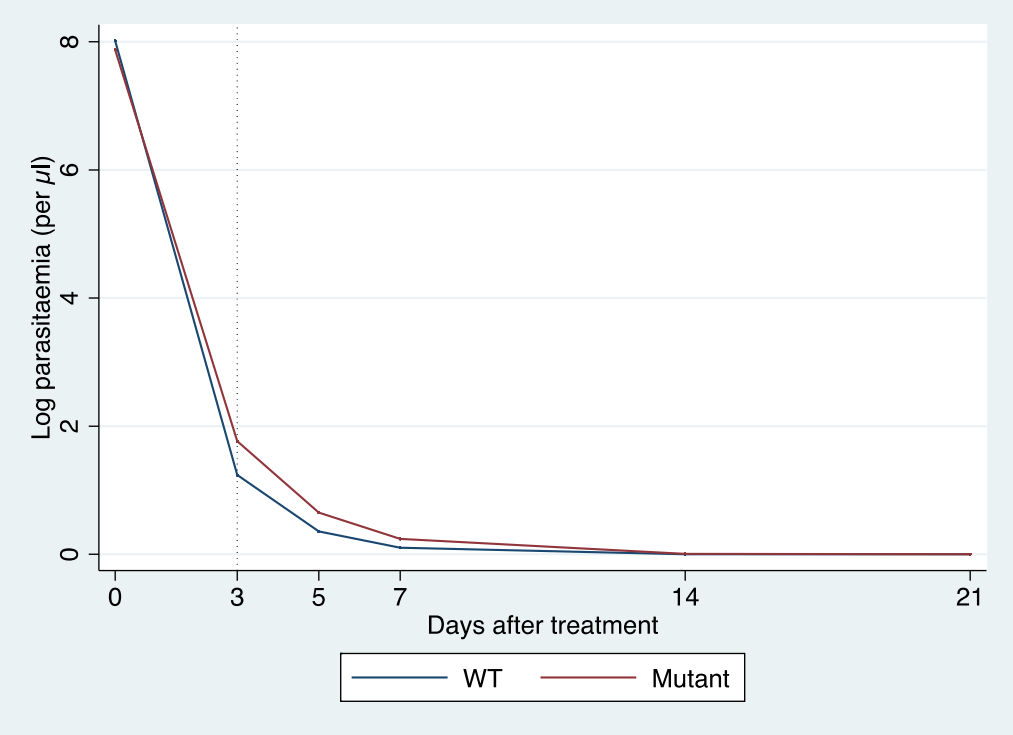


Figure 5.6 Parasitaemia and days after treatment using uqPCR by wild type and kelch13 mutations

Nonlinear exponential regression was used to fit the model for the reduction in parasitaemia per µl over time after treatment as shown in Figure 5.6. For the first 3 days there is rapid reduction of parasites, presumably due mainly to the action of artemether. The subsequent slower reduction of residual parasites is probably due to partner drug action. After adjusting for admission parasitaemia, no significant difference in reduction of parasite density by day 3 in patients with wild type was found compared with kelch13 propeller mutants (ANCOVA, p=0.48).

Table 5.9 Random effects maximum likelihood linear regression analysis examining relationship between residual parasite density over time using uqPCR methods and treatment arm and kelch13 mutations

Variables		**Coefficient**	**95% CI**	**p**
Day		-0.8	-0.8 to -0.7	<0.001*
Treatment arm	3D AL	Ref		
	5D AL	-0.1	-1.1 to 0.9	0.9
Kelch13	WT	Ref		
mutations	Mutant(>440)	0.2	-0.8 to 1.2	0.7

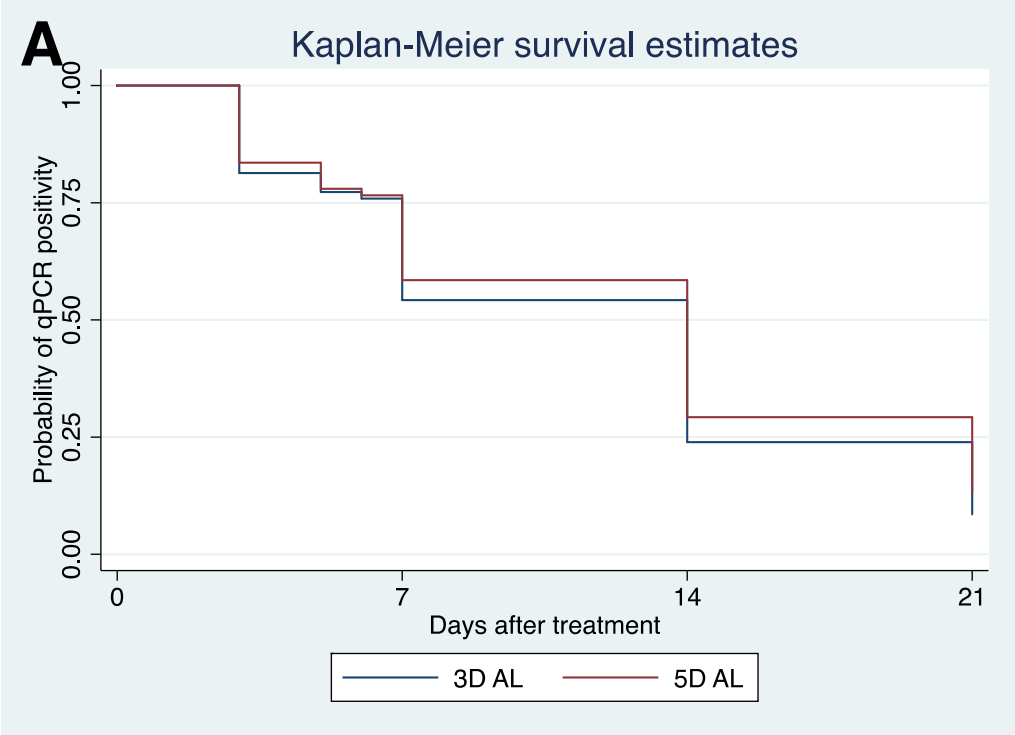
To assess whether kelch13 propeller mutations modify the effect of treatment on parasitaemia reduction, a random effects maximum likelihood regression model of residual parasitaemia was fitted with an interaction term for treatment and kelch13. Day of measurement was included as a covariate in this model. The total number of observations over time was 546 and average observations per patient was 3.6 (Range 1 to 6). As shown in Table 5.9, the numbers of parasite were significantly decreasing over time due to the effect of treatment ($p<0.001$) after adjusting for kelch13 status. However, there was no significant difference between the two treatment groups ($p=0.9$). Patients with kelch13 propeller mutations had more parasites over time compared to patients with wild type but this was also not statistically significant ($p=0.7$).

5.6.6 Risk factors for uqPCR positivity at 21 days after treatment

A total of 146 patients were included in this survival analysis. Total time at risk was 1,616 days. The percentages of patients with a positive uqPCR result at day 21 were 8% (3 to 17) and 13% (6 to 23) respectively, and the hazard ratio was 1.1 (0.8 to 1.6) for treatment arm. The effects of mutations and treatment regimen on uqPCR positivity at 21 days after treatment had hazard ratios of 1.0 (0.7 to 1.4) and 1.1(0.7 to 1.5) respectively. The Kaplan-Meier curves showed that both treatment arms, and presence or absence of mutations have the same positivity rates (logrank tests gave p>0.05) (Table 5.10) (Figure 5.7).

Table 5.10 Positivity rates and hazard ratios for risk factors of uqPCR positivity at 21 days after treatment

Risk factor		*Percentage uqPCR positive*	*Hazard Ratio (95%CI)*
Treatment arm	3D AL	8 (3 to 17)	1.1 (0.8 to 1.6)
	5D AL	13 (6 to 23)	
Kelch13	WT or <440	10 (4 to 20)	1.0 (0.7 to 1.4)
	Mutation >440	11(5 to 20)	



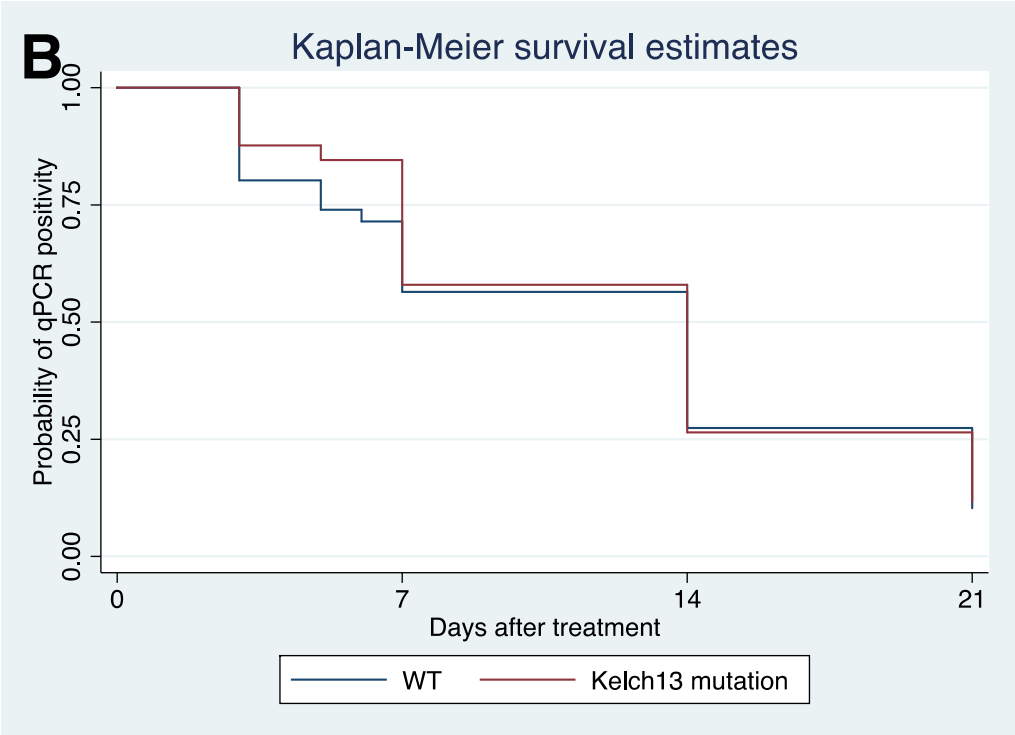


Figure 5.7 Kaplan-Meier survival curves of (A) treatment arms (B) kelch13 propeller mutations.

5.6.7 Gametocytaemia

Twenty two percent (17/78) of the 3 day AL treatment arm and 21% (16/76) of the 5 day AL treatment arm presented with gametocytes on admission (Table 5.1). The median duration of gametocyte carriage in the 3 day AL and 5 day AL treatment arms were 36 (36 to 92.5) and 60 (36 to 95) hours respectively. No significant differences were found between the two treatment groups although the 5 day AL treatment arm showed slightly higher median duration of gametocytaemia than the 3 day AL arm. For patients with wild type or non-propeller kelch13 alleles, the median gametocyte carriage was 36 (36 to 76.5) hours, and for those with kelch13 propeller mutant alleles it was 92.5 (36 to 95) hours (p=0.12)(Table 5.11). The mean (95%CI) log10 AUCs were 4.91 (4.2 to 5.6) and 4.94 (4.0 to 5.8) for the 3 day AL and 5 day AL treatment groups respectively. The corresponding values of AUC by presence of kelch13 propeller mutation were 4.78 (4.05 to 5.51) and 5.11 (4.29 to 5.94) for wild type and kelch13 propeller mutations respectively. The differences between the log10 gametocytaemia AUCs between treatment arms and between wild type and kelch13 propeller mutations were both not statistically significant (p=0.83 and p=0.38, respectively)(Figure 5.8). After excluding gametocytaemia on admission, the person gametocytaemia weeks (PGW) per 1,000 person-weeks of follow up in the 3 day and 5 day AL treatment groups were the same (3.0/1,000 person-weeks) (Table 5.12). The PGWs of both treatment arms were not also statistically significant when compared with 3 days treatment with AL plus single dose primaquine 0.75 mg/kg in a previous study in Myanmar reported in 2010 by Smithius *et al.* (63)(p=0.5 in both cases). However, there was a significant difference between the two treatment arms of the current study and the AL alone

arm in the Smithius *et al.* study (63)(Table 5.12). Overall, these results suggest that kelch13 propeller mutations and duration of treatment had no impact on gametocytaemia, but in the current study the use of low dose primaquine 0.25 mg/kg is responsible for the short gametocyte carriage time seen in both arms.

Table 5.11 Gametocyte carriage time (GCT)

Variable	*n*	*Median GCT (IQR)(hour)*	*P value*[#]
Treatment arm			
3 Day AL	18	36 (36-92.5)	0.11
5 Day AL	17	60 (36-95)	
Mutation			
Wild type	20	36 (36-76.5)	0.12
Kelch13 mutation	15	92.5 (36-95)	

[#]Wilcoxon rank-sum (Mann-Whitney) test

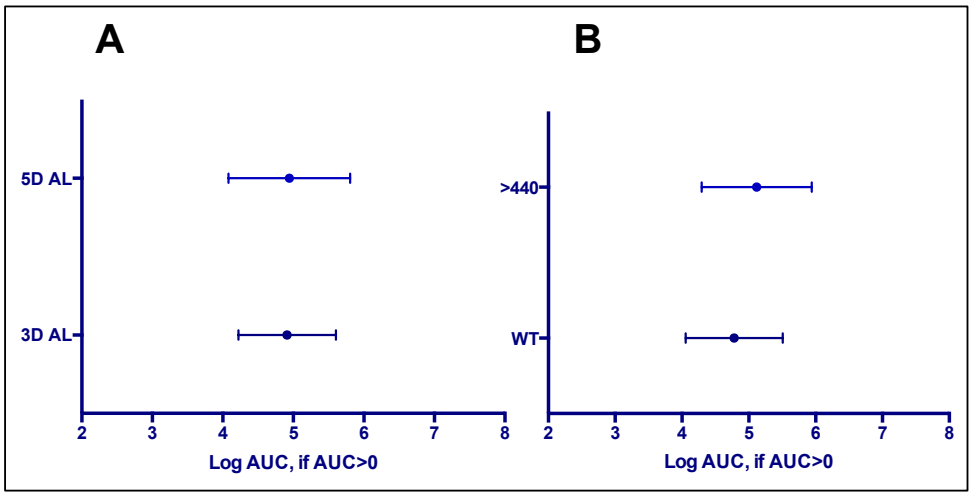


Figure 5.8 Association between (A) length of treatment with AL, (B) kelch13 propeller mutation and the area under the gametocytaemia curve (point estimates and 95% CI)

Table 5.12 Comparison of *Plasmodium falciparum* gametocyte carriage by person gametocytaemia weeks (PGW)

Drugs	*Treatment duration*	*n*	*PGW*	*P value*
AL plus primaquine 0.25 mg/kg	3 day	78	3.0	
AL plus primaquine 0.25 mg/kg	5 day	76	3.0	
AL plus primaquine 0.75 mg/kg (63)	3 day	78	4.7	0.5 #
AL (63)	3 day	84	58.2	<0.001*, **

compared with AL plus primaquine 0.25 mg/kg (both 3 day and 5 day)

* compared with AL plus primaquine 0.25 mg/kg (both 3 day and 5 day)

** compared with AL plus primaquine 0.75 mg/kg

[p value calculated by using incidence-rate ratio calculator]

5.6.8 Haematological changes

The mean (SD) haemoglobin concentration on admission of the patients in the 3 day AL and 5 day AL treatment arms were 12.2 (0.2) and 12.0 (0.3) respectively (p=0.6). After 28 days of treatment, the mean (SD) haemoglobin values were 12.3 (0.2) for the 3 day and 12.5 (0.19) for the 5 day treatment arms. In the 3 day arm, the haemoglobin concentrations on admission and after 28 days were not significantly different, but in the 5 day arm the haemoglobin concentration was slightly increased after 28 days of treatment compared to the admission values (paired t test, p<0.05) (Figure 5.9).

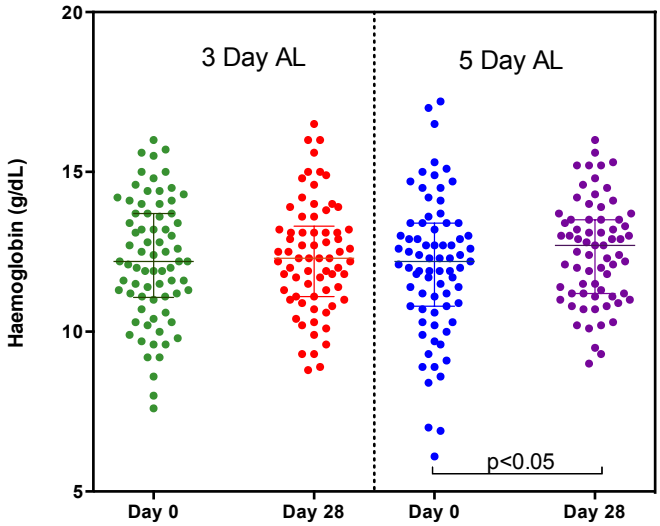


Figure 5.9 Haemoglobin concentrations on day 0 and day 28 of 3 day and 5 day AL treatment arms

5.6.9 Curative efficacy

There were two treatment failures with recurrence of *P. falciparum* parasitaemia on blood smear in this study, one at day 28 and the other at day 42. Both were in the 5 day treatment arm. Unfortunately, DNA extraction for these two recurrent *P. falciparum* cases was not successful and genotyping by MSP 1, MSP 2 and GLURP could not be carried out. One case was a 6 years old child who on admission had parasites with the F446I kelch13 propeller mutation, and the other a 24-year-old adult with G449A-carrying parasites on admission. The overall treatment failure rate for the study was 1.4% (2.6% in the 5 day AL arm and 0% in the 3 day AL arm)(Table 5.13).

Three new cases of *P. vivax* occurred during follow up period, one case on day 35 and two cases on day 42. One patient with mixed infection on admission was had *P. vivax* on the blood smear at the day 35 follow up assessment; this case had F446I P. falciparum infection on admission and was in the 5 day AL treatment arm.

Table 5.13 Treatment efficacy analysis

Outcome	*3 day AL*	*5 day AL*	*Total*
Number of patients	78	77	155
Discontinued, n (%)	8 (10.3)	7 (9.1)	15 (9.7)
Per protocol population, n	70	70	140
Treatment failure (PCR-uncorrected) n (%)	0 (0)	2 (2.6)	2 (1.4)
ACPR on day 42 % (95% CI)	100 (94.9 to 100)	97.1 (90.1 to 99.7)	98.6 (94.9 to 99.8)

ACPR, adequate clinical and parasitological response

5.6.10 Adverse events

A total of 35 adverse events were reported during the study involving 14 and 10 participants from the 3 day and 5 day AL treatment groups respectively (Table 5.14). Among these events, 18 were related to the nervous system (dizziness, headache), 8 were gastrointestinal (abdominal pain, diarrhoea), 5 were respiratory (sneezing, cough), 2 cardiovascular (palpitation, tiredness) and 1 musculoskeletal (muscle and joint pain).

There was no significant difference between the two treatment regimens in terms of adverse events. Among these events, 14 (40%) were judged to be “not related” to the treatment drugs, 7 (20%) “possibly related”, 9 (25.7%) “probably related” and 5 (14.3%) “definitely related”. All adverse events were only of mild and moderate severity. All 24 patients recovered from these adverse events. No serious adverse events were reported.

Table 5.14 Adverse events

	Treatment arm	
Event	3 Day AL	5 Day AL
Abdominal pain	2	2
Cough	2	2
Diarrhoea	1	1
Dizziness	5	5
Headache	4	4
Joint/muscle pain	3	-
Palpitation	1	-
Sneezing	1	-
Tiredness	1	-
Toothache	1	-
Total	21	14

Two sample test of proportions were calculated and there was statistically no significant difference between treatment arms. ($p>0.05$)

5.7 Discussion

In this study conducted in the south-eastern part of Myanmar, an area considered to have established artemisinin resistance, the overall treatment efficacy was 98.7% (95%CI 95.3 to 99.8). A total of 7 patients were lost to follow up before the end of the 42-day follow-up period, but all had negative blood films at their last follow up visit. All cases from the 3 day AL arm remained parasite free until 42 days. Two treatment failures were identified in the 5 day AL treatment arm. It is possible that the presence of kelch13 propeller mutations, the relatively low dose of lumefantrine for children in AL (203) and young age (204) increased the risk of recrudescence.

Although AL was effective in terms of cure, day 3 parasite positivity on blood smear occurred in 23% in the 3 day AL and 17% of the 5 day AL group. More than half of these infections carried kelch13 propeller mutations, confirming the presence of artemisinin resistance infections in Kayin state according to the recent WHO definition (60).

P. vivax relapses often occur around 21 days after AL treatment and thereafter at 3 weeks intervals (20, 205, 206). In this study, one patient (mixed infection on admission) was diagnosed with *P. vivax* on blood smear at day 35. This would be most consistent with a presumed relapse, as artemisinin derivatives have no hypnozoitocidal activity (207, 208). The treatment efficacy of artemether-lumefantrine for uncomplicated *P. vivax* varies according to geographical region and the fastest time to relapse occurs in Southeast Asia compared with other malaria endemic areas (209).

While there was 20% day 3 *P. falciparum* positivity on blood smear, 80% day 3 parasite positivity on uqPCR was observed in this study. A high prevalence of

submicroscopic parasitaemia is common in Southeast Asia and other regions (210-213), and patients harbouring submicroscopic parasitaemia have a longer duration of gametocyte carriage, thereby increasing the likelihood of onward transmission by infecting mosquitoes (189). However, in this study the median gametocyte carriage times in the 3 day and 5 day AL groups were 36 (36 to 92) and 60 (36 to 96) hours respectively, significantly less than that in the TRAC study (160(<66 to 252) where primaquine was not used (93). Recent reviews suggested that AL clears asexual parasites and also reduces gametocyte carriage in symptomatic and asymptomatic carriers (214) but that this effect depends on the daily dose of artemether (203) and that this is a predictor of recrudescence in Southeast Asia. Consistent with previous studies (215-218), the addition of a single low dose primaquine reduced the gametocyte carriage time and haematological recovery was rapid in both the 3 day and 5 day AL arms in this current study.

Routine microscopic examination of blood smears can detect half of the cases identified by PCR methods (219). In this study, as expected, highly sensitive qPCR methods detected more positive cases than microscopy. The patients with day 3 parasitaemia on blood smear were found to have a higher rate of uqPCR positivity during the first week of follow up. It is possible that higher admission parasitaemia can lead to a higher chance of day 3 positivity on blood smear and these patients probably carry submicroscopic parasitaemia on uqPCR during the first week of follow up. Only 52% of the patients with day 3 parasitaemia on blood smear had kelch13 propeller mutations, and kelch13 wild type infections remaining positive at day 3 on blood smear have been found in other parts of Myanmar (112). This most likely represents a stochastic effect with long half-life

infections simply presenting the top end of the log-normal distribution of the parasite half lives observed with kelch13 wild type infections (103).

A few studies have been published on sequential monitoring of residual parasitaemia by highly sensitive qPCR methods (189, 190). The estimated parasite reduction ratio *in vivo* for artemether is 10^3 – 10^5 after 48 hours of the start of treatment (220). In this study, we measured parasite density after 72 hours using uqPCR and the median percentage parasite remaining (PPR) in the 5 day AL group was slightly higher than in the 3 day group, though this difference was not statistically significant. It can therefore be assumed that the efficacy of standard 3 day AL treatment for uncomplicated falciparum malaria remains high in the south-eastern part of Myanmar.

Overall, 47% of patients had infections with kelch13 mutations; 13 different propeller mutations were found, of which P441L, F446I, G449A, P553L, R561H and P574L have previously been associated with resistance and C580Y had been confirmed to be associated with resistance *in vivo* (60). Twenty-eight percent of mutant parasites carried F446I, which has been reported previously as the predominant mutation in the north, east and south east of Myanmar (149, 179). The second-most prevalent mutation was G449A (13%), which has been found in west and south east of Myanmar (112, 149). The third-most prevalent one was C580Y (11%), which has been reported as the dominant resistance mutation in Southeast Asia (83, 93). Compared to other states and regions in Myanmar, C580Y can be found mostly in Kayin state (149).

An effect of kelch13 propeller mutations compared to wild type on longitudinal uqPCR positivity could not be identified in this study. F446I and G449A were the predominant kelch13 propeller mutations in this study and these mutations are

associated with slow parasite clearance (60) on blood smear but have a milder resistance phenotype compared with C580Y. A recent study in Vietnam reported that nearly 25% of samples showed positive results on uqPCR at day 21 and the prevalence of kelch13 propeller mutation was 80% (I543T and Y493H). The authors suggested that the submicroscopic parasites seen during follow up were probably circulating gametocytes (190). However, in this study we used an ACT plus low dose primaquine in all patients and these submicroscopic infections during follow up might be “sleeping forms” (221, 222) or “dead parasites”. Teuscher *et al.* found a temporary halt (dormancy) of up to 20 days in the growth of parasites following a single exposure of DHA *in vitro (223).* It is possible that multiple exposures to DHA (as in a treatment course) might extend dormancy for more than 20 days. This might explain why most of the patients with submicroscopic parasitaemia post-treatment in this study remained ‘silent’ for the full 42 days of follow up. To date, the role of sub-microscopic parasitaemia detected by uqPCR is not completely understood and additional studies such as transcriptomic studies will be needed find any link between dormancy and submicroscopic parasitaemia. Such a link would explain why there was no effect of treatment arm on uqPCR positivity from day 3 to day 21 during follow up. Regarding adverse effects, there was no significant difference between the extended course of 5 day AL compared with routine 3 day AL, however to be certain that there is no difference in tolerability between the arms further studies would need to be conducted with a larger sample size.

Regarding safety, dizziness was the most commonly reported adverse event, followed by headache. For both symptoms the severity was assessed as mild or moderate, and these symptoms are anyway common in acute malaria. If partner

drug resistance rapidly follows the emergence of artemisinin resistance as in the case of piperaquine resistance in DP in Cambodia (97, 98, 154), the efficacy of ACT will decline. In such a scenario, lumefantrine resistance needs to be monitored carefully and alternative choice of drugs should be considered if lumefantrine resistance emerges.

Limitations of the study

Day 7 lumefantrine drug levels are still being measured. Further work is needed to understand the nature and implications of submicroscopic parasitaemia post-treatment, including longitudinal studies with longer follow up with uqPCR and parasite genotyping and transcriptomics.

5.8 Conclusion

Despite a nearly 50% prevalence of kelch13 mutations, the treatment efficacy of artemether-lumefantrine remains high in the south-eastern part of Myanmar. The 3 day treatment regimen is still efficacious. The 5 day regimen was well tolerated though its use is not currently indicated.

5.9 Summary of findings

The salient findings of chapter 5 are as follows:

- The differences in the percentages of parasites remaining between the two treatment arms at all the time points assessed were not statistically significant.

- At 5 days post-treatment most of the patients had negative malaria blood smears, but 50% of patients in the 3 day AL treatment arm and 44.4% in the 5 day AL treatment arm had detectable parasitaemia on uqPCR.
- Overall, 47% of all patients had kelch13 propeller mutations.
- 20% of all patients were still parasitaemic on blood smear on day 3 after AL treatment, but this positivity was not associated with kelch13 propeller mutations.
- No effect of kelch13 mutations on uqPCR positivity post-treatment could be identified in this study.
- Overall, presence of kelch13 propeller mutations and duration of treatment had no impact on gametocytaemia. Adding low dose primaquine 0.25 mg/kg was likely responsible for the short gametocyte carriage time in this study.
- In the 3 day AL arm, the haemoglobin concentration on admission and after 28 days was not significantly different but in the 5 day AL arm the haemoglobin concentration was slightly increased after 28 days of treatment compared to admission values.
- Treatment efficacy with both AL regimens was high, with a 1.4% treatment failure rate in this study overall.
- The percentage 21 day parasite positivity on uqPCR was 8% (3 to17) and 13% (6 to 23) in the 3 day and 5 day AL treatment arms, respectively (hazard ratio 1.1 (95%CI, 0.8 to 1.6)).
- There was no significant difference between the two treatment regimens in terms of tolerability.

6 Spatial and temporal distribution of kelch13, Day 3 blood smear positivity, uqPCR positivity and gametocytaemia across studies

6.1 Chapter 6 abstract

Background: Artemisinin resistance in *Plasmodium falciparum* extends across Southeast Asia. This compromises both the long-term effectiveness of first-line treatments for falciparum malaria (dihydroartemisinin-piperaquine (DP) and artemether-lumefantrine (AL)) by exposing the partner drug to selective pressures leading to resistance, and consequently treatment failure.

Methods: Three datasets from my survey and two clinical trials were combined and analysed to describe the geographical distributions of kelch13 mutations and where possible trends over time. In addition, I compared parasite clearance half-lives from Myanmar with those described from elsewhere in Southeast Asia, the parasitocidal efficacies of DP and AL, and examined correlations between day 3 parasitaemia and kelch13 mutations.

Results: Overall, 35% of all patients had kelch13 propeller mutations. One "confirmed" molecular marker of artemisinin resistance (C580Y) and nine "associated" markers (P441L, F446I, G449A, N458Y, P553L, R561H, P574L and A675V) were found. The percentage of infections with kelch13 mutant parasites increased from 2013 to 2015 (25% to 68%) ($p<0.0001$). In Upper Myanmar, F446I was the most prevalent kelch13 mutation. Comparisons of the parasite clearance half-lives between infections with WT and F446I, WT and C580Y, F446I and C580Y were all statistically significant (Bonferroni corrected, $p <0.001$). The median parasite clearance half-lives were 2.6 hours in Thabeikkyin to 4.4 hours in Myitkyina. There were no treatment failures in the DP trial and only two cases of treatment failure with *P. falciparum* in the AL trial. Patients treated with DP showed a higher probability of uqPCR positivity at day 21 than those treated with AL. After adjusting for admission parasitaemia, the risk of

positivity was nearly one and half fold in patients treated with DP compared those with AL (hazard ratio of 1.6, 95% CI 1.1 to 2.2, p <0.001). There was no significant difference between kelch13 propeller mutation-carrying infections compared to wild type infections in the parasite density over time as measured by uqPCR (p=0.8).

Conclusion: Overall one third of patients had kelch13 propeller mutations, and this proportion was observed to be increasing over time. These findings highlighted that artemisinin resistance is increasingly widespread in northern, central and southeastern Myanmar.

6.2 Background

Between 2013 and 2015 I conducted one patient survey and two clinical trials in Myanmar. These three studies identified *Plasmodium falciparum* kelch13 mutations in different geographical areas at different time points. In this chapter, the data relating to kelch13 mutations, highly sensitive PCR and clinical outcomes were combined and analyzed to describe geographical distributions, associations and where possible trends over time. The parasite clearance half-life data of C580Y mutation carrying infections from Cambodia (93) were used as a reference “confirmed” molecular maker of resistant infections, and compared with the wild type and F446I alleles from the DP trial. Other clinical and laboratory data obtained in the two clinical trials and combined for analysis include uqPCR measurements, day 3 parasitaemia, gametocytaemia and treatment efficacy. To compare parasite clearance half-lives across Southeast Asian countries, data from the TRAC study were used (93). To compare day 3 parasitaemia and kelch13 mutations and person gametocytaemia weeks after ACT or ACT plus PQ

treatment, relevant data from published papers were used (63, 93, 112, 115, 187, 188, 224, 225).

6.3 Hypotheses

The following hypotheses were tested in this chapter:

1. The prevalence of kelch13 mutant parasites is increasing over time in multiple regions of Myanmar.

2. The geographical range of kelch13 mutant parasites is extending within Myanmar

3. The F446I kelch13 mutation, common in northern and central Myanmar and in Yunnan, China, is significantly associated with a slow parasite clearance phenotype.

4. There is an epidemiologically useful relationship between Day 3 blood smear positivity and the prevalence of kelch13 mutant parasites.

5. Use of highly sensitive uqPCR to assess parasitaemia post treatment in cases of acute malaria is more sensitive than using blood films and may provide additional drug efficacy information.

6. Day 3 uqPCR at a study level is more informative about the prevalence and nature of kelch mutations than Day 3 blood smear positivity.

7. Single low dose primaquine is safe and effective in clearing gametocytes in falciparum malaria.

6.4 Objectives

The objectives were:

- To study the spatial and temporal distribution of *P. falciparum* kelch13 mutants causing malaria in Myanmar
- To compare the parasite clearance half-lives of infections with wild type and F446I mutant alleles in Myanmar with those of infections carrying the reference “confirmed” mutation (C580Y) in Cambodia
- To compare the parasite clearance rates of infections with and without kelch13 mutations across countries in Southeast Asia
- To compare the paracitocidal efficacy of artemether-lumefantrine and dihydroartemisinin-piperaquine using uqPCR methods
- To determine the correlation between day 3 parasitaemia and kelch13 mutations
- To study the effect of artemisinin-based combination therapy and low dose primaquine on gametocytaemia in infections with and without kelch13 mutations

6.5 Statistical Analysis

Three datasets from one survey and two clinical trials were combined. Continuous variables were summarized by their mean value and standard deviation, or if the distribution was non-normal, by their median value and interquartile range. Tests of association between two categorical variables were performed using the chi-squared test or chi-squared for trend if necessary. The

ANOVA F test was used to compare the three groups of continuous variables and a Bonferroni correction was used for post hoc analyses.

Survival rates are expressed as the percentage of uqPCR negativity over 21 days calculated using the Kaplan-Meier method. Kaplan-Meier curves comparing AL and DP are shown for up to 21 days of follow-up. Cox regression was used to calculate the hazard ratio and the 95% confidence interval for uqPCR positivity. Admission parasitaemia was used as a covariate.

Simple logistic regression was used to test associations between each potential risk factor and prolonged parasite clearance half-life, and multiple logistic regression used to analyse risk factors significant on univariable analysis.

The strength of the relationship between day 3 parasitaemia and prevalence of kelch13 mutations was estimated using Pearson's correlation coefficient. Multiple logistic regression was used to test associations between potential risk factors and blood smear and uqPCR persistent parasitaemia at 3 days post treatment.

Person gametocytaemia weeks (PGW) were calculated for each case as the number of weeks in which gametocytaemia was present on the peripheral blood smear (excluding admission) divided by the duration of follow-up, expressed per 1,000 person-weeks. The prevalence of gametocytaemia after treatment with DP, AL or DP/AL plus PQ was evaluated using meta-analyses.

All analysis were carried out in Stata statistical software version 14.0 (StataCorp, College Station, Tx, USA) and Graphpad prism software version 6.0 (Graphpad Software Inc., La Jolla, CA, USA). A p value less than 0.05 was considered statistically significant.

6.6 Results

6.6.1 Spatial and temporal distribution of kelch13 mutations in Myanmar

After combining the datasets, infections with parasites with non-synonymous kelch13 SNPs situated between amino acid positions 210 - 440 were pooled in a 'stem' category, and those with SNPs in amino acid positions >440 were categorized as separate groups by amino acid position. In a countrywide survey, a total of 940 samples were successfully sequenced but some of the survey samples overlapped with samples from the DP trial and some were from neighbouring countries (Thailand and Bangladesh). After removing overlapping samples and samples from neighbouring countries, a total of 697 samples remained. Mon and Kayah states each have fewer than 10 samples, and therefore these were not included in the analysis, leaving 687 samples from survey, 114 from DP trial and 152 from AL trial. In total 953 patient samples were analysed by state and region to understand the spatial and temporal distribution of kelch13 mutations.

Figure 6.1 shows the distribution of kelch13 mutations in Myanmar after combining the datasets from the survey and the clinical trials. The distribution of kelch13 mutations found varies between states and regions. The F446I mutation was found in 12.3% (117/953) of all samples. It is the most common kelch13 mutation in Myanmar, widespread throughout five regions. The second most prevalent mutation is C580Y, present in 4.5% (43/953) of samples. C580Y, which is dominant in Cambodia and associated there with a median parasite clearance half-life of 8.7 hours (83), only occurred in southeastern Myanmar close to Tak

province of Thailand. The P574L mutation is widely spread across six regions but unfortunately the parasite clearance half-life of P574L was only measured in the DP trial; it was found to be 7.5 hours in a single patient. The prevalence of kelch13 mutations is higher in border areas such as Kayin and Kachin states than in inland regions such as Mandalay. Overall in my studies 35% of all patients (332/953) were infected with parasites harbouring kelch13 propeller mutations. Using the recent WHO definition of artemisinin resistance we found one kelch13 mutation (C580Y) ‘confirmed’ as conferring artemisinin resistance and nine mutations (P441L, F446I, G449A, N458Y, P553L, R561H, P574L and A675V) ‘associated’ with resistance (60).

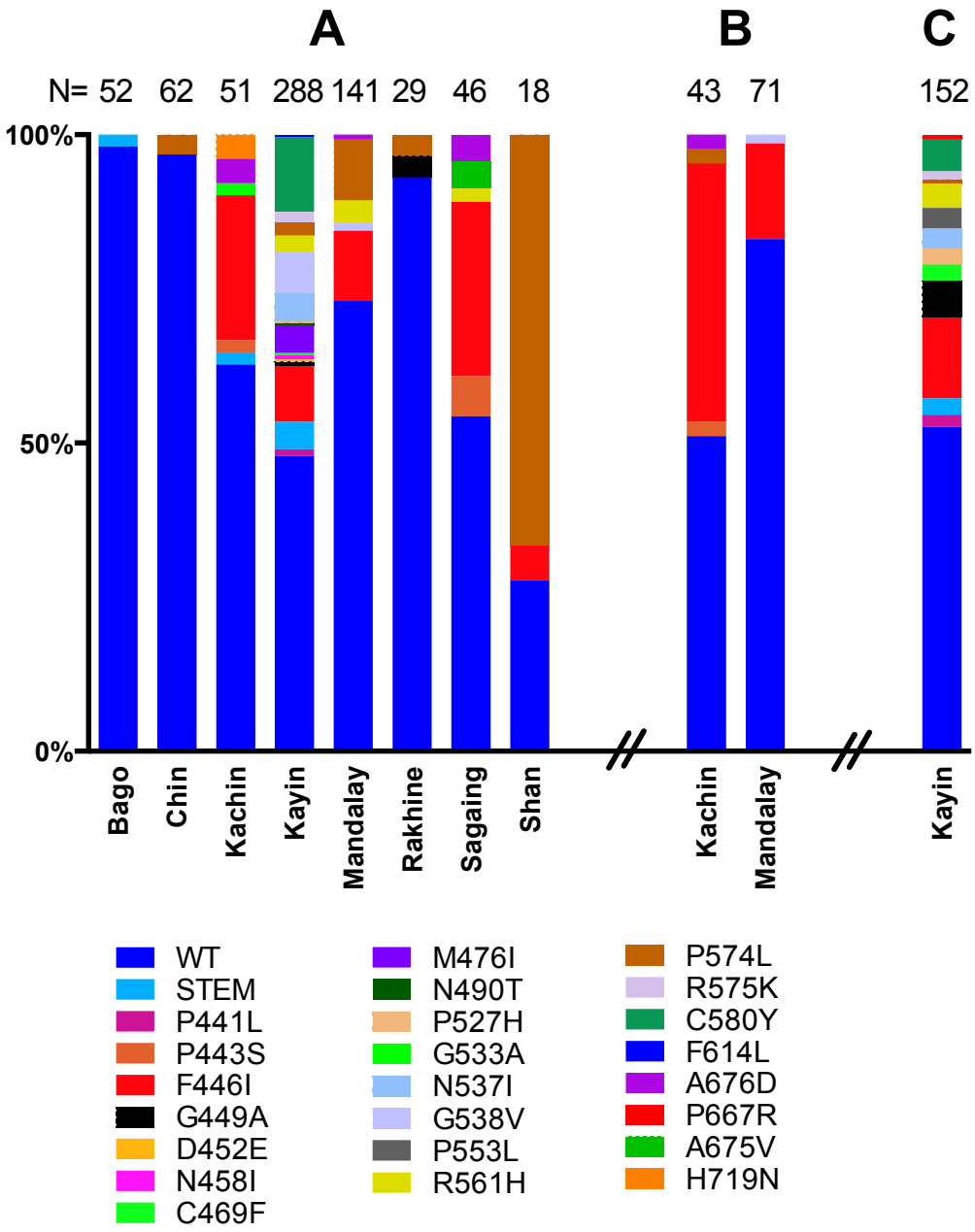


Figure 6.1 Kelch13 mutation prevalence in all studies: (A) country-wide molecular survey; (B) DP trial; and (C) 3 vs. 5 AL trial.

In the combined datasets there are four geographical areas with samples from three years (2013 to 2015), allowing some analysis of temporal trends in the distribution of kelch13 mutations. In Myitkyina, northern Myanmar, a total of 28 and 33 patients were studied in the years 2013 and 2014 respectively. The percent positivity of F446I increased from 25% to 45.5%, however this increase was statistically not significant (p=0.09). In Thabeikkyin, central Myanmar, the percent positivity of F446I increased slightly from 13.3% in 2013 to 22% in 2014. In upper Kyainseikgyi township, which includes Kyaikdon sub-township and Kyainseikgyi town, the percent positivity showed a different pattern from the other areas. In lower Kyainseikgyi township, which includes Thanpaya and three pagoda pass sub-townships, the percent positivity of F446I was similar in 2014 and 2015 (Figure 6.2).

When the infections were dichotomised into two groups as either wild type + stem mutations or kelch13 propeller mutations (>440), and analysed by year, there was a significant increase in kelch13 propeller mutations (pooled across all regions) from 2013 to 2015; 25% in 2013, 32% in 2014 and 68% in 2015 ($p<0.0001$, Chi-squared for trend) (Figure 6.3).

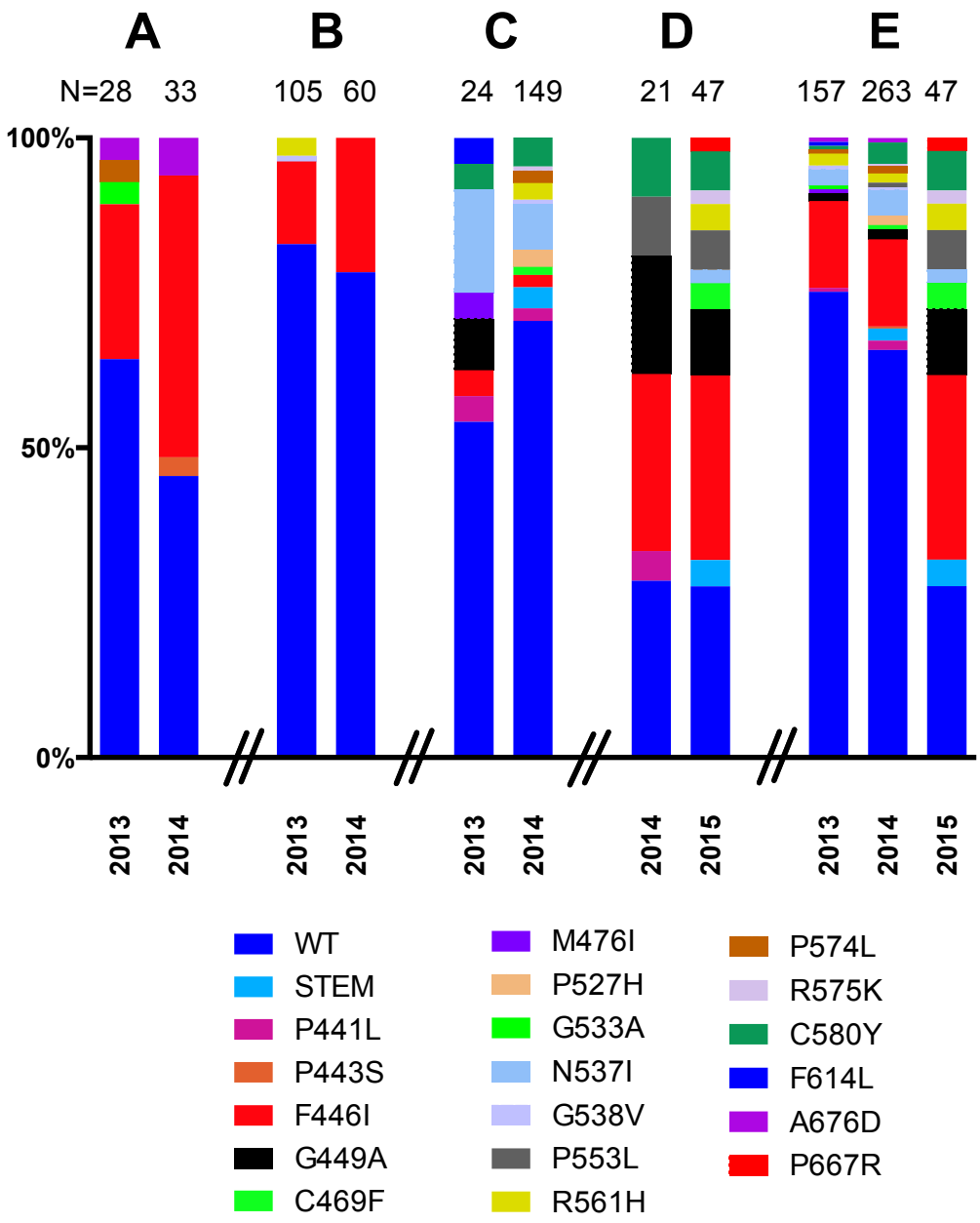


Figure 6.2 Kelch13 mutation prevalence by year in (A) Myitkyina, Kachin state (B) Thabeikkyin, Mandalay region (C) Upper Kyainseikgyi (D) Lower Kyainseikgyi, Kayin state and (E) overall.

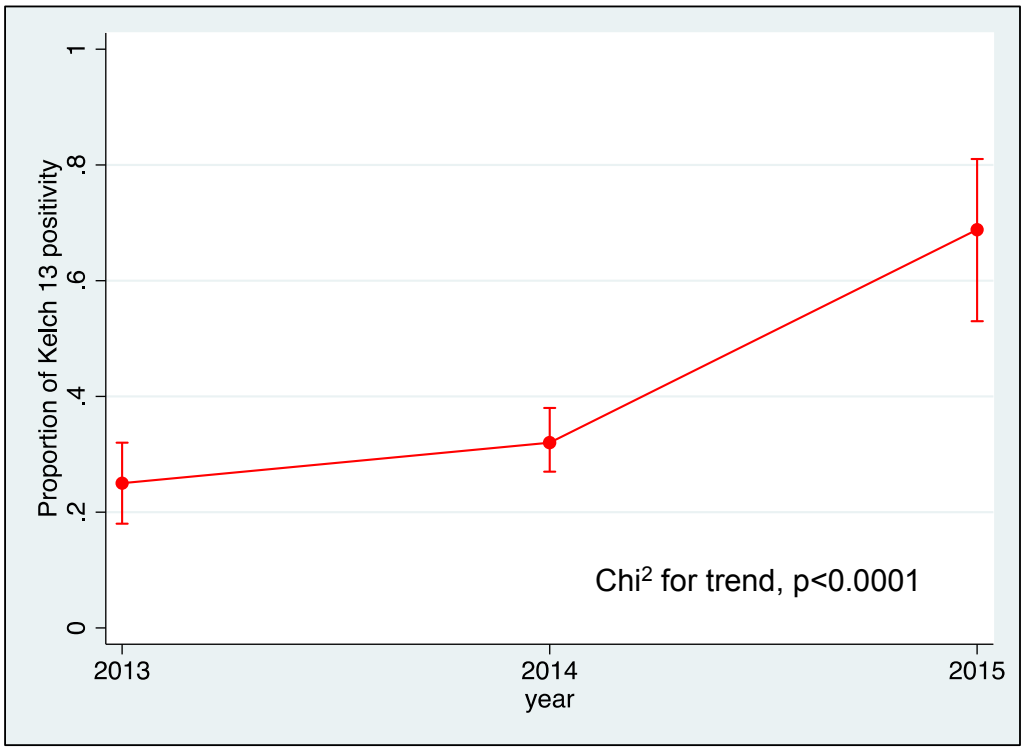


Figure 6.3 Trend of kelch13 mutations and wild type in four different geographical regions of Myanmar (Myitkyina, Thabeikkyin, upper and lower Kyainseikgyi townships) (combined results from the thesis studies; 2013 to 2015).

6.6.2 *Pf* kelch13 and parasite clearance half-life

In general, the clinical phenotype of artemisinin resistance is expressed as prolonged parasite clearance half-life (93, 226) which can be obtained counting parasitized RBC in peripheral blood smears six hourly until two consecutive slides were negative, and the slope half-lives were estimated by using the WWARN parasite clearance estimator (101). To confirm artemisinin resistance the parasite clearance half-life need to be measured, and to confirm the association of a particular kelch13 mutation with resistance the association between genotype and parasite clearance phenotype established. In a recent update from WHO (60) only four kelch13 mutations (Y493H, R539T, I543T and C580Y) were validated as confirmed mutations and there was lack of clarity about F446I.

To assess the parasite clearance rate phenotypes of the mutations found in Myanmar during my research, and compare this to artemisinin resistant phenotypes observed elsewhere, I analysed the parasite clearance half-lives of infections from the DP trial alongside those of the reference “confirmed” molecular marker (C580Y) in closely studied Cambodian patients (with permission of Ashley et al (93)). The F446I mutation was associated with a median clearance half-life of 4.7 hours, relatively lower than that of C580Y (6.4 hours)(Figure 6.4).

A global ANOVA F-test was used to compare the three groups: WT and F446I from the DP study, and C580Y from the TRAC 1 study. There was a significant difference between the three groups ($p<0.001$), and on post-hoc analysis the differences in parasite half lives between infections with WT and F446I, WT and

C580Y, and F446I and C580Y were all statistically significant (Bonferroni corrected, p <0.001 for all comparisons) (Figure 6.5).

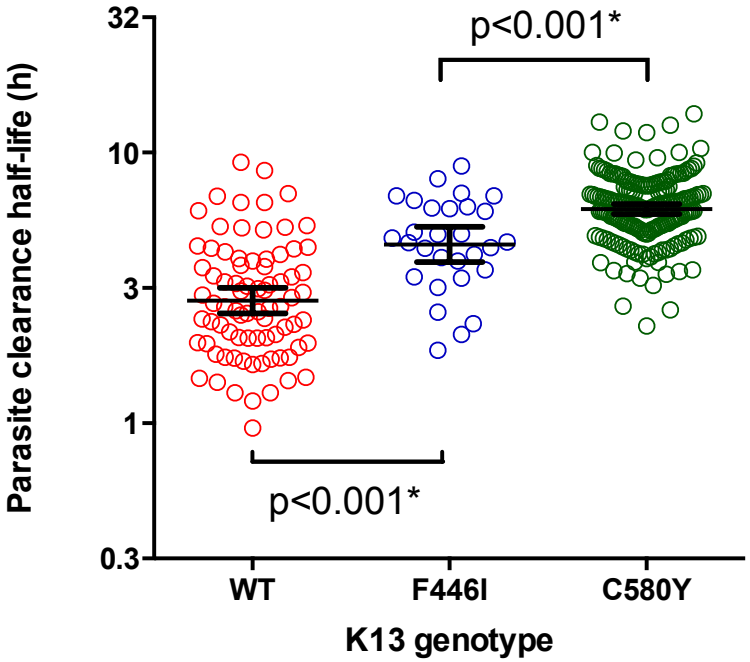


Figure 6.4 Parasite clearance half-life and kelch13 mutation: WT and F446I data from the DP trial compared to C580Y parasite clearance half-lives from Cambodia. These latter data have been published in Ashley *et al.* (93). One circle indicates one patient.

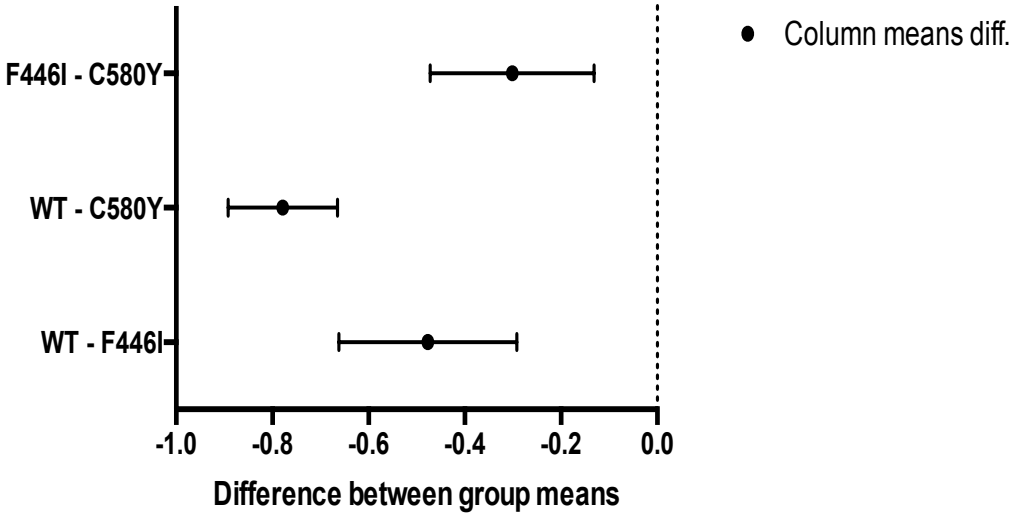


Figure 6.5 Differences between group means of parasite clearance half life, using wild type and F446I data from the DP trial and C580Y half-life data from Cambodia. These latter data have been published in Ashley *et al.* (93). Black circles represent differences between group means and bars represent the 95% confidence intervals.

6.6.3 Parasite clearance half-life across the countries

As shown in Figure 6.6, median parasite clearance half-lives range from 2.5 hours in Ramu, Bangladesh to 6.1 hours in Pailin, Cambodia. Among the three sites in Myanmar, the Thabeikkyin patients had the fastest clearance (median parasite clearance half-life 2.6 hours), with Shwe Kyin and Myitkyina patients significantly slower (4.2 and 4.4 hours respectively). For comparison, in Bangladesh, on Myanmar's western border, the median parasite clearance half-life in the TRAC study was 2.5 hours. Only 1.8% of these Bangladeshi patients had a prolonged parasite clearance half-life (>5hr). This compares with 12.7, 12.5 and 39.5 percent in Thabeikkyin, Shwe Kyin and Myitkyina, respectively, in Myanmar. At the other extreme, 71.4 % of all patients from Pailin, Cambodia showed a delayed clearance half-life (Table 6.1). For multivariate analyses of factors associated with parasite clearance, half-life cut-off values of 4 and 5 hours were used. With the 4 hour cut-off, the presence of kelch13 propeller mutation had an odds ratio for prolonged parasite clearance half-life of 13.0 (95%CI 8.0 to 20.3, $p<0.001$). Using a cut-off of 5 hours, the odds ratio was 8.3 (95%CI 5.3 to 12.8, $p<0.001$). The type of treatment (drug) and parasitaemia on admission were not significant risk factors for prolonged parasite clearance half-life (Table 6.2).

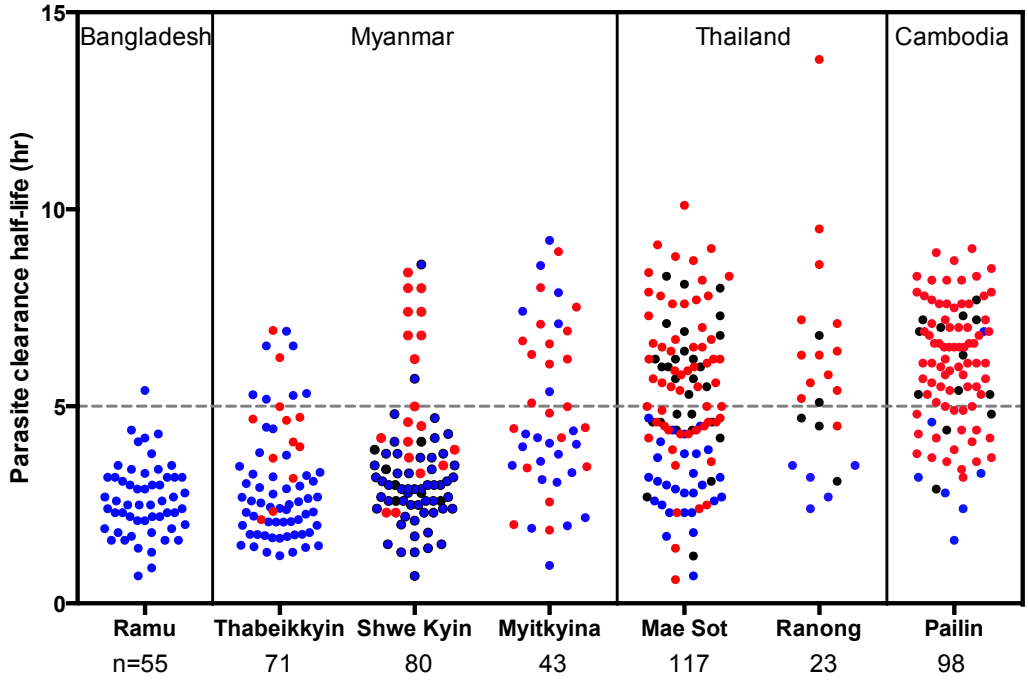


Figure 6.6 Parasite clearance half-life across study sites in Myanmar, Bangladesh, Thailand and Cambodia. One circle represents one patient. Red circles represent a kelch13 mutation after position 440, black circles represent at least part of kelch13 sequence missing or heterozygous, and blue circles are wild type or mutation before position 441. Thabeikkyin and Myitkyina data are from the current study, and other sites are from the TRAC 1 study. These latter data have been published previously in Ashley *et al.* (93)

Table 6.1 Parasite clearance and kelch13 propeller mutations across the sites

Study site	n	Year	Median parasite clearance half-life (IQR) hours	Parasite clearance half-life >5hr No. of patients/total no. (%)	Parasite clearance half-life >4hr No. of patients/total no. (%)	kelch13 propeller mutation[§] No. of patients/total no. (%)
Ramu	55	2011 to 2013	2.5 (2-3.2)	1/55 (2)	5/55 (9)	0/55 (0)
Thabeikkyin[#]	71	2013 to 2014	2.6 (2.0-3.8)	9/71 (13)	16/71 (23)	12/71 (17)
Shwe Kyin	80	2011 to 2013	3.1 (2.6-4.1)	10/80 (13)	21/80 (26)	19/73 (26)
Myitkyina[#]	43	2013 to 2014	4.4 (3.4-6.6)	17/43 (40)	27/43 (63)	21/43 (49)
Mae sot	117	2011 to 2013	4.9 (3.7-6.4)	55/117 (47)	83/117 (71)	61/88 (69)
Ranong	23	2011 to 2013	5.4 (3.5-6.8)	14/23 (61)	17/23 (74)	13/18 (72)
Pailin	89	2011 to 2013	6.1 (4.9-7.2)	70/98 (72)	84/98 (86)	78/85 (92)

[§] The denominator excludes missing and heterozygous genotypes.
[#] Two sites from current study and others from TRAC 1 study by Ashley *et al.* (93). Patients in the current study received dihydroartemisinin-piperaquine for three days and the others (Ashley *et al.* (93)) received artesunate 2 or 4 mg/kg for 3 days followed by 3 day course of ACTs.

Table 6.2 Multivariable analysis examining relationship between clearance half-life and the covariates admission parasitaemia, type of treatment, and kelch13 propeller mutations

	Odds ratio	95% CI	P value
Half-life cutoff 4 hours			
Parasitaemia on admission (>1%)	1.3	0.9-2.0	0.20
Drug: AS2	Ref		
AS4	0.9	0.5-1.5	0.71
DP	0.6	0.3-1.0	0.07
Kelch 13 (>440)	12.8	8.0-20.3	<0.001*
Half-life cutoff 5 hours			
Parasitaemia on admission (>1%)	1.5	1.0-2.3	0.07
Drug: AS2	Ref		
AS4	1.6	1.0-2.7	0.07
DP	0.8	0.4-1.5	0.43
Kelch 13 (>440)	8.3	5.3-12.8	<0.001*

* statistically significant

6.6.4 Ultrasensitive quantitative PCR findings and treatment regimens

6.6.4.1 *Kaplan-Meier analysis of uqPCR by two treatment regimens*

A total of 258 patients were included in this analysis with 114 patients from DP trial and 154 from 3 vs. 5 AL trial. The Kaplan-Meier curves show that patients treated with AL have higher rates of uqPCR negativity, however this is an uncontrolled comparison between two trials carried out in different geographical settings and the level of population immunity may affect uqPCR negativity. uqPCR negativity at day 14 was 44% (95%CI 38% to 50%) in those receiving AL and 12% (7% to 21%) in those receiving DP (Figure 6.7).

The risk of positivity at day 21 was twofold in patients treated with DP compared to those treated with AL. The Cox regression gave a hazard ratio of 1.9 (95%CI 1.4 to 2.7, p <0.001). After adjusting for admission parasitaemia, the risk of positivity at day 21 was nearly one and half fold in patients treated with DP compared those with AL. The Cox regression gave a hazard ratio of 1.6 (95% CI 1.1 to 2.2, p <0.001)(Table 6.3).

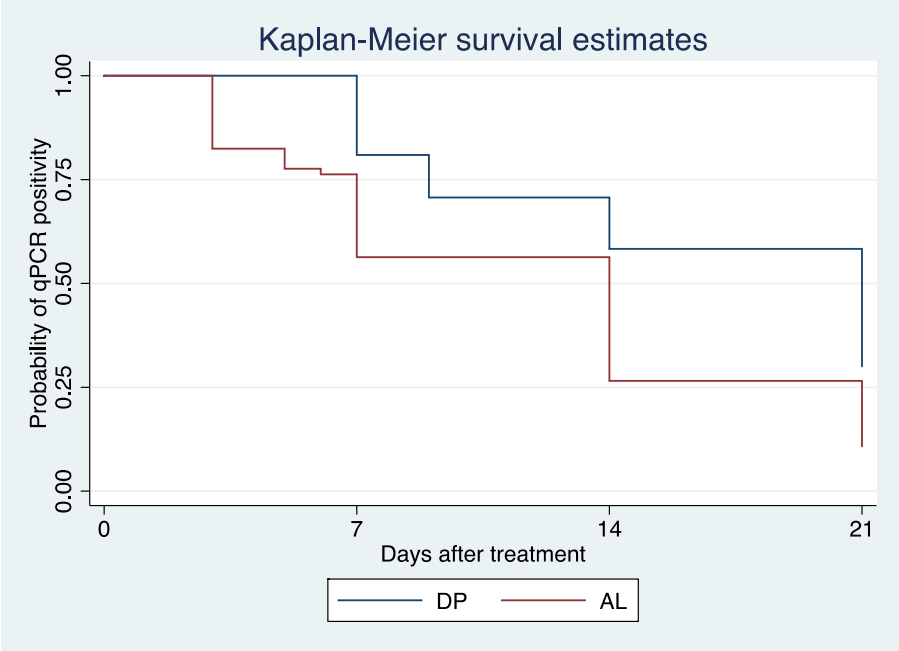


Figure 6.7 Kaplan-Meier survival estimates for becoming uqPCR negative, by treatment regimens. DP refers dihydroartemisinin-piperaquine and AL to artemether-lumefantrine.

Table 6.3 Hazard ratios for risk factors of uqPCR negativity in patients treated with artemisinin-based combination therapy

Risk factor		**Percentage of uqPCR negativity 21 days**	**Hazard ratio (95% CI)**	**Hazard ratio adjusted for admission parasitaemia (95% CI)**
Admission Parasitaemia	<1%	89 (83 to 93)	0.4 (0.3 to 0.6)	
	>=1%	65 (51 to 79)		
Drugs	DP	70 (58 to 81)	1.9 (1.4 to 2.7)	1.6 (1.1 to 2.2)
	AL	89 (83 to 94)		

6.6.4.2 *Percent positivity of uqPCR*

After combining the dataset, every participant data collection point was checked. If the uqPCR collection was only up to day 9, these participants were dropped from the main data set. This left 151 participants in the repeated measures analysis of uqPCR. A total of 42, 54 and 55 participants from DP, 3 day AL and 5 day AL treatment arms were included, respectively.

Table 6.4 illustrates the breakdown of uqPCR positivity in three different treatment groups. The percent positivity of uqPCR after treatment with DP was 40% at day 21 whereas for the 3 day and 5 day AL arms this was 10% and 15% respectively. However, the two clinical trials had different inclusion criteria and the initial admission parasitaemia in DP trial was higher than that of the AL trial.

Table 6.4 Percent positivity of uqPCR by three treatment regimens

uqPCR	*Day 0*	*Day 3*	*Day 7*	*Day 14*	*Day 21*
	No (%)	*No (%)*	*No (%)*	*No (%)*	*No (%)*
Negative	0 (0)	13(8.7)	40(27.0)	85(57.8)	117(79.6)
Positive	151(100)	137(91.3)	108(73.0)	62(42.2)	30(20.4)
Total (N)	151	150	148	147	147
DP					
Negative	0(0)	0(0)	2(4.9)	9(23)	25(60)
Positive	42(100)	42(100)	39(95)	31(78)	17(40)
Subtotal (n)	42	42	41	40	42
3 day AL					
Negative	0(0)	7(13)	19(35)	39(72)	47(90)
Positive	54(100)	47(87)	35(65)	15(28)	5(10)
Subtotal (n)	54	54	54	54	52
5 day AL					
Negative	0(0)	6(11)	19(36)	37(70)	45(85)
Positive	55(100)	48(89)	34(64)	16(30)	8(15)
Subtotal (n)	55	54	53	53	53

6.6.4.3 *Regression analysis of parasite density and treatment regimens*

As Table 6.5 shows, it is apparent that the number of parasites as assessed by uqPCR remaining at day 3 after the treatment of DP is higher than that in the 3 day AL and 5 day AL arms after adjusting for admission parasitaemia. These result are significant at p=0.002 and p<0.001 respectively. However, there is no difference between the 3 day and 5 day AL treatment arm (p=0.3). These results suggest that AL is better than DP in relation to parasite clearance at day 3 using uqPCR in this study, though differences in immunity between the sites cannot be excluded as a possibility.

Table 6.5 Regression analysis of log parasite density on day 3 after treatment measured by uqPCR by treatment arm after adjusting for admission parasitaemia

Treatment	*Coefficient*	*p*	*95% CI*
DP	Ref		
3D AL	-1.3	0.002*	-2.1 to -0.5
5D AL	-1.7	<0.001*	-2.5 to -0.9
3D AL	Ref		
DP	1.3	0.002*	0.5 to 2.1
5D AL	-0.4	0.3	-1.2 to 0.4

* statistically significant

6.6.4.4 *Relationship between kelch13 and residual parasite density using uqPCR*

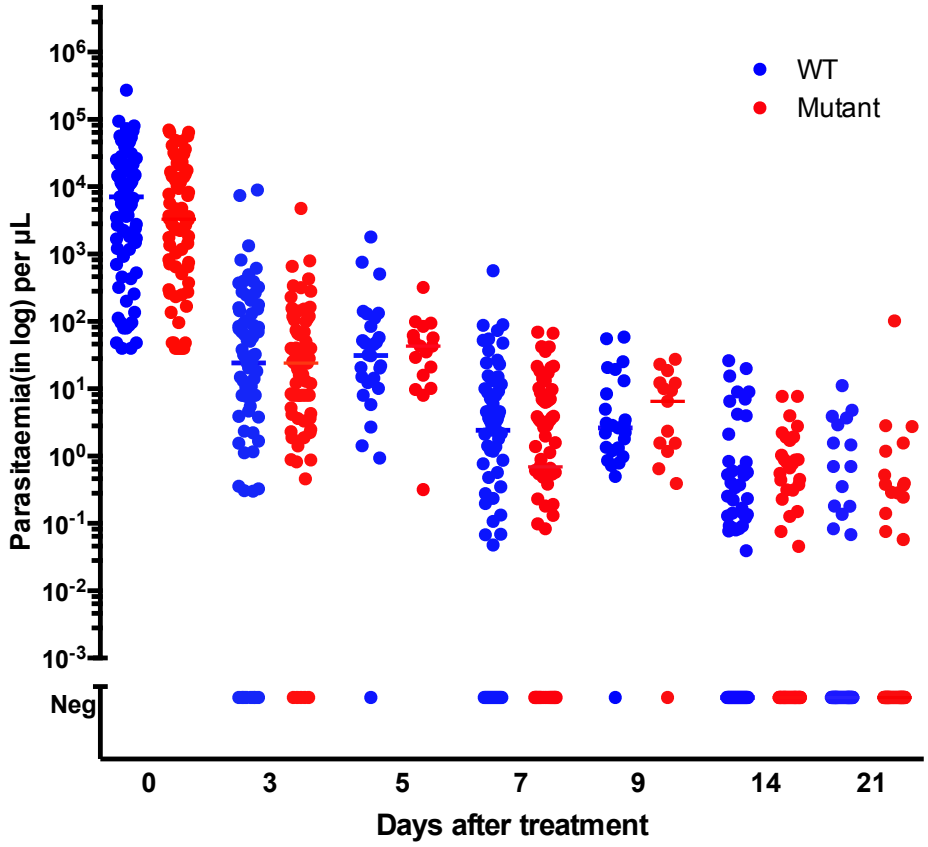


Figure 6.8 Residual parasitaemia using uqPCR by kelch13 propeller mutation and days after treatment. Blue circles represent patients with kelch13 wild type and red circles represent mutation after position 440.

Individual patients' parasite density over time using uqPCR was shown in Figure 6.8.

Table 6.6 Random effects maximum likelihood linear regression analysis examining relationship between residual parasite density overtime using uqPCR methods and treatment arm and kelch13 mutations after adjusting for admission parasitaemia

Variable		*Coefficient*	*p*	*95% CI*
Day		-0.7	<0.001*	-0.7 to -0.6
Drug	DP	Ref		
	AL	-2.9	<0.001*	-3.6 to -2.1
Kelch13	Wild type	Ref		
	Mutations (>440)	-0.1	0.8	-0.8 to 0.6

The total number of observations over time was 693 and the average number of observations per patient was 4.6 (Range 2 to 7). Unsurprisingly the parasite density decreased significantly over time due to the effect of treatment ($p<0.001$) after adjusting for the admission parasitaemia (Table 6.6). The AL treatment effect is greater than that for DP after adjusting the admission parasitaemia ($p<0.001$). There was no effect of presence of a kelch13 propeller mutation on parasite density over time compared to patients with wild type infections ($p=0.8$).

Regression analysis of log-transformed parasitaemia by kelch13 mutations was performed and stratified by site. No significant effect of kelch13 propeller mutation on parasitaemia was found. The slope of the parasitaemia curve for

infections with kelch13 propeller mutations was slightly different from that of those with kelch 13 wild type but was not statistically significant.

6.6.5 Day 3 parasite positivity after treatment

Although day 3 positivity on blood smear is only a rough guide to the prevalence of artemisinin resistance, it is a measurement common to both of our two clinical trials (in the AL study frequent sampling for parasitaemia was not possible). Therefore, data on day 3 blood smear positivity and kelch13 propeller mutant frequency from these two clinical trials and from other publications (93, 112, 115) could be plotted together (Figure 6.9). The Pearson correlation coefficient was 0.89 ($p<0.0001$). It can be seen that if there is high prevalence of kelch13 mutations, day 3 parasite positivity will be high. In areas like Cambodia, the C580Y mutation is predominant and it was found that there was a high frequency of kelch13 mutation-bearing infections in which parasites are not cleared by day 3. But in areas such as northern Myanmar and the China-Myanmar border, where the F446I mutation is predominant, the day 3 parasitaemia rate is relatively low because of the "intermediate" nature of the mutation phenotype. In addition, day 3 parasitaemia is influenced by the admission parasitaemia (180).

As discussed in section 6.5.4, 3 days of AL appears superior to DP in relation to parasite clearance at day 3 as measured by uqPCR. By microscopy, day 3 positivity in the 3 day AL arm was 23% (18/78), compared to 18% (20/114) in the DP study ($p>0.05$). As noted above, this analysis does not take into account geographical differences in immunity – which could materially affect parasite clearance rates.

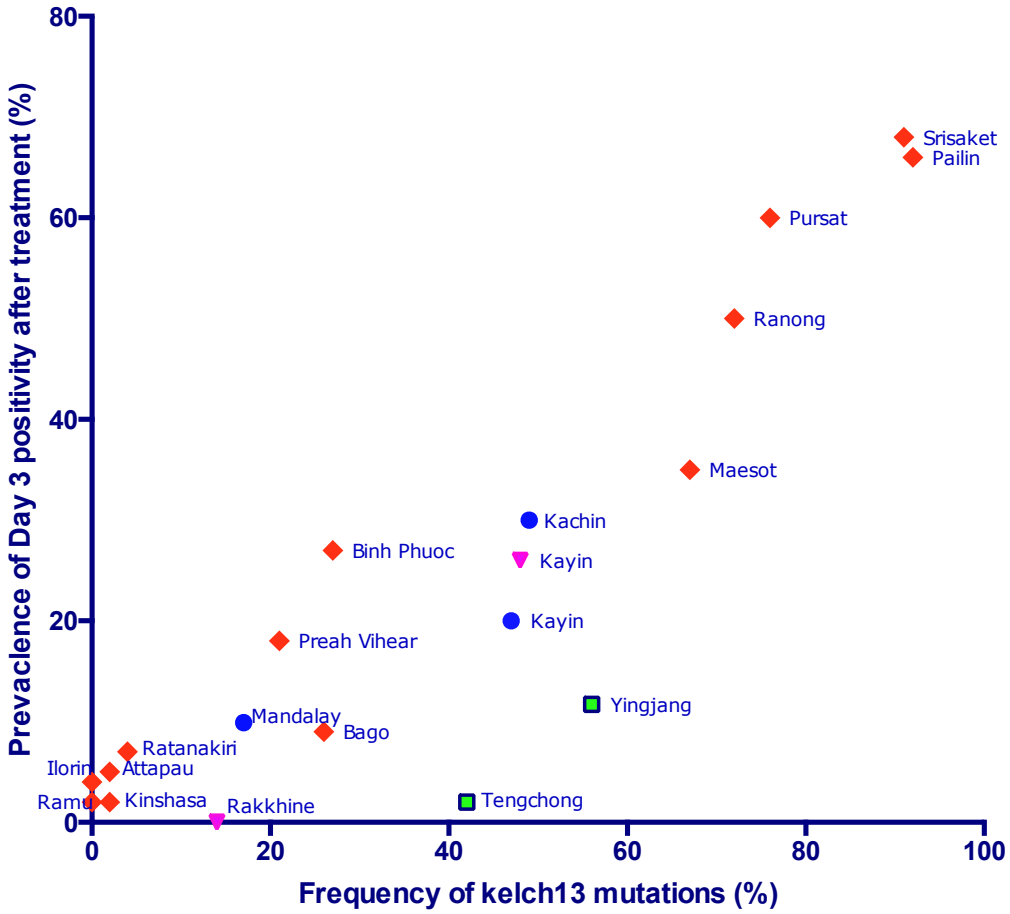


Figure 6.9 Correlation between frequency of non-synonymous kelch13 mutations and the prevalence of day 3 blood smear positivity after artemisinin combination therapy. Circles are from current study, diamonds represent data from Ashley *et al.* (93), triangles are from Nyunt *et al.* (112) and squares represent data from Huang *et al.* (115).

(KEY; **Myanmar**: Mandalay, Kachin, Kayin, Bago, Rakkhine; **Bangladesh**: Ramu; **Thailand**: Mesot, Srisaket, Ranong; **Laos**: Attapeu; **Cambodia**: Pailin, Preah Vihear, Ratanakiri, Pursat; **Vietnam**: Binh Phuoc; **Nigeria**: Ilorin; **DR Congo**: Kinshasa; **China**: Tengchong, Yinjang)

6.6.5.1 *Relationship between blood smear, uqPCR day 3 parasitaemia and presence of kelch13 propeller mutations*

There was a discrepancy between blood smear and uqPCR day 3 positive parasitaemia in this study.

In the DP trial, 18% of patients were identified as blood smear positive while 95% of patients were identified as uqPCR positive at day 3. In the 3 vs. 5 AL trial, 20% of patients were identified as blood smear positive while 84% of patients were identified as uqPCR positive at day 3. Multivariable logistic regression analyses were carried out using both blood smear and uqPCR day 3 positivity as the dependent variable. With blood smear day 3 positivity, parasitaemia on admission (day 0) as an explanatory variable was associated with an odds ratio of 3.4 (95%CI 1.2 to 9.6, p=0.02) while the presence of a kelch13 propeller mutation was a weak predictor (Table 6.7).

With uqPCR day 3 positivity as the dependent variable, treatment with artemether-lumefantrine as an explanatory variable was associated with an odds ratio of 0.3 (95% CI 0.1 to 0.9, p=0.03), while parasitaemia on admission and presence of a kelch13 propeller mutation were not significant factors (Table 6.7).

Table 6.7 Multivariable analysis examining the relationship between blood smear or uqPCR day 3 positivity and the covariates admission parasitaemia, type of treatment, and kelch13 propeller mutations

Day 3 positivity		*Odds ratio*	*95% CI*	*P value*
Blood smear				
Parasitaemia on admission	<1%	Ref		
	≥1%	3.4	1.2 to 9.6	0.02*
Drug	DP	Ref		
	AL	2.2	0.9 to 5.7	0.1
Kelch13	WT	Ref		
	Mutation (>440)	1.9	1.0 to 3.5	0.05
uqPCR				
Parasitaemia on admission	<1%	Ref		
	≥1%	1.0	0.2 to 4.8	1.0
Drug	DP	Ref		
	AL	0.3	0.1 to 0.9	0.03*
Kelch13	WT	Ref		
	Mutation (>440)	1.8	0.8 to 4.1	0.2

DP, dihydroartemisinin-piperaquine; AL, artemether-lumefantrine; WT, wild type.

6.6.6 Person gametocytaemia week

In the DP trial, in which all patients received single low dose primaquine, gametocytaemia was detected post-treatment at a rate of 26 PGW per 1,000 weeks. In comparison to other studies done in Myanmar or in Africa (63, 187,

188), our gametocytaemia duration was shorter than with DP alone but three times longer than with DP plus 0.75 mg primaquine. Our DP trial used a three fold lower dose of primaquine than Smithuis *et al.* (Table 6.1).

On meta analysis, the prevalence of gametocytaemia after treatment with DP alone was higher compared to DP plus a single dose primaquine ($Z=60.52$, $p<0.001$)(Figure 6.10). However, the Grande *et al.* study conducted in 5 countries in Africa using DP alone (224) showed similar results to the Myanmar DP plus single dose primaquine studies. This may be due to geographical differences in gametocyte rate and immunity.

In the 3 vs 5 AL trial, in which all patients received single low dose primaquine, we found 3 PGW per 1,000 weeks in both the 3 day and 5 day AL arms. In other studies in which patients were randomised to AL plus low dose primaquine or AL alone, the PGW was shorter in the primaquine group (63, 187). However, contrary to expectations, AL plus 0.75 mg primaquine in the 2010 Smithuis *et al.* study in Myanmar showed a longer PGW duration than in our study. This may be related to differences in study design (in the timing and frequency of measurements of gametocytes by microscopy), or to differences in the study populations (malaria transmission and baseline gametocytaemia). The prevalence of gametocytaemia (summarized as PGW) after treatment with AL alone was shown to be higher than in studies with AL plus single dose primaquine ($Z=23.38$, $p<0.001$) (Figure 6.11).

There was no effect of kelch13 propeller mutation on gametocytaemia expressed as PGW (PGW/1,000 weeks of follow up) and there was no significant difference between wild type and kelch13 propeller mutations after adjusting for treatment arm ($p=0.12$).

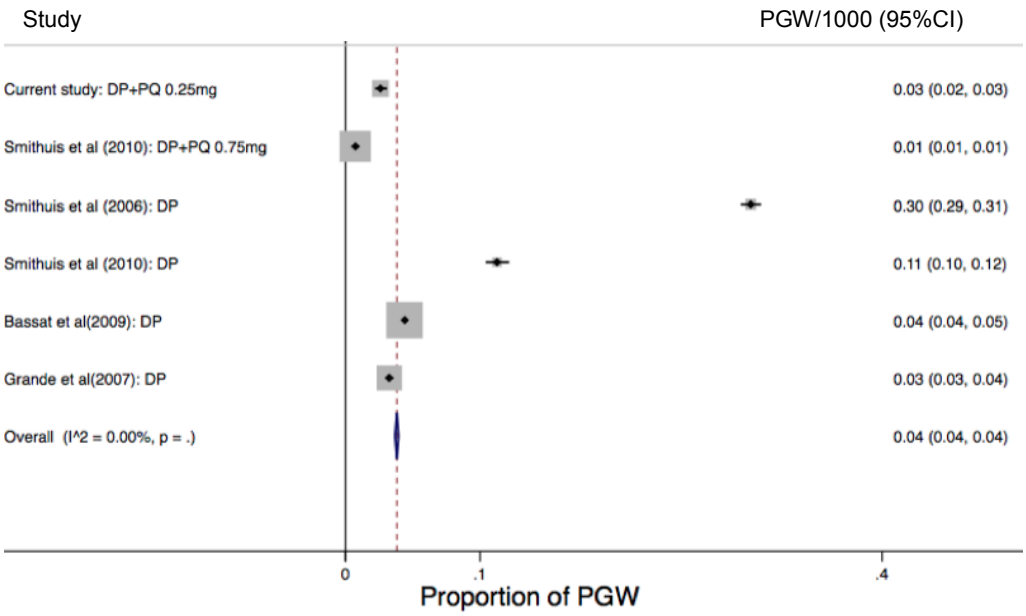


Figure 6.10 Forest plot displaying the duration of gametocytaemia (Person gametocytaemia week, PGW) per 1000 person-weeks after treatment with DP and DP plus PQ. Red dashed line represents PGW across studies. Size of box is proportional to the size of individual trial.

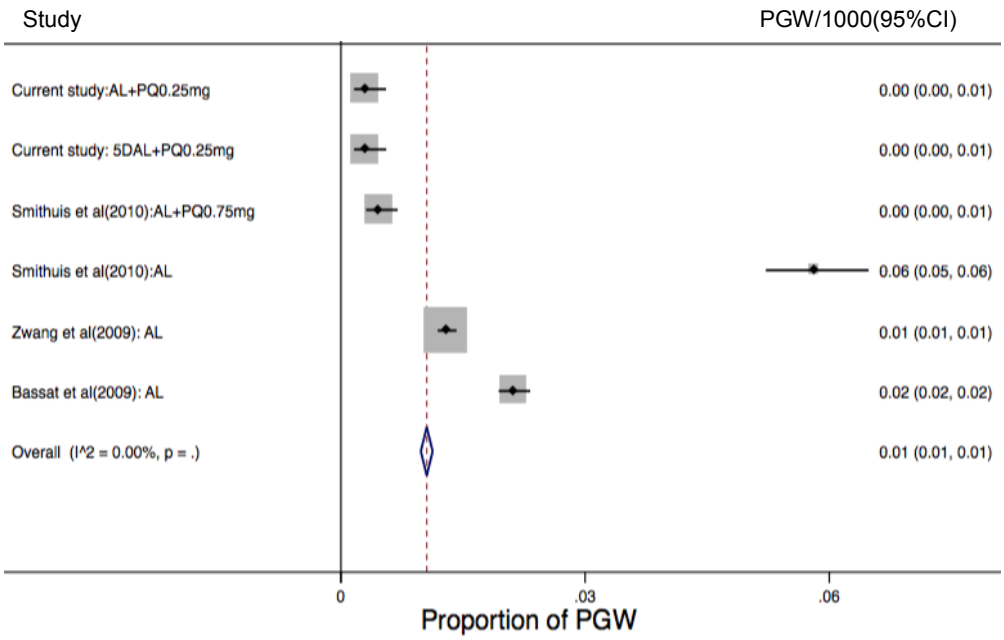


Figure 6.11 Forest plot displaying the duration of gametocytaemia (Person gametocytaemia week, PGW) per 100 person-weeks after treatment with AL and AL plus PQ. Red dashed line represents PGW across studies. Size of box is proportional to size of individual trial.

Table 6.8 Comparison of person gametocytaemia weeks (PGW) per 1,000 person weeks in DP/AL and DP/AL plus single dose PQ

Study	PGW (96% CI)	Follow up days	n	Design	Population	Country
DP + PQ 0.25 mg	26 (16,31)	42	114	Open label	>5 years to 65 years	Current study
DP + PQ 0.75 mg	7.17	63	83	Randomized open label trial	>1 years to 65 years	Myanmar (2010)(63)
DP	302	42	334	Randomized open label trial	>1 years to 65 years	Myanmar (2006)(188)
DP	112.8	63	78	Randomized open label trial	>1 years to 65 years	Myanmar (2010)(63)
DP	43.97	42	1,038	Randomized open label trial	6-59 months children	Burkina Faso, Kenya, Mozambique, Uganda and Zambia(187)
DP	32.5	63	230	Randomized open label trial	>5-60 years	Peru (224)
3D AL+PQ 0.25 mg	3 (1.3,10.7)	42	78	Randomized open label trial	>5 years to 65 years	Current study
5D AL+PQ 0.25 mg	3 (1.3,10.8)	42	77	Randomized open label trial	>5 years to 65 years	Current study
3D AL+PQ 0.75 mg	4.65	63	78	Randomized open label trial	>1 years to 65 years	Myanmar (2010)(63)
3D AL	58.2	63	84	Randomized open label trial	>1 years to 65 years	Myanmar (2010)(63)
3D AL	13	28	1,319	Randomized, comparative and open label	Children and adults	Sub-Saharan Africa (225)
3D AL	21.23	42	510	Randomized open label trial	6-59 months children	Burkina Faso, Kenya, Mozambique, Uganda and Zambia (187)

6.6.7 Treatment efficacy in two clinical trials

After initial parasite clearance on blood smear, in the DP trial all patients remained parasite free by blood smear until 42 days; two patients were lost to follow-up before 42 days with negative blood films at their last follow-up visit.

After initial blood smear negativity two cases in the 3 vs. 5 AL trial became *P. falciparum* positive by blood smear, one at day 28 and the other at day 42. PCR genotyping could not be performed because of low parasite density in the two recrudescent infections. Both cases were infected with parasites carrying a kelch13 propeller mutation (one F446I, the other G449A). There were also four cases of symptomatic *P. vivax* infection in the 3 vs. 5 AL trial, detected at day 35 (n=3) and day 42 (n=1). Among these, three cases an initial falciparum monoinfection, and one was a recurrent parasitaemia in a patient with mixed falciparum and vivax infection on admission. PCR genotyping for *P. vivax* could not be performed.

6.7 Discussion

6.7.1 Kelch13 mutations, including F446I

This study conducted in ten administrative states and regions of Myanmar found that *P. falciparum* kelch13 mutations are widespread throughout the north, central and southeastern parts of the country. Overall 35% of patients had kelch13 propeller mutations. The day 3 parasite positivity rate ranged from 18% to 23%. The kelch13 F446I mutation is predominant in the Yunnan province of China along the Myanmar border (115, 116, 179), which is 100 km from the Myitkyina site, and has increased over time along the Myanmar-China border area as well as in Upper Myanmar. From 2013 to 2015, the percent positivity of kelch13 propeller mutations in my studies sharply increased (25% to 68%). There was a higher prevalence of kelch13 molecular markers in eastern border states and regions compared to the inland regions. This suggests that to achieve the ultimate goal of elimination in the region, cross border malaria control should focus on migrant workers, the military population, and ethnic minorities.

From the population genetics perspective it has been proposed that the spread of resistance is geographically compartmentalized. A study conducted by Miotto *et al.* found that parasites harbouring the most common kelch13 alleles (C580Y, Y493H, and R539T) have spread across borders in the region containing Cambodia, Vietnam and Eastern Thailand (85). Other research has emphasised independent emergence of kelch13 mutations, particularly in regions of Southeast Asia more geographically distant from the Western Cambodian epicentre (119). From a public health perspective, whether artemisinin resistance has spread from somewhere else or emerged independently in specific areas, all

stakeholders in the global health community have to take responsibility to halt the problem of artemisinin resistance by all means.

6.7.2 Parasite clearance half-life

Comparing the results of the DP study with other published studies I was able to make a detailed assessment of parasite clearance half-life, which remains the gold standard method of measurement for determining artemisinin resistance phenotypes needed for the characterization of artemisinin resistance (80, 82, 85, 171). The use of clearance half-life to define artemisinin resistance was validated in western Cambodia, where C580Y is predominant (103). Along the Thai-Myanmar border, where longitudinal studies have been undertaken for more than two decades, the C580Y mutation only recently replaced earlier kelch13 mutations associated with lesser effects on parasite clearance (93, 170). At the China-Myanmar border, F446I was the predominant kelch13 mutation during the study period (115, 149, 179). This mutation is associated with a significant slowing in parasite clearance, but not to the same extent as that associated with the mutations now prevalent along the Thai-Myanmar border or in western and northern Cambodia and southern Laos. The median parasite clearance half-life of F446I infections in this study was 4.7 hours. However, the median clearance half-life in Ramu (Bangladesh) was only 2.5 hours, as kelch13 mutations and the artemisinin resistance problem are not yet present (93, 113). The parasite clearance half-life data suggests that Myanmar is the front line of the extent of artemisinin resistance, and the region where efforts to halt the spread of artemisinin resistance from Southeast Asia to the India subcontinent and Africa should be focused.

6.7.3 Persistent parasitaemia at day 3 on blood smear and uqPCR

In this study, parasitaemia on admission was significantly associated with day 3 blood smear persistent parasitaemia while the presence of a kelch13 propeller mutation was only a weak predictor. Type of treatment was also significantly associated with day 3 uqPCR persistent parasitaemia, independent of kelch13 propeller mutations. It may be due to the intermediate nature of kelch13 propeller mutations (e.g. F446I) predominant in this study. This finding suggests that as expected uqPCR measurements can identify more positive cases than microscopy. Once calibrated against treatment failures, in larger studies where treatment failures are a problem, day 3 uqPCR positivity may be a useful tool to predict the presence of resistance. However, day 3 blood smear positivity remains useful as a rough epidemiological tool for use where frequent blood sampling to determine parasite clearance half life is not possible.

6.7.4 Quantitative PCR to detect sub-microscopic parasitaemia

Residual parasitaemia after ACT treatment is an important determinant of the transmission potential of malaria infection. There is very little published data on the time course of parasitaemia measured by uqPCR following the treatment of symptomatic malaria. To date, only Thriemer *et al.* (190) from central Vietnam and Beshir *et al.* (189) from Kenya reported prevalence of standard (not ultrasensitive) qPCR parasitaemia after ACT treatment. Thriemer *et al.* reported that nearly 25% of samples showed positive results on uqPCR at day 21 and the prevalence of kelch13 propeller mutation was 80% (I543T and Y493H). The authors suggested that the submicroscopic parasites seen during follow up were probably circulating gametocytes (190). Beshir *et al.* reported that 32% of

children showed residual parasitaemia on day 3 by duplex quantitative PCR (189). Although all patients in the two Myanmar clinical trials had cleared parasitaemia by day 5 as measured by microscopy, when using uqPCR it was found that 10 to 40 % of patients still carried parasites on day 21 despite treatment with a full course of an ACT.

Despite this there were two treatment failures by the standard WHO criteria in my AL study (i.e. less than two percent of all patients except for the 12 lost to follow-up failed to achieve adequate clinical and parasitological response – ACPR). Further research is necessary to determine whether this submicroscopic infection consists of asexual parasites, possibly in a dormant or 'sleeping' form (221, 223), or gametocytes.

6.7.5 Treatment efficacy

Many factors can compromise the treatment efficacy of antimalarial drugs, such as incorrect dosing, counterfeit or substandard drugs, poor absorption, and poor compliance of patients in following the treatment regimen (227). One study found that in Southeast Asia 32% of antimalarial drugs tested were poor quality or substandard (outside 85-115% API)(228). A recent study in Myanmar on the availability and quality of antimalarial drugs in the private sector outlets found that artemisinin based mono-therapy was commonly found in retailers (71). The cost of authentic drugs in developing countries is beyond the means of poor people, and this is one of the reasons why cheaper drugs (often substandard or counterfeit) can be found in the market.

At the time of recruitment into the trials reported in this thesis (2013-2015) therapeutic efficacy of AL and DP was still high within Myanmar, but with the

spread of artemisinin resistance lumefantrine and piperaquine are now less well protected; this could be “the calm before the storm” (182).

6.7.6 Gametocytaemia

Sawa *et al.* (229) suggested that AL is most appropriate for low endemicity settings to reduce community transmission of *P. falciparum.* In addition, single low dose primaquine is safe and there were no complications in my studies. The person gametocytaemia week data support the addition of single low dose primaquine to kill gametocytes in areas of artemisinin resistance, although mosquito infectivity studies need to be performed to confirm the effect of the drug in blocking transmission. There is evidence that malaria caused millions of deaths in Africa following the spread of chloroquine and sulphadoxine-pyrimethamine resistance from Asia (172, 230). Recently, it was demonstrated that artemisinin-resistant *P. falciparum* isolates from Cambodia could develop and produce sporozoites in both Asian and African vectors (231). My studies suggest that for both clinical efficacy and transmission blocking the Myanmar authorities should continue to use the standard three day AL regimen plus primaquine 0.25 mg/kg for the first line treatment of uncomplicated falciparum malaria. However, careful monitoring of drug efficacy and strengthening of surveillance activity throughout the country will be needed.

During the study period the treatment efficacy of AL and DP remained high. However, we need to prepare for the future if the efficacy of first line drugs drop below the threshold of 10% treatment failure (the threshold recommended by WHO) (60). Future research should be undertaken to investigate longer durations of ACT, triple combinations, and other newer combinations (for example Arterolane, a synthetic endoperoxide compound, plus piperaquine phosphate) in

the Greater Mekong Subregion. Treatment studies including uqPCR measurements up to day 42 after treatment and beyond would be useful to evaluate further the relationship between treated acute malaria infections and asymptomatic parasitaemia, and to determine whether post-treatment submicroscopic infection consists of asexual parasites, possibly in a dormant or 'sleeping' form, or gametocytes.

6.8 Conclusions

Surveying the temporal and spatial distribution of artemisinin resistance markers is crucial for rapid assessment of the artemisinin resistance situation in Southeast Asia. In this study, the phenotypic and genotypic data support the role of the F446I mutation in causing slow parasite clearance in northern and central Myanmar. Its phenotype appears to be 'intermediate', with a faster median clearance half-life than confirmed established mutations such as C580Y associated with artemisinin resistance in Cambodia and Thailand.

Submicroscopic parasitaemia is an important element in the transmission of malaria. Our uqPCR data suggests that a three day regimen of artemether-lumefantrine may be better than dihydroartemisinin-piperaquine in clearing these parasites, though a direct randomised comparison with longitudinal uqPCR measurements is needed to confirm this. However, neither drug regimen clears 100% of parasitaemias by day 21 after treatment. The day 3 blood smear positivity of 3 day AL was 23% and that of DP was 18%, and 35% of patients in my studies were infected with parasites bearing kelch13 propeller mutations. This suggests that in accordance with the recent WHO criteria for artemisinin resistance artemisinin resistance is established in Myanmar (60).

6.9 Summary of findings

The salient findings of chapter 6 are as follows:

- Overall, 35 % of all patients had kelch13 propeller mutations.
- The percentage of positive kelch13 mutations increased between 2013 to 2015 (25% to 68%).
- The prevalence of kelch13 propeller mutation-bearing parasites is increasing over time and their geographical range is extending from south-eastern to northern Myanmar.
- The median parasite clearance half-life of F446I was 4.7 hours. This supports an artemisinin resistance phenotype intermediate in nature when compared to the C580Y mutation.
- Patients treated with DP showed a higher rate of day 21 parasite positivity by uqPCR post treatment compared with those treated with AL. After adjusting for admission parasitaemia, the risk of positivity was nearly two fold higher in patients treated with DP compared to those treated with AL.
- High prevalence of kelch13 mutations was correlated with high day 3 parasite positivity on blood smear.
- Treatment efficacy of both DP and AL remains high in Myanmar. There were only two cases of treatment failures with *P. falciparum*, both in the AL trial.
- Single low dose primaquine (0.25 mg/kg) is safe and effective in clearing gametocytes in falciparum malaria and there were no complications in our studies.

7 Summary and discussion

The aims of the research reported in this thesis were to examine the spread in Myanmar of resistance of *Plasmodium falciparum* malaria to artemisinin derivatives through epidemiological, clinical, parasitological and pharmacological investigations, to determine geographical extent, prevalence and severity of artemisinin resistance in Myanmar and to find new treatment solutions for artemisinin resistance falciparum malaria.

7.1 Prevalence of kelch13 molecular markers for artemesinin resistance

In the molecular survey reported in Chapter 3 I found that 39% of the malaria parasite samples taken from cases across Myanmar and neigbouring border regions carried a kelch13 propeller mutation. In seven of ten administrative regions in Myanmar the overall kelch13 prevalence was more than 20%. Twenty six different non-synonymous kelch13 mutations were found, including nine mutations not described previously in Southeast Asia. Geospatial mapping showed that the overall prevalence of kelch13 mutations exceeded 10% in much of the east and north of the country. In Homalin, Sagaing region, 25 km from the India border, 47% of samples carried kelch13 propeller mutations. Much of lower Myanmar, and Chin and Rakhine states in the west, had a very low prevalence of kelch13 mutations. The raw data from my survey was shared at an early stage with the World Health Organization Global Malaria Program (WHO GMP), the Worldwide Antimalarial Resistance Network (WWARN) and the Myanmar National Malaria Control Program, so that policy makers could be informed of the latest information regarding the extent and nature of artemisinin resistance in the

region. Appropriate therapeutic regimens and malaria control measures should be tested urgently and implemented comprehensively if spread of artemisinin resistance to other regions is to be avoided. A vigorous international effort to contain this enormous threat is needed.

There has recently been considerable controversy about the nature of this threat, and in particular whether artemisinin resistant parasites are ‘jumping’ or ‘popping’ (232, 233). It has been convincingly shown that unlike the spread of other resistance determinants in *P. falciparum* and other pathogens, the kelch13 determinants of artemisinin resistance have arisen as multiple mutations on multiple genetic backgrounds (85, 119). The results of my survey confirm that multiple kelch13 mutations are present in Myanmar, many of which have been proven to be associated with clinical artemisinin resistance. At the same time there is also evidence of local clonal expansion and geographical of parasite strains with particular kelch13 mutations. Why the phenomenon of artemisinin resistance has only arisen in the last 10 years, and only in Southeast Asia, despite the use of artemisinins in the region for over 30 years, is still not understood. What is undeniable is that phenotypic artemisinin resistance has expanded inexorably from its initial epicentre in Western Cambodia in a geographically contiguous manner. So despite the unusual multiple mutation, multiple genetic background, molecular nature of kelch13 mediated artemisinin resistance, we should remain vigilant and continue to work to stop the ‘spread’ of artemisinin resistance to South Asia and Africa. Although ACTs have now been used extensively in Africa for nearly a decade, no convincing evidence of artemisinin resistance as a clinical and programmatic problem has arisen, and there is no population genetic evidence of selection of kelch13 mutants (85).

Other factors are clearly at play; these may include transmission intensity, population immunity, and currently unknown parasite factors.

7.2 Dihydroartemisinin-piperaquine and detailed characterization of artemisinin resistance phenotype

The clinical trial reported in Chapter 4 demonstrated that the median parasite clearance half-life was prolonged in northern and central Myanmar. In 21% of patients the parasite clearance half-life was more than 5 hours. Delayed parasite clearance was significantly associated with single point mutations in the propeller region of the Pf kelch13 gene. Mutation at amino acid position 446 (F446I) was found in 25% of all patients and was associated with a median clearance half-life of 4.7 hours compared with 2.7 hours for infections without kelch13 mutations. Overall, 18% of patients had persistent parasitaemia by blood smear at day 3. By highly sensitive qPCR methods 40% of patients still carried parasites 21 days after treatment with dihydroartemisinin-piperaquine. However, all patients followed remained parasite free for 42 days on blood smear and treatment efficacy as assessed by WHO criteria was 100%.

Although treatment efficacy was high with DP, nearly one-fifth of infections remained parasite positive on blood smear at day 3, and one-third of the patients had kelch13 mutations with a prolonged parasite clearance half-life, providing further evidence that artemisinin resistance is established in northern and central Myanmar. The dominant kelch13 mutations observed in Upper Myanmar, F446I, appears to be associated with an intermediate rate of parasite clearance compared to other common mutations described elsewhere in the Greater Mekong Subregion. The findings highlight the relevance of carefully measuring

parasite clearance rate when assessing whether artemisinin resistance is present in previously uncharacterised locations. Previous therapeutic efficacy studies only reported day three positivity rates, which have been shown to depend greatly on initial parasitaemia and to be a relative insensitive marker of the presence of artemisinin resistance (101, 180).

The high cure rate seen in the DP trial despite the presence of artemisinin resistance may lead to complacency about the threat of artemisinin resistance. The role of the artemisinin component of ACTs is to reduce rapidly the parasite biomass, reducing the number of parasites exposed to partner drug monotherapy. With artemisinin failing the partner drugs are vulnerable to the development of resistance. This problem has been dramatically demonstrated to be a real concern with the recent increase in failure rates of artesunate-mefloquine on the Thai-Myanmar border and the development of piperaquine resistance in Cambodia (97, 121). Although DP is currently efficacious in central and northern Myanmar, increased use of DP now that artemisinin resistance is established will expose piperaquine to the development of resistance. Currently DP is only used in these areas by the Myanmar army; ideally if wider deployment becomes necessary (if AL begins to fail) DP should be combined with a third drug – in other words a triple artemisinin combination therapy. Studies of triple ACTs are underway across the region.

7.33 day versus 5 day course of Artemether-lumefantrine

The clinical trial reported in Chapter 5 demonstrated that the percentages of parasites remaining at any of time points were not statistically significant difference between two treatment groups. The prevalence of kelch13 mutations in the enrolled cases was 47.4%, with the F446I mutation observed in 28% of all

those with mutations. After adjusting for admission parasitaemia, there was no significant difference in the reduction in parasite density at day 3 in patients with wild type compared with those with kelch13 propeller mutant parasites. The presence of kelch13 propeller mutations and the duration of AL had no impact on duration or AUC of gametocytaemia, almost certainly because the addition of low dose primaquine 0.25 mg/kg in both arms led to a shortened gametocyte carriage time. There was no significant difference between the two treatment regimens in terms of tolerability. Adequate clinical and parasitological responses on day 42 of 3 day and 5 day arm were 100 (95%CI, 94.9 to 100) and 97.1 (95%CI, 90.1 to 99.7) respectively. Although treatment efficacy of artemether-lumefantrine remains high, artemisinin resistance, as determined by study of molecular markers, has emerged and is established in south-eastern part of Myanmar.

The lack of difference in day 3 parasite positivity on blood smear supports the contention that this parameter is insensitive for the detection of artemisinin resistance. However, novel use of a highly sensitive qPCR to track the decline in parasitaemia demonstrated no association with kelch13 mutations, which was a slightly unexpected result. Possible explanations for this include the current high efficacy of lumefantrine in southeastern Kayin state, the intermediate parasite clearance phenotype of the kelch mutations detected, and possibly the level of population immunity in this endemic area. It is also possible that low residual parasite densities seen after blood smear negativity may represent a ‘dormant’ or ‘sleeping’ parasite population (221, 223). To assess fully the resistance phenotype of malaria in this area we would ideally have used initial parasite reduction ratios by taking frequent blood smears, but for logistical reasons this was not possible for this trial.

The standard three day AL treatment regimen was still efficacious, and though the 5 day treatment regimen was well tolerated it was not associated with any significant clinical or parasitological advantages, even in those infected with kelch13 mutation-carrying parasites. An extended ACT regimen is one possible clinical solution if failure rates with artemisinin resistant malaria are high, though further research would be needed to confirm this. However high ACT failure rates in areas of artemisinin resistance are usually due to the development of partner drug resistance, and in this scenario triple ACTs would theoretically be a better solution.

7.4 Spatial and temporal distribution of kelch13, day 3 blood smear positivity qPCR positivity and gametocytaemia across studies

In Chapter 6 data on molecular and parasitological features of the infections studied in the previous three chapters were combined and analysed. The resulting analysis demonstrated clearly that kelch13 mutation-carrying parasites are widely spread across Myanmar. One “confirmed” molecular marker of artemisinin resistance (C580Y) and nine “associated” markers (P441L, F446I, G449A, N458Y, P553L, R561H, P574L and A675V) were found throughout the country. The percentage of infections with kelch13 mutant parasites increased from 2013 to 2015 (25% to 68%). In Upper Myanmar, F446I was the most prevalent kelch13 mutation. Comparisons of the parasite clearance half-lives between infections with WT and F446I, WT and C580Y, and F446I and C580Y were all statistically significant. The median parasite clearance half-lives ranged from 2.6 hours in Thabeikkyin to 4.4 hours in Myitkyina.

Submicroscopic parasitaemia is an increasingly recognised factor in the transmission of malaria. Its application to the assessment of treatment though is new, and its role, if any, in understanding efficacy as yet poorly defined. On the basis of serial uqPCR measurements, it can be argued that from our study findings that a 3 day regimen of artemether-lumefantrine is better than dihydroartemesinin-piperaquine at reducing parasitaemia. This however was not a randomised comparison and the studies were carried out in different populations with potentially different levels of immunity. DP showed high probability of uqPCR positivity at day 21 post-treatment than AL. After adjusting for admission parasitaemia, the risk of positivity was nearly one and half fold in

patients treated with DP compared to those with AL (hazard ratio of 1.6, 95% CI 1.1 to 2.2). There was no association between the presence of kelch13 propeller mutations and residual parasite density and at day 21. The percent positivity of uqPCR after treatment for AL and DP were 15% and 40% respectively by uqPCR at day 21.

The implications of this are currently unclear, as it is unknown what the contribution is of these parasites to recrudescence, transmission, or longstanding sub-microscopic carriage. Further longitudinal studies will need to be done to determine the pathophysiological relevance of these parasites.

Day 3 positivity on blood smear of 3 days of AL was 23% compared to 18% with 3 days of DP, suggesting according to the currently imperfect WHO criteria that artemisinin resistance is established in northern, central and south-eastern Myanmar .

There were no failures in the DP trial and only two cases of treatment failure with *P. falciparum* in the AL trial. Single low dose primaquine is safe and appears effective in reducing gametocyte carriage. There were no complications in our two clinical trials in northern, central and south-eastern Myanmar. The person gametocytaemia week data supported the addition of single low dose primaquine to kill gametocytes in area of artemisinin resistance, although mosquito infection studies would need to be performed to confirm the impact on transmission.

7.5 Weaknesses and limitations of the studies

7.5.1 Molecular survey

There are a number of weaknesses and limitations in the molecular survey.

1. The geospatial mapping methodology is greatly relies on the accuracy of the map surface generated, which in turn is dependent on the data available together with underlying assumptions of the geospatial model employed.
2. There are major areas of uncertainty in the Kelch13 distribution map generated (as highlighted in the uncertainty maps), highlighting the paucity of information from some areas of the country. Further sampling would be needed for more accurate estimates of the spatial distribution of artemisinin resistance markers in these locations
3. The samples analysed were 'convenience' samples, obtained where there were studies being conducted or clinics linked to the investigators operating. In addition the DNA extraction and sequencing success rate with those samples consisting of used RDTs was low. I have no reason to believe that either of these factors introduced any major bias (except in producing the heterogeneity in data availability by region in 2. above.), but the possibility of bias remains.

7.5.2 Dihydroartemisinin-piperaquine and detailed characterization of artemisinin resistance phenotype

The main weakness of this study is its size. The sample size at the two sites was small, both less than the original recruitment target. This limits the accuracy of the point prevalence estimates for Kelch13 allele frequency and the parasite clearance half lives associated with each allele. It may also limit the power of comparative analyses, such as examining differences in parasite clearance half-life between the two sites.

7.5.3 3 day versus 5 day course of Artemether-lumefantrine

This clinical trial has a number of weaknesses and limitations. The primary end point was prospectively defined as detectable parasitaemia by ultra-sensitive PCR, but as this the first time this has been used as an outcome measure in a clinical trial the implications and interpretation of the results are limited by our necessarily restricted understanding of the biology of very low levels of parasite DNA. For example, it is unclear what proportion of the very low level 'parasitaemia' represents live and reproducing asexual parasites – rather than dead parasites or free parasite DNA, gametocytes, or parasites which have gone into cell cycle arrest (sleeping beauties)(21, 234). A further related weakness is that it was not possible to clarify the longitudinal changes in the uqPCR results with the conventional parasite clearance half life (by blood film microscopy), as logistical limitations prevented the taking of frequent blood films over the first three days post-treatment.

7.6 General conclusion

In conclusion, the aim of this thesis was successfully executed. The findings of this study have a number of important implications for treatment policy of uncomplicated falciparum malaria in Myanmar.

The information related to *Pf*kelch13 molecular markers in our studies suggested that artemisinin resistance is widespread throughout the country and the F446I mutation is prevalent across the country from south-eastern to northern Myanmar. The F446I mutation-carrying parasites showed delayed clearance half-lives, especially in central and northern Myanmar. Regional laboratories need to

be strengthened to perform real-time monitoring of kelch13, and data sharing between government, INGOs, research institutes and WHO is vital if artemisinin resistance is to be effectively countered.

Greater efforts are needed to assess regularly the treatment efficacy of the three first line antimalarial drugs (AL, DP and AS-MQ), and triple artemisinin combinations should be studied to ready them for early deployment to prevent the worse case scenario of widespread artemisinin and partner drug resistance – which has already come to pass in Cambodia.

Apart from drug treatment and vector control, targeted use of mass drug administration and the novel use of vaccines such as RTS,S should be studied to determine the best tools to use for malaria elimination. More studies using uqPCR are needed on submicroscopic parasitaemia, both in asymptomatic individuals and post-treatment, in order to assess its contribution to long term treatment efficacy and malaria transmission.

The Myanmar National Malaria Control Programme is currently in a ‘control’ phase, but the widespread emergence of artemisinin resistance in the country should encourage them to move rapidly to the next step - pre elimination then elimination. All tools currently available, along with those under development, will need to be deployed. Malaria control and elimination activities will need to focus on migrant workers, military population, and ethnic minorities, and on collaborative cross border malaria control, if the ultimate goal of malaria elimination in the region is to be achieved.

8 Related publications

A cross-sectional survey on molecular markers of artemisinin resistance have been published in Lancet Infectious Diseases journal. A clinical trial on dihydroartemisinin-piperaquine was submitted to Malaria journal on 14th October 2015.

Paper

Tun KM, Imwong M, Lwin KM, Win AA, Hlaing TM, Hlaing T, et al. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect Dis. 2015;15(4):415-21.

Tun KM, Jeeyapant A, Imwong M, Thein M, Aung SSM, Hlaing TM, et al. Parasite clearance rates in Upper Myanmar indicate a distinctive artemisinin resistance phenotype: a therapeutic efficacy study.(Submitted)

Grist E, Flegg J, Humphreys G, Mas I, Anderson J, Ashley E, **Tun KM** et al. Smart surveillance to charaterise optimally the geographical distribution of artemisinin resistant *Plasmodium falciparum*. (Submitted)

Poster

Tun KM, Hlaing TM, Thein M, Aung SSM, Imwong M, Smithuis F, et al., editors. Demonstrating artemisinin resistance falciparum malaria Kelch13 molecular marker. Myanmar Military Medical Conference; 2015; Yangon, Myanmar: Directorate of Medical Services; Feb, 2015.

Tun KM, Ashley E, Hlaing TM, Thein M, Aung SSM, Imwong M, et al., editors. Detection of artemisinin-resistant falciparum malaria by measuring parasite clearance half-life and determining kelch13 propeller mutations. Myanmar Military Medical Conference; 2016; Yangon, Myanmar: Directorate of Medical Services; Feb, 2016.

9 Acknowledgements

This thesis is supported by Li Ka Shing Oxford DPhil Fellowship programme which is managed through the Nuffield Department of Medicine, University of Oxford. Apart from main funding, Department of International Development (DFID) and Three Millennium Development Goal Fund (3MDGF) supported for the clinical trials. Wellcome Trust and Bill and Melinda Gates Foundation supported for the countrywide molecular epidemiological survey. Many people have guided and supported me for my long DPhil journey and I would like to thank them all for their support especially for:

Prof. Nicholas Day for welcoming me to be a part of Mahidol-Oxford Tropical Medicine Research Unit, providing mentorship to understand drug resistance malaria research, project management and valuable discussion. Prof. Nick Day has been central to all the work I have done and I am very grateful for all your constant encouragement throughout my study period.

Prof. Jeremy Farrar for letting me explores the world outside Myanmar and careful guidance for applying funding.

Prof. Nicholas White, heartfelt thank for invaluable idea concerning analysis and writing my thesis and papers. Attending your lectures and talks and being part of your team were invaluable lifetime experiences for me.

Dr. Frank Smithuis, for supervising and sharing your wealth of knowledge and experience in malaria and tropical medicine throughout my study period in Myanmar and indispensable input and guidance for my “transfer and confirmation exam”.

Prof. Peter Horby, for your guidance from my application to thesis writing. I am truly grateful for all your support.

I am particularly grateful for the assistance given by Prof. Tin Maung Hlaing, without his guidance and persistent help this thesis would not happen to be possible. Prof. Myo Myint Thein, former director of DMS, thank you for your kind permission to attend University of Oxford. Prof. Kyaw Soe, for providing valuable assistance during my data collection period in Myanmar. Prof. Soe Win, director of DMS, for permission to extend my study period and encouragement.

Dr. Charles Woodrow and Dr. Mallika Imwong, thank you for introducing me into molecular and genetics malariology. Your discussions were a source of inspiration for writing up my thesis chapters.

My intellectual debt is to Prof. Arjen Dondorp who gave insightful comments and suggestions from protocols development to final thesis writing.

Dr. Elizabeth Ashley: for your moral support in my first year, excellence advices and allowing me to work as a part of TRAC project in Myanmar.

Dr. Phaikyeong Cheah and Prayoon, thank you for planning and managing all studies through out my study period.

Dr. Bob Taylor, for answering my questions and curiosity during writing up thesis.

Dr. Win Htay Aung and Dr. Aung Ban from Myitkyina and Thabekkyin hospitals for you collaboration and coordination during my clinical trials at your hospitals.

Drs. Min Thein, Sai Soe Moe Aung, Aung Hpone Myint and Zwe Thiha Kyaw and all staff involved at Mahidol-Oxford Tropical Medicine Research Unit, Worldwide Antimalarial Resistance Network, Defence Services Medical Research Centre, Myanmar Oxford Clinical Research Unit and Medical Action Myanmar (Myanmar

and Thailand) thank you for your contribution and hard work performing in our countrywide survey and two clinical trials.

All patients who participated in kelch13 epidemiological survey and two clinical trials.

I am indebted to my many colleagues who supported me especially from malaria laboratory, molecular laboratory, pharmacology laboratory and clinical trial support group.

Atthanee Jeeyapant and Dr. Mavuto Mukaka for statistical help and teaching. Dr. Mehul Dhorda and Chompoo for your support to sample management. Boy, thank you for your help to keep my computer hardware and software running. Prapass Wannapinij (Aon) thanks for your kind contribution to make GIS maps of my study sites. Tic, Ant, Ko Kyaw Soe, Ko Soe Tha, Br Kaung Myat Moe, Br Yin Aung San, Be Be and Naw Naw: for you incredible hard work in the lab at very remote areas in Myanmar. Padd and Bee for your assistance in data management. Drs. Khin Maung Lwin and Aung Pyae Phyo for constant supply of Myanmar food in Bangkok.

Dr. James Watson, Dr. Xin Hui Chan and Dr. Aung Pyae Phyo for your kind contribution of proof reading of my chapters.

In addition, I would like to thank all people I have met in Bangkok unit and Green Templeton College for their warm welcome.

To my parents, thank you for the constant encouragement throughout my life and education. To my younger brother, Thiha, thank you for your encouragement to apply DPhil programme in University of Oxford.

Last but not the least, I cannot find words to express my gratitude to my wife Htet Htet Aung for your dedicated endurance and love. My kids, Htet Alinkar Kyaw

and Hpone Myat Maung for your understanding and love throughout the long DPhil journey.

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Appendix

11 Appendix A

11.1 Geospatial mapping

To estimate the geographical distribution of drug resistance, the molecular marker data were analysed using geospatial modelling techniques (85). These models can be used to identify geographical areas where data are currently insufficient for policy makers to determine whether drug resistance is present or not. In this study, for the purposes of predictive geospatial mapping, the total proportion of samples in each location with a non-synonymous mutation after amino acid position 440 of the K13 gene was calculated. These data along with the total number of samples and geographical information system (GIS) co-ordinates for each sampling site were entered as input into a geostatistical model, yielding a predictive map of estimated mutation prevalence on a 5x5 km grid in Myanmar. To reduce model uncertainty and achieve a more robust estimate of the K13 mutation prevalence two alternative geostatistical modelling approaches were utilized. The first approach involved the use of a regression model implemented within a Bayesian framework, producing a posterior distribution of prevalence summarized by the median to create a single continuous surface as described previously (235) (see also Supplementary Information). Secondly, the well established spatial statistical interpolation methodology of 'ordinary kriging' was applied (236). With both models a corresponding ‘uncertainty map’ was generated to reflect the confidence associated with the predictions across the map domain. In the Bayesian model this was represented by a corresponding standard deviation surface whereas in the kriging model this was shown by the kriging variance. With the kriging

approach, a variogram was also employed to describe the strength of spatial dependence observed in the data (Figure 11.1).

11.1.1 Model-based Method

A Bayesian geostatistical model of the proportion of individuals with any marker (>440 amino acids into the protein) was developed. The number of individuals in each study with a resistance mutation (N_i^+) was assumed to be binomially distributed, given the number of individuals in the study (N_i) and the probability $P(\underline{x}_i)$:

$$N_i^+ \left| N_i, P(\underline{x}_i) \sim Binomial(N_i, P(\underline{x}_i)) \right. .$$

The probability, $P(\underline{x})$, at an arbitrary location $\underline{x}$, was modelled as the inverse logit transformation of the sum of a random field, $f(\underline{x})$, and an unstructured random component, $\varepsilon(\underline{x})$:

$$P(\underline{x}) = \text{logit}^{-1}(f(\underline{x}) + \varepsilon(\underline{x})) .$$

The unstructured components, $\varepsilon(\underline{x})$, were assumed to be independent and identically distributed with zero mean and variance V while the random field, $f(\underline{x})$, was modelled as a stationary Gaussian process, with constant mean function $\mu(\underline{x}) = \beta_0$ and Matern covariance function, $C(\underline{x})$

$$f(\underline{x}) \left| \beta_0, \sigma, \varphi_x, \gamma \sim GP(\mu(\underline{x}), C(\underline{x})) \right.$$

where β_0 is the mean parameter, φ_x is the spatial scale parameter, σ is the partial sill and γ is the degree of differentiability parameter. The covariance between a study conducted at location $\underline{x}_i$ and a study performed at $\underline{x}_j$ was given by

$$C(\underline{x}_i, \underline{x}_j) = \sigma^2 \frac{\Delta x^\gamma \kappa_\gamma(\Delta x)}{2^{\gamma-1}\Gamma(\gamma)},$$
$$\Delta x = \frac{2\sqrt{\gamma} D_{GC}(\underline{x}_i, \underline{x}_j)}{\varphi_x}$$

where $D_{GC}(\underline{x}_i, \underline{x}_j)$ is the great circle distance, Γ is the gamma function and κ_γ is the modified Bessel function of the second kind of order γ .The following priors were imposed:

$$p(\beta_0) \propto 1$$
$$\phi_x \sim \text{Exponential}(1)$$
$$V \sim \text{Exponential}(0.1)$$
$$\sigma \sim \text{Exponential}(0.1)$$
$$\gamma \sim \text{Uniform}(0, 3)$$

Using the Python module PyMC, the model was fitted with Markov chain Monte Carlo (MCMC) (237, 238). Predictive maps were generated on a 5 x 5 km grid from the MCMC samples. For each prediction location, prevalences were drawn and the distribution summarized with the median and standard deviation of this set.

11.1.2 Kriging Method

Let $z(x_k)$, k =1, 2 … n be the observed values of the variable z at locations x_1, x_2,… x_n. We require to find coefficients λ_k for k = 1,2, … n, such that the estimate $\hat{z}_0$ of z_0 (=$z(x_0)$) at any point x_0

$$\hat{z}_0 = \sum_{k=1}^{n} \lambda_k z(x_k) \qquad (1)$$

minimize the mean squared prediction error (referred to as ‘the kriging variance’)

$$\sigma_e{}^2 = E[(\, z_0 - \hat{z}_0)^2] \tag{2}$$

subject to the constraint (to ensure unbiased)

$$1 = \sum_{k=1}^{n} \lambda_k \tag{3}$$

Kriging coefficients wk.

Denoting z_0 as a random variable we first note that

$$\sigma_e{}^2 = E[(\, z_0 - \hat{z}_0)^2] = Var\,(z_0 - \hat{z}_0) + E[(z_0 - \hat{z}_0)]^2$$

and because by unbiasedness $E[(z_0 - \hat{z}_0)]^2 = 0$,

$$= Var(z_0) + Var\,(\hat{z}_0) - 2Cov(\hat{z}_0, z_0) \tag{4}$$

Using the method of Lagrange multipliers we therefore require to minimize the Lagrangian

$$L = Var\,(z_0 - \hat{z}_0) + 2\beta(\textstyle\sum_{k=1}^{n} \lambda_k - 1)$$

$$= \sigma^2 + \textstyle\sum_{k=1}^{n} \sum_{j=1}^{n} \lambda_k\, \lambda_j Cov\left(z(x_k), z\left(x_j\right)\right) - 2(\sum_{k=1}^{n} \lambda_k Cov(z(x_k)\,, z_0)) + 2\beta(\sum_{k=1}^{n} \lambda_k - 1) \tag{5}$$

where σ^2 is the variance of z_0 and *Cov(x,y)* is the covariance of *x* and *y*. This is achieved by taking partial derivatives of *L* with respect to λ_k for k= 1, 2, … *n* and the β and then setting each to zero, which yields a homogeneous system of *n* simultaneous equations which can be written in matrix form as

$$\begin{bmatrix} Cov\big(z(x_1), z(x_1)\big) & \dots & Cov\big(z(x_1), z(x_n)\big) & 1 \\ \vdots & \ddots & \vdots & \vdots \\ Cov\big(z(x_n), z(x_1)\big) & \dots & Cov\big(z(x_n), z(x_n)\big) & 1 \\ 1 & \dots & 1 & 0 \end{bmatrix} \begin{bmatrix} \lambda_1 \\ \vdots \\ \lambda_n \\ \beta \end{bmatrix} = \begin{bmatrix} Cov(z(x_1), z_0) \\ \vdots \\ Cov(z(x_n), z_0) \\ 1 \end{bmatrix} \quad (5)$$

or more compactly as

$$\boldsymbol{C\lambda = D} \quad (6)$$

The kriging coefficients λ_k and the Lagrange multiplier β are then found by multiplying the right hand side vector in equation (8) by the inverse matrix $\boldsymbol{C^{-1}}$

$$\boldsymbol{\lambda = C^{-1}D}$$

11.1.3 Kriging coefficients λ_k in terms of the variogram

The variogram can be written in terms of the covariance function as

$$\gamma_{ij} = \frac{1}{2} Var\left(z(x_i) - z\big(x_j\big)\right) = 1/2(\,\sigma^2 + \sigma^2 - 2Cov\big(z(x_i) - z\big(x_j\big)\,\big)$$

$$= \sigma^2 Cov\big(z(x_i) - z\big(x_j\big)\,\big)$$

So we have that

$$Cov\big(z(x_i) - z\big(x_j\big)\,\big) = \sigma^2 - \gamma_{ij}$$

and the kriging equations (5) can be rewritten equivalently in terms of the variogram as

$$\begin{bmatrix} -\gamma_{11} & \dots & -\gamma_{1n} & 1 \\ \vdots & \ddots & \vdots & \vdots \\ -\gamma_{n1} & \dots & -\gamma_{nn} & 1 \\ 1 & \dots & 1 & 0 \end{bmatrix} \begin{bmatrix} \lambda_1 \\ \vdots \\ \lambda_n \\ \beta \end{bmatrix} = \begin{bmatrix} -\gamma_{10} \\ \vdots \\ -\gamma_{n0} \\ 1 \end{bmatrix} \quad (7)$$

or more compactly

$$\boldsymbol{\Gamma\lambda} = \boldsymbol{\Gamma}_0 \qquad (8)$$

Kriging coefficients λ_k and the Lagrange multiplier β are found multiplying the right hand side by the inverse matrix $\boldsymbol{\Gamma^{-1}}$

$$\boldsymbol{\lambda} = \boldsymbol{\Gamma^{-1}\Gamma_0}$$

11.1.3.1 ***Kriging variance*** $\boldsymbol{\sigma_e{}^2}$

Multiplying *i*th row of matrix **C** and **D** in equation (5) by λ_i for *i* =1 …*n* and forming their sum gives

$$\sum_{i=1}^{n} \lambda_i \sum_{j=1}^{n} \lambda_j Cov\left(z(x_i), z(x_j)\right) + \sum_{i=1}^{n} \lambda_i \beta = \sum_{i=1}^{n} \lambda_i Cov(z(x_i), z_0)$$

From equation (4) the kriging variance is then given by

$$\sigma_e{}^2 = \sigma^2 + \sum_{k=1}^{n}\sum_{j=1}^{n} \lambda_k \lambda_j Cov\left(z(x_k), z(x_j)\right) - 2\left(\sum_{k=1}^{n} \lambda_k Cov(z(x_k), z_0)\right)$$

$$= \sigma^2 + \left(\sum_{i=1}^{n} \lambda_i Cov(z(x_i), z_0) - \beta\right) - 2\left(\sum_{k=1}^{n} \lambda_k Cov(z(x_k), z_0)\right)$$

$$= \sigma^2 - \boldsymbol{\lambda^T}\mathrm{D} \qquad (9)$$

where $\boldsymbol{\lambda^T}$ denotes the transpose of vector **λ** from equation (6)**.**

11.1.4 Kriging variance $\boldsymbol{\sigma_e{}^2}$ in terms of the variogram

The kriging variance $\sigma_e{}^2$ can similarly be expressed in terms of the variogram by replacing covariance terms in vector **D** of equation (8)

$$\boldsymbol{D} = \begin{bmatrix} Cov(z(x_1), z_0) \\ \vdots \\ Cov(z(x_n), z_0) \\ 1 \end{bmatrix} = \begin{bmatrix} \sigma^2 - \gamma_{10} \\ \vdots \\ \sigma^2 - \gamma_{n0} \\ 1 \end{bmatrix}$$

so that from equation (9)

$$\sigma_e{}^2 = \sigma^2 - \boldsymbol{\lambda^T D}$$

$$= \sigma^2 - [\lambda_1 \quad \dots \quad \lambda_n \quad \beta\,] \begin{bmatrix} \sigma^2 - \gamma_{10} \\ \vdots \\ \sigma^2 - \gamma_{n0} \\ 1 \end{bmatrix}$$

$$= \sigma^2 - \sigma^2 \sum_{i=1}^{n} \lambda_i - \sum_{i=1}^{n} \lambda_i \gamma_{i0} + \beta$$

$$= \sum_{i=1}^{n} \lambda_i \gamma_{i0} + \beta$$

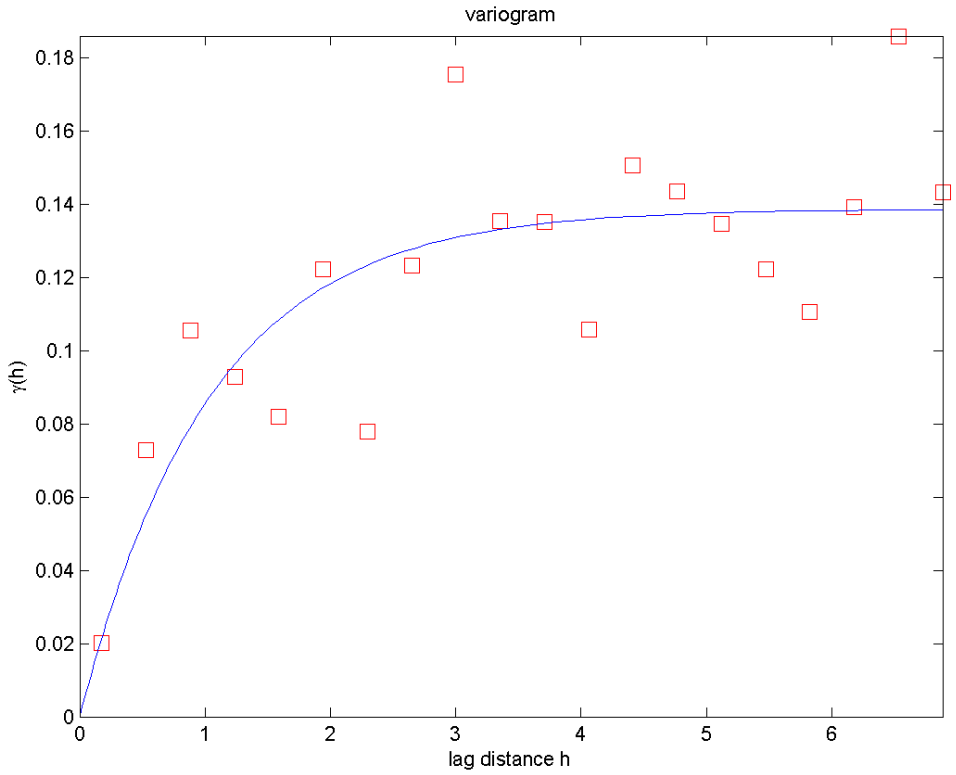


Figure 11.1 Variogram produced by the kriging approach.

An empirical variogram was constructed as a scatter plot (squares) of semivariance $\gamma(h)$ versus lag distance h to describe spatial dependence in the data collected. The plot describes how variation in estimated K13 mutation prevalence observed between different sample sites is dependent on (Euclidean) separation distance between those sites. The plot was constructed by binning all pairwise site distances into a default 20 bins, each at a fixed increment apart. Spatial dependence can be seen to decrease as the distance between the sample data sites is increased. Such a plot will typically achieve a plateau (referred to as a 'sill') after a certain separation distance (referred to as the 'range') exhibited by a ‘flattening of the curve’ as is illustrated in the plot after a lag distance of approximately 4 separation units (20 km).

12Appendix B

12.1 Ethical Approval (OXTREC)

Oxford Tropical Research Ethics Committee

University of Oxford
Joint Research Office
Churchill Hospital, Oxford OX3 7LE
Tel. +44 (0) 1865 (5)72348 fax +44 (0) 1865 (5)72228
E-mail: jacqueline.gerencser@admin.ox.ac.uk

Professor Nick White
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine
3rd Floor, 60th Anniversary Chalermprakiat Building.
420/6 Ratchawithi Road, Ratchathewi District, Bangkok
10400, Thailand

17 November 2014

Dear Professor White

Full Title of Study: Mapping markers of antimalarial drug resistance.

OxTREC Reference: 553-14

Other documents reviewed were:

Documentation	Version	Date
Minimal Risk Application Form		
Protocol	V2.0	10/11/14
Scientific Peer Review Form by Dr Phaik Yeong Cheah		22/09/14
PI Signatory Page		10/10/14

The OxTREC executive team reviewed the above application on the 13 November 2014 and gave approval for this study.

Approval is given for the first five years and is subject to receiving the local ethical approval.

Any subsequent changes to the application must be submitted to the Committee as an Amendment. This should include a letter to give the reasons for the proposed modifications and all modified documents with changes tracked.

Please submit a completed Annual Report form on every anniversary of this approval and a final End of Study Report.

Yours sincerely

Dr Mary Warrell
OxTREC Chairman

Direct Line Tel: +44 (0)1865 (5)723436
Fax: +44 (0)1865 (5)72228 Email: jacqueline.gerencser@admin.ox.ac.uk Web: www.admin.ox.ac.uk/tso/

12.2 Ethical Approval (Myanmar)

Office of the Commander in Chief (Army)

Directorate of Medical Services

Defence Services Medical Research Centre

DSMRC,TatKon Township

Nay Pyi Taw

Telephone: +95-9402700750

Document No: 1/2/IRB-7

Dated:7 .1.2014

The Institutional Review Board on biomedical research involving human subjects, Defence Services Medical Research Centre, approves to conduct the following proposed research project.

Epidemiology of Malaria in Low Transmission Season, Myanmar

Principal Investigator: Dr. Kyaw Myo Tun

Colonel Tin Maung Hlaing

(Chair)

Institutional Review Board

Defence Services Medical Research Centre

ay Pyi Taw

epublic of the Union of Myanmar

13Appendix C

13.1 Participant Information Sheet (English version)

Note: If you are a parent or guardian of a child below 18 years old, please read "you" as "your child".

You have been diagnosed with falciparum malaria. Malaria is caused by parasites which infect the blood and is transmitted by mosquitoes. We are conducting a study to determine how well an antimalarial drug called DHA-PIP is working in treating malaria. We would like to ask you to participate in this study.
In this leaflet, we will give you information about the study to help you decide whether or not you want to take part. If you have any questions or concerns, you will have a chance to discuss them with the study doctor.

13.1.1 What is the study about?

• We are studying a drug called DHA-PIP in about 160 patients who have been diagnosed with falciparum malaria in Myanmar.
• DHA-PIP is an artemisinin type drug. These drugs are recommended and used all around the world to treat people with malaria. DHA-PIP usually works very quickly and is very safe and effective at curing the disease.
• In 2009, a study found that in some parts of Cambodia artemisinin derivative was not working as quickly as before - it was taking a longer time for malaria parasites to disappear from the blood after treatment. We call this problem artemisinin “resistance”.

• We are now conducting a similar study here and several other countries in Asia, as well as 2 countries in Africa. We want to measure how quickly the parasites disappear from your blood after you have been treated with DHA-PIP.

• This is important to know, because new treatments might be necessary and we need to know how far this problem of artemisinin resistance has spread.

13.1.2 Do I have to take part?

• No. You should only take part if you decide you want to. We want to give you enough information to help you make an informed decision. Once you have this information, it will be your decision to take part or not.

We will assess you to see if you can take part in this study. You will be asked a number of questions about your health, both now and in the past, been examined and have blood taken. If you are female, please tell us if you are pregnant or think you may be pregnant.

• If you do not agree to take part in the study or we find you are unsuitable, you will be given the standard treatment for your malaria. If you agree to the study but later change your mind, you can withdraw without giving any reason, without affecting your treatment.

• You may ask questions at any time. You may also want to talk to your family and friends about your decision.

13.1.3 What will happen if I take part in the study?

• You will need to stay in hospital for at least 3 nights while you are treated and observed.

• It is important for you stay in hospital to be sure the malaria had gone from your body. For some patients, this may mean staying in hospital for more than 3 nights.

• A doctor will examine you on the day you are admitted and regularly during your stay in hospital.

• We will need to take a sample of your blood when you arrive, and also a number of times during your stay.

• You will then be given DHA-PIP once daily for 3 days. Primaquine will be given on Day 0 after DHA-PIP first dose.

13.1.4 What does giving blood involve?

• When you first arrive, the extra blood needed for the study will be up to 5 mL (up to 0.5-1 mL for children). One teaspoon is 5 mL of blood.

• After admission sample, we will collect small drops of blood to look for malaria in your blood and measure your red blood count at 6 hourly until you are discharged from the hospital. We will also take blood for special tests on the malaria itself and measure the DHA-PIP in your blood.

13.1.5 What will the blood sample be used for?

• The blood sample will be used to assess how quickly the malaria is cleared from your blood. In addition to this, the blood sample will be used to see if your blood test if you have any types of blood that resistance of the parasite to antimalarial drugs, to measure drug concentrations, and to do genetic studies on the parasites to try and explain why they can resist the drug. For this reason, the

samples will be transferred to the laboratories of Mahidol-Oxford Tropical Medicine Research Unit in Thailand and sent on to other specialist laboratories in other parts of the world e.g. UK, USA.

If you consent some of your leftover blood samples will be stored and may be used for further studies. Any new tests not covered in the present protocol will not be carried out unless a separate approval is obtained from the relevant ethics committees.

13.1.6 How much blood will be given and is it harmful to give this amount of blood?

For adults, the maximum blood volume will be 18 mL (42 days of follow up, less than 4 teaspoon). For children, the corresponding volumes are 4 mL (1 teaspoon). Allowing for the possibility that we may need to repeat blood tests, we will add 1 mL to these maximum blood volumes.

• It is not harmful to your body as your body can easily cope with giving this much blood. It will not affect your health, and most people do not feel any different after giving this amount of blood.

• To keep the risk of this as small as possible, well-trained and experienced doctors and nurses will collect the blood.

13.1.7 What will happen after I leave hospital?

• After you return home, you will need to visit the hospital clinic for your follow up treatment and assessment on Days 5, 7, 9 and weekly until for up to Day 42. At

each visit, we will check that you are still in good health, and take a small amount of blood to make sure that the malaria parasites have not come back.

• We will pay for a reasonable amount of travel costs, and for your lost time in making these visits.

• These visits are important for the study, as they allow us to make sure the disease is really cured. They will also give us a chance to make sure you are still feeling well.

• If you agree, we might also ask to visit you at home so that we can observe the environment and understand the risks for malaria.

13.1.8 What will happen to the information you collect?

• The information we collect from speaking to you and from analyzing your blood samples will be kept confidential by the study team. We will not share your personal information with anyone outside the study. No one other than the study team and authorised personnel from the study sponsor and regulatory authorities are allowed direct access to your personally identified medical records.

• When the study is completed, we will combine your test results with those of the other participants, and the overall results will be analyzed.

• We would like to share these overall results, including with a group called the World Wide Anti Malarial Resistance Network (WWARN) and the World Health Organisation (WHO) who are monitoring how well antimalarial drugs are working worldwide. The National Malaria Control Programme in your country will be responsible for how these data are used. We will also publish the results in the medical literature. In both these cases we will make sure that you cannot be personally identified.

13.1.9 What are the advantages of taking part?

• If you decide to take part, we will treat your disease, but we will be happy to do this even if you decide not to take part. In either case, the drugs we use to treat your malaria (and any other drugs you need) will be given to you for free.

We will monitor you closely to make sure you have cleared the malaria from your blood.

• Although you may not get a direct benefit from taking part, the results of the study will improve our understanding of malaria in your area, and whether DHA-PIP is still working well or not.

13.1.10 What are the disadvantages of taking part?

• You will need to stay in the clinic for at least 3 nights and then come back once daily for 3 days to receive the rest of the treatment. You will also need to come to the hospital once a week for at least 2 weeks after you return home.

• You will need to give blood more frequently than would usually be required to treat your disease. Although this may be uncomfortable, we do not expect it to be harmful to your health. During the first 3 days when frequent blood taking will be required, you will not be able to leave the hospital compound. Therefore, you may need to make arrangements with your relatives/friends to help you.

DHA-PIP is very well tolerated, side effect that are common are mild diarrhoea and abdominal pain. Serious hypersensitivity reaction can occur rarely. Otherwise, at the doses used for the treatment of acute malaria, there are no known toxicities. The other malaria medicines used in this study have been

studied thoroughly and used routinely to treat malarial and their toxicities are well described. In general, they are all well tolerated.

• If you do have any side effects or any unexpected problems during your stay with us, we will treat these problems fully and with no charge to you or your family. In the unlikely event that you are harmed as a direct result of participation in the study, compensation will be available from the University of Oxford's insurance scheme.

• We appreciate that taking part in this study may be inconvenient for you and your relatives. We will also give you a small amount of money to compensate you for your lost time and to cover the cost of food.

13.1.11 What if I decide not to take part?

• You do not have to take part in this study. You can also stop at any time after the study has started.

• If you decide not to take part, or if you withdraw after the study begins, we will still treat your illness for free. You can withdraw from the study for any reason, and this will not affect your further treatment.

13.1.12 Who is conducting this study and who has approved it?

The study is conducted by a group of doctors and scientists in the Tracking Resistance to Artemisinin Collaboration (TRAC). It is sponsored by and insured via the University of Oxford and funded by the UK Department for International Development. The study has been approved by the Oxford Tropical Research Ethics Committee and the ethics committees in your country.

13.1.13 What if I have questions?

• If you have any questions or concerns after reading this leaflet, you will have a chance to discuss them fully with a member of our team before you decide whether or not to take part. We will also be available throughout the study to answer any questions or address any concerns that you may have later on.

• While you are in hospital, you can contact your attending doctors and nurses 24 hours a day with any questions or concerns.

• If you have any questions while you are at home, you can contact Dr. Kyaw Myo Tun by telephone at 095025584.

13.2 Informed consent form (Myanmar translation)

သဘောတူညီချက်ပုံစံ

အဓိကသုတေသီ - ဒေါက်တာကျော်မျိုးထွန်း

အဖွဲ့အစည်းအမည် - တပ်မတော်ဆေးတက္ကသိုလ် နှင့်အောက်စဖို့တက္ကသိုလ်

ငွေကြေးထောက်ပံ့သည့် အဖွဲ့အစည်း - University of Oxford

သုတေသနအဆိုပြုလွှာအမည် - မြန်မာနိုင်ငံရှိ မပြင်းထန်သောဌက်ဖျားလူနာများတွင် အာတီဆူနိတ် ဌက်ဖျားဆေးယဉ်ပါးမှု ကိုလေ့လာခြင်း။

အပိုင်း(က) သုတေသနနှင့်ပါတ်သက်သည့်အကြောင်းအရာ

၁။ မိတ်ဆက်ခြင်း

ကျွန်တော်ကတော့ တပ်မတော်ဆေးတက္ကသိုလ် မှာ တာဝန်ထမ်းဆောင်နေတဲ့ဒေါက်တာ ကျော်မျိုးထွန်း ဖြစ်ပါသည်။ ကျွန်တော်တို့အနေနဲ့ မြန်မာနိုင်ငံမှာ အဖြစ်များတဲ့ဌက်ဖျားရောဂါနဲ့ ပါတ်သက်ပြီး သုတေသန လုပ်ငန်းတစ်ခု ကိုဆောင်ရွက်လိုပါတယ်။ ကျွန်တော်အနေနဲ့ သုတေသနနှင့်ပါတ်သက်သည့် အချက်အလက်များကို ပြည့်စုံစွာ ပြောပြပြီး သင့်အား သုတေသနမှာ ပါဝင်ရန် ဖိတ်ခေါ်လိုပါသည်။သင့်အနေနှင့် ယခုချက်ချင်းဆုံးဖြတ်ရန် မလိုပါ။ ဒီသုတေသန အကြောင်း စဉ်းစား၊ ဆွေးနွေးတိုင်ပင်ပြီးမှ ဆုံးဖြတ်နိုင်ပါတယ်။ သင့်အနေနဲ့ နားမလည်တဲ့အချက်များရှိရင် ကျွန်တော့်ကိုသော်လည်းကောင်း၊ သုတေသနအဖွဲ့မှ အခြားသူများကိုသော် လည်းကောင်း မေးမြန်းနိုင်ပါတယ်။

၂။ သုတေသန၏ရည်ရွယ်ချက်

ဌက်ဖျားရောဂါဟာဒီဒေသမှာ အဖြစ်အများဆုံးနဲ့ သေဆုံးမှုအန္တရယ်အရှိဆုံး ရောဂါတစ်မျိုး ဖြစ်ပါသည်။ ဌက်ဖျားရောဂါ ကုသဆေးများအနက် အာတီဆူနိတ် ဆေးဟာ ဌက်ဖျားရောဂါလူနာများကို 80 မှ 90 ရာခိုင်နှုန်း အထိလုံးဝ ပျောက်ကင်းအောင် ကုသနိုင်ပါသည်။ ဒါပေမယ့် ဒီဒေသမှာ အာတီဆူနိတ်ဆေးဟာ ဆေးမတိုးတဲ့ ပြဿနာရှိနေပြီ ဖြစ်ပါသည်။ ဒါကြောင့် ကျွန်တော်တို့နဲ့ သုတေသန လုပ်ခြင်းဖြင့် ဌက်ဖျားရောဂါ တိုက်ဖျက်ရေးမှာ နည်းလမ်းကောင်းတွေကို ရှာဖွေနိုင်ပြီး၊ အာတီဆူနိတ် ဆေးမတိုးသည့် ဌက်ဖျားရောဂါကိုပါ တိုက်ဖျက်နိုင်ရန် ရည်ရွယ်ပါသည်။

၃။ သုတေသန ဆောင်ရွက်ပုံ အမျိုးအစား

ဤသုတေသနသည်လေးဆယ့်နှစ်ရက်အတွင်း သင်၏လက်မောင်းမှ ငါးကြိမ်ခန့် သွေးနမူနာ ရယူခြင်း၊ လက်ဖျားထိပ်မှ သွေးဖောက်စစ်ဆေးခြင်း၊ တစ်ဦးချင်း (၅)မိနစ်ခန့်မေးမြန်းခြင်း တို့ပါဝင်ပါမည်။

၄။ သုတေသနတွင် ပါဝင်မည့် သူရွေးချယ်ခြင်း

ဤသုတေသနတွင် အသက်၆လအထက်မှ စ၍ ပါဝင်နိုင်ပါတယ်။ ကိုယ်ဝန်ဆောင် မိခင်လောင်းများကို တော့ပါဝင်ခွင့်မရှိပါဘူး။ ဒါကြောင့် သင့်အား ဤသုတေသနတွင် ပါဝင်ရန် ဖိတ်ခေါ်ခြင်းဖြစ်ပါတယ်။

၅။ မိမိဆန္ဒအလျောက်ပါဝင်ခြင်း

ဤသုတေသနတွင် သင်ကူညီပါဝင်ခြင်းသည် သင်၏ သဘောဆန္ဒအလျောက်သာဖြစ်ရပါမည်။ ပါဝင်ခြင်း/ မပါဝင်ခြင်းမှာ သင်၏ ဆန္ဒအတိုင်းရွေးချယ်မှုသာ ဖြစ်ပါသည်။ အကယ်၍ဤသုတေသနတွင် သင်မပါဝင်ပါက ကျန်းမာရေးဌာနတွင် သင်ရရှိခံစားနေသော ဆေးကုသမှုများ ပြောင်းလဲမှုမရှိဘဲ ဆက်လက်ခံစားရရှိမည် ဖြစ်ပါသည်။

၆။ အသုံးပြုမည့်ဆေးဝါးအကြောင်း

ဤသုတေသနတွင် Dihydroartemisinin+Piperaquine (DHA+PPQ) ဆေးနှင့် Primaquine (PQ) ဆေးဝါးများကို အသုံးပြုပါမည်။ ၎င်းဆေးဝါးများသည် ၎က်ဖျားရောဂါ ကုသဆေးအဖြစ် လက်ရှိသုံးစွဲနေသောဆေးဖြစ်ပြီး ကမ္ဘာ့ကျန်းမာရေး အဖွဲ့ကြီးမှ အတည်ပြုထားသော ဆေးဝါးများ ဖြစ်ပါသည်။ DHA+PPQ ဆေးသည် ဘေးထွက်ဆိုးကျိုးအဖြစ် အခန်မ့သင့်ပါက အနည်းငယ်မူးဝေခြင်း၊ ဝမ်းဗိုက်အောင့်ခြင်း တို့ဖြစ်နိုင်ပါသည်။ PQ ဆေးသည်ဘေးထွက်ဆိုးကျိုးအနေဖြင့် အခန်မ့သင့်ပါက မအီမသာခံစားရခြင်း၊ ဝမ်းဗိုက်အောင့်ခြင်းနှင့် G6PD လူနာများတွင် သွေးအားနည်းရောဂါများ ဖြစ်ပွားနိုင်ပါသည်။

၇။ လုပ်ဆောင်ပုံ

ကျွန်တော်တို့အနေနဲ့ မပြင်းထန်သောငှက်ဖျားလူနာများတွင် DHA+PPQ နှင့် PQ ဆေးဝါးများ ပေးပြီး အာတီဆူနိတ် ၎က်ဖျားဆေးယဉ်ပါးမှု ကိုလေ့လာသုတေသန ပြုသွားပါမည်။၎က်ဖျားလူနာ တစ်ဦလျှင် (၄၂) ရက်ခန့် စောင့်ကြည့် သွားမည် ဖြစ်ပါသည်။

သုတေသနကာလအတွင်း ကျန်းမာရေးဝန်ထမ်း များမှ သင်နှင့်အခြားသော ဝါဝင်သူများအား ဂရုတစိုက် စောင့်ကြည့် သွားမည်ဖြစ်ပါသည်။ အကယ်၍ သင့်အနေဖြင့် သုတေသနနှင့်ပါတ်သက်၍ တစ်စုံတစ်ရာ မေးမြန်းရန်ရှိပါက သုတေသနအဖွဲ့ဝင်များထံသို့ ဆက်သွယ် မေးမြန်း/ပြောကြားနိုင်ပါသည်။

ကျွန်တော်တို့အနေဖြင့် သင်၏လက်မောင်းမှ သွေးနမူနာရယူရာတွင် ပိုးသန့်စင်ထားသော တစ်ခါသုံးအပ်များ/ဆေးထိုးပြွန်များ အသုံးပြုပါမည်။ တစ်ကြိမ်လျှင်သွေးပမာဏ အနည်းငယ် လက်ဖျားထိပ်မှ ရယူမည်ဖြစ်ပြီး သုတေသနပြီးဆုံးပါက ဖျက်ဆီးမည်ဖြစ်ပါသည်။

ထိုအပြင်သင်အား ကျွမ်းကျင်သော မေးမြန်းသူများက (၅)မိနစ်ခန့်တွေ့ဆုံမေးမြန်းပါမည်။ မေးမြန်းစဉ်အချိန် အတွင်း အချို့သော မေးခွန်းများကို သင့်အနေဖြင့် ဖြေကြားရန် ဆန္ဒမရှိပါက သင့်အနေဖြင့် မဖြေဆိုဘဲ နေနိုင်ပါသည်။ မေးမြန်းရရှိသည့် အချက်အလက်များကို လျှို့ဝှက်ထားရှိမည် ဖြစ်ပါသည်။

၈။ သုတေသန ဆောင်ရွက်ပုံအဆင့်ဆင့်အားရှင်းလင်းခြင်း

ဤသုတေသနတွင် သင်ပါဝင်လိုသည့် ဆန္ဒရှိပါက အနည်းဆုံး ဆေးရုံ(၃) ရက်ခန့် တက်ရောက်ကုသ ပြီး (၄၂)ရက်တာကာလအတွင်း ကျန်းမာရေး ဌာနသို့ ခြောက်ကြိမ်ခန့် လာရောက်ရမည် ဖြစ်ပါသည်။

အကြိမ်တိုင်းတွင် လက်ဖျားထိပ်မှ သွေးဖောက်စစ်ဆေးခြင်း ဖြင့် သင်ခန္ဓာကိုယ်ကို ဖျားနာစေနိုင်သော ငှက်ဖျားရောဂါပိုး ရှိ/မရှိကို ရှာဖွေစစ်ဆေးပါမည်။ ကျွန်တော်တို့မှ သင့်အား မေးခွန်းအနည်းငယ်မေး၍ သင့်၏အရပ်၊ ကိုယ်အလေးချိန်နှင့် ကိုယ်အပူချိန်တို့ တိုင်းတာပါမည်။

အကယ်၍ သင်၏သွေးနမူနာတွင် ငှက်ဖျားရောဂါပိုးထပ်မံတွေ့ရှိပါက လိုအပ်သော ငှက်ဖျား ရောဂါ ကုသဆေးများ တိုက်ကျွေး၍ ပျောက်ကင်းသည် အထိကုသ ပေးသွားပါမည်။

၉။ အချိန်ကာလ

သုတေသနသည် အချိန်ကာလအားဖြင့် အနည်းဆုံး ဆေးရုံ(၃) ရက်ခန့် တက်ရောက်ကုသ ပြီး (၄၂)ရက်ခန့် ကြာမြင့်မည်ဖြစ်ပါသည်။ ဤအချိန်ကာလ အတွင်း သင့်အနေဖြင့် ကျန်းမာရေးဌာန (၆) ကြိမ်ခန့် လာရောက် ရမည်ဖြစ်ပါသည်။ တစ်ကြိမ်လာလျှင် မိနစ်(၂၀)ခန့်ကြာမြင့်နိုင်ပြီး သင်၏လက်မောင်းမှ သွေးနမူနာရယူခြင်း၊ လက်ဖျားထိပ်မှ သွေးဖောက်စစ်ဆေးခြင်း နှင့်သင့်အားဖေးခွန်း အနည်းငယ်မေးခြင်း ပြုလုပ်ပါမည်။ (၄၂)ရက်တာကာလ အပြီးတွင် သုတေသနပြီးဆုံး ပါမည်။

၁၀။ ဖြစ်နိုင်သည့်ဘေးထွက်ဆိုးကျိုးများ

ယခုအသုံးပြုမည့်ဆေးဝါးများသည် ကမ္ဘာ့ကျန်းမာရေးအဖွဲ့မှ အတည်ပြုထားသော ဆေးဝါးများဖြစ်ပြီး ဘေးထွက်ဆိုးကျိုး အချို့ရှိနိုင်ပါသည်။ သွေးနမူနာရယူသည့် သင်၏လက်မောင်းတွင် ခဏတာခဏာရောင်ရမ်းခြင်း ဖြစ်နိုင်ပါသည်။ အချို့သော လူနာများတွင် PQ ဆေးကြောင့် သွေးယိုစီးခြင်းနှင့်သွေးအားနည်းရောဂါများဖြစ်ပွားနိုင်ပါသည်။ သို့ရာတွင် ကျွန်ုပ်တို့၏ ကျန်းမာရေး ဝန်ထမ်း များက သင့်အားအနီးကပ်ကြည့်ရှုစောင့်ရှောက်ပြီး မလိုလားအပ်သော ဘေးထွက် ဆိုးကျိုးများကို စောင့်ကြည့်သွားပါမည်။ဌင်းဆိုးကျိုးများဖြစ်လာပါက သင့်တော်သည့်ဆေးဝါးများ ဖြင့်ကုသ ပေးသွားမည် ဖြစ်ပြီး သင့်သောက်သုံးနေသောဆေးဝါးများကို ရပ်တန့်သွားမည်ဖြစ်ပါသည်။ နောက်တစ်ဆင့် လွှဲပြောင်း ကုသရန် လိုအပ်ပါက လွှဲပြောင်းကုသမှုပေးသွားမည်ဖြစ်ပြီး လုပ်ဆောင်မှုအဆင့်တိုင်းအတွက် သင်နှင့်အမြဲ တိုင်ပင်ဆွေးနွေးသွားပါမည်။

၁၁။ ရရှိနိုင်သောဆိုးကျိုးများ

ဤသုတေသနတွင်သင်ပါဝင်ခြင်းသည် မပါဝင်ခြင်းထက် ဆိုးကျိုးများဖြစ်နိုင်ပါသည်။ဥပမာ- ဆေးများကြောင့် ဘေးထွက်ဆိုးကျိုးများရရှိခြင်း။ သို့ရာတွင် ဘေးထွက်ဆိုးကျိုးဖြစ်နိုင်ချေမှာ အလွန်နည်းပါးသော်လည်း သင့်အနေဖြင့် ဤသို့သော ဖြစ်နိုင်ချေများကို သတိပြုရပါမည်။ ကျွန်ုပ်တို့ အနေဖြင့် ဆိုးကျိုးများကို လျော့နည်းနိုင်သမျှ လျော့နည်းအောင် ဆောင်ရွက်မည်ဖြစ်ပြီး သင့်အနေဖြင့်

မမျော်လင့်နိုင်သော ဆိုးကျိုးတစ်စုံတစ်ရာဖြစ်လာပါက ကောင်းမွန်သော ကျန်းမာရေး စောင့်ရှောက်မှု ကိုပေးပါမည်။

၁၂။ ကိုယ်စိတ်အနှောက်အယှက်

ဤသုတေသနတွင်ပါဝင်ခြင်းဖြင့်သင့်အနေနှင့် တစ်စုံတစ်ရာကိုယ်စိတ် အနှောက်အယှက် ဖြစ်စေနိုင်ပါသည်။ ဥပမာ သင်၏လက်မောင်းမှ သွေးနမူနာယူခြင်း ကြောင့်လည်းကောင်း၊ အချို့သောမေးခွန်းများကို ဖြေကြားခြင်းဖြင့်လည်းကောင်း သင့်အားစိတ်အနှောက်အယှက် ဖြစ်စေနိုင် ပါသည်။

၁၃။ အကျိုးကျေးဇူး

ဤသုတေသနတွင်ပါဝင်ခြင်းဖြင့်သင့်အားငှက်ဖျားဆေးစံပြုကုထုံး နှင့်စနစ်တကျ ကုသပေးခြင်း ကြောင့် သင်၏ငှက်ဖျားရောဂါပျောက်ကင်း မည့်အကျိုးခံစား ရမည်ဖြစ်ပါသည်။ ထို့အပြင် သင်ပါဝင်ခြင်းသည် အာတီဆူနိတ် ဆေးမတိုးသော ငှက်ဖျားရောဂါတိုက်ဖျက်ရေးအတွက် ပါတ်သက်သော အချက်အလက်များကို ရရှိစေပြီး အနာဂါတ်တွင် ဤဒေသ၊ ဤနိုင်ငံ၌ ငှက်ဖျားရောဂါတိုက်ဖျက်ရေးအတွက် အကျိုးကျေးဇူးများ ရရှိနိုင်မည် ဖြစ်ပါသည်။

၁၄။ လျှို့ဝှက်ထားရှိခြင်း

ဤသုတေသနတွင် သင်ပါဝင်ခြင်းဖြင့် သင့်ပါတ်ဝန်းကျင်မှ သင့်နှင့်ပတ်သက်သော အချက်အလက်များကို ကျွန်ုပ်တို့အား မေးမြန်းလာပါက လျှို့ဝှက်ထားမည်ဖြစ်ပါသည်။

ဤသုတေသနမှ ကောက်ယူရရှိသည့် အချက်အလက်များကို လျှို့ဝှက်ထားရှိမည်ဖြစ်ပါသည်။ ဤသုတေသနတွင် သင့်ထံမှ မေးမြန်းသိရှိရသည့် အချက်အလက်များကို သင်၏ အမည်ဖော်ပြခြင်းမရှိဘဲ သတ်မှတ်နံပါတ်စဉ်ကိုသာ အသုံးပြု၍ဖိုင်တွဲပြီး သိမ်းဆည်းထားပါမည်။ သတ်မှတ်သည့် နံပါတ်စဉ်ဆိုင်ရာအတွက် အမည်များကို သော့ခတ် သိမ်းထားမည်ဖြစ်ပြီး သုတေသနအဖွဲ့မှလွဲ၍ မည်သူတစ်ဦးတစ်ယောက်မှ သိရှိစေမည် မဟုတ်ပါ။

၁၅။ သုတေသနရလာဒ်များကို ဖြန့်ဝေခြင်း

ဤသုတေသနတွေ့ရှိချက်များကို အများပြည်သူအတွင်းကျယ်ကျယ်ပြန်ပြန့်. ဖြန့်ဝေခြင်းမပြုမီ သင်နှင့် သင့်ပါတ်ဝန်း ကျင်လူထုအတွင်း ဖြန့်ဝေသိရှိစေပါမည်။

၁၆။ သုတေသနမှ နှုတ်ထွက်ပိုင်ခွင့်

ဤသုတေသနတွင်ပါဝင်ရန် သင့်အနေနှင့်ဆန္ဒ မရှိပါက မပါဝင်ဘဲ နေနိုင်ပါသည်။ ထိုသို့ပါဝင်ရန်ငြင်းဆန်ခြင်းဖြင့် သင့်အနေနှင့်နောင်အခါ ကျန်းမာရေးဌာနများတွင် ဆေးကုသခွင့်အပေါ် မည်သို့မျှ သက်ရောက်စေခြင်းမျိုးမရှိပါ။ ကျန်းမာရေးဌာနများတွင် သင့်အနေဖြင့် ဆေးကုသခွင့်များ ဆက်လက်ရရှိခံစားရရှိပါမည်။ဤသုတေသနမှ အချိန်မရွေး နှုတ်ထွက်နိုင်ပါသည်။ထိုသို့နှုတ်ထွက်ခြင်းဖြင့် လူနာတစ်ဦးအနေဖြင့် သင်၏ အကျိုးခံစားခွင့် တစ်ရာ ကိုဆုံးရှုံးစေမည် မဟုတ်ပါ။

၁၇။ ဆက်သွယ်ရန် ပုဂ္ဂိုလ်

အကယ်၍ မေးခွန်းမေးစရာရှိပါက ယခု(သို့မဟုတ်) နောက်မှ မေးမြန်းနိုင်ပါသည်။ နောက်တွင် မေးမြန်းရန်ရှိပါက ဒေါက်တာကျော်မျိုးထွန်း ၊ ၀၉၅၀၂၅၅၄၄ သို့ဆက်သွယ်မေးမြန်းနိုင်ပါသည်။ ဤသုတေသန အဆိုပြုလွှာကို ဆေးဝန်ထမ်းညွှန်ကြားရေးမူးရုံး၊ ဆေးသုတေသနတပ် ၏ လူပုဂ္ဂိုလ်များအပေါ် သုတေသနပြုခြင်း ဆိုင်ရာ ဘုတ်အဖွဲ့မှ ခွင့်ပြုချက်ရယူပြီး ဖြစ်ပါသည်။ အကယ်၍သင့်အနေဖြင့် မေးခွန်းမေးစရာရှိပါက လူပုဂ္ဂိုလ်များအပေါ် သုတေသနပြုခြင်းနှင့် ဖြစ်နိုင်သည့် အန္တရာယ်ကို ကာကွယ်ပေးရန် ဖွဲ့စည်းထားသည့် နေပြည်တော် ရှိ ဆေးသုတေသနတပ် ၏ လူပုဂ္ဂိုလ်များအပေါ် သုတေသနပြုခြင်း ဆိုင်ရာဘုတ်အဖွဲ့ အတွင်းရေးမှူးထံသို့ ဆယ်သွယ် မေးမြန်းနိုင်ပါသည်။

အပိုင်း (ခ) သဘောတူညီကြောင်းလက်မှတ်ရေးထိုးသည့်နှုံစံ

ကျွန်ုပ်ကို ၄င်ဖျားရောဂါ တိုက်ဖျက်ရေးသုတေသနတွင် ပါဝင်ရန် ဖိတ်ကြားခြင်း ခံရပါသည်။ ဤသုတေသနတွင်ပါဝင်ခြင်းဖြင့် ၄င်ဖျားရောဂါ ကုသဆေးများ သောက်သုံးရမည်ဖြစ်ပြီး သွေးနမူနာယူခြင်းများကိုလည်း ခံယူရမည် ဖြစ်ပါသည်။ ဖြစ်နိုင်ချေ အန္တရာယ်အနည်းငယ် ရှိပြီးလူမှုရေးနှင့် စိတ်ပိုင်းဆိုင်ရာ ကသိကအောက်ဖြစ်မှုများ ဖြစ်နိုင်သည်ဟု သိရပါသည်။ ဤသုတေသနတွင် ပါဝင်ခြင်းဖြင့် ကျွန်ုပ်၏ကိုယ်ကျိုးတစ်စုံတစ်ရာမှ ရရှိခံစားမည် မဟုတ်ဟုသိရှိပါသည်။ ဤသုတေသနကို ဆောင်ရွက်မည့် တာဝန်ခံဆရာဝန်၏ အမည်နှင့် ဆက်သွယ်ရန်လိပ်စာ၊ ဖုန်းနံပါတ်များကို သိရှိပြီးဖြစ်ပါသည်။

ကျွန်ုပ်အနေဖြင့် သက်ဆိုင်သည့်အချက်အလက်များကို ဖတ်ရှုပြီး (သို့) တစ်ဦးတစ်ယောက်မှ ကျွန်ုပ်ကိုဖတ်ပြပြီးဖြစ်ပါသည်။ ကျွန်ုပ်အနေဖြင့် သိရှိလို သည်များရှိပါက မေးခွန်းများမေးမြန်းနိုင်ပြီး ဤသုတေသနတွင် ပါဝင်ရန်အတွက် ကျွန်ုပ်၏ သဘောဆန္ဒအလျောက် ပါဝင်ခြင်းဖြစ်ကြောင်း သဘောတူဆန္ဒ ပြုပါသည်။ ဤသုတေသနမှ ကျွန်ုပ်ပါဝင်လိုသည့်ဆန္ဒမရှိပါက နှုတ်ထွက်နိုင်ပြီး၊

ဤသုတေသန ပါဝင်သည်ဖြစ်စေ၊ မပါဝင်သည်ဖြစ်စေ ကျန်းမာရေးဌာနများတွင် ဆေးကုသမှုခံယူခွင့်အပေါ် မည်သို့မျှထိရောက်စေခြင်းမျိုးမရှိပါ။

သုတေသနတွင်ပါဝင်သူ၏အမည် .....

လက်မှတ် .....

ရက်စွဲ .....

အကယ်၍စာမတတ်မြောက်ပါက

စာတတ်မြောက်သူ တစ်ဦးတစ်ယောက်က လက်မှတ်ရေးထိုးပေးရမည် (ဖြစ်နိုင်လျှင်သုတေသန အဖွဲ့နှင့် ဆက်စပ်သူမဖြစ်စေရပါ) စာမတတ်သူအနေဖြင့် ၎င်း၏လက်ဗွေပုံစံကို ဤစာရွက်ပေါ်တွင် ရိုက်နှိပ်ရပါမည်။

ကျွန်ုပ်အနေဖြင့် ဤသုတေသနတွင်ပါဝင်ခွင့်ရှိသူ စာမတတ်မြောက်သူ၏ ကိုယ်စား သဘောတူညီမှု ပုံစံကို အသေအချာဖတ်ရှုပြီး ကိုယ်စားလက်မှတ် ရေးထိုးခြင်းဖြစ်ပါသည်။ သုတေသနတွင်ပါဝင်သူအနေဖြင့် သိလိုသည့်မေးခွန်းများ မေးမြန်းရန်အခွင့်အရေးရှိကြောင်းသိရှိရပါသည်။ ကျွန်ုပ်အနေဖြင့် အတိအကျ ပြောနိုင်သည်မှာ သုတေသနတွင်ပါဝင်လိုသူမှ သဘောတူညီကြောင်းဆန္ဒမှာ ၎င်းကိုယ်တိုင် လွတ်လွတ်လပ်လပ်ပေးကြောင်း အတည်ပြုပါသည်။

စာမတတ်သူဖြစ်ပါက အသိသက်သေ၏အမည် ..... သုတေသနတွင်ပါဝင်သူ၏လက်ဗွေ

လက်မှတ် .....

ရက်စွဲ .....

သုတေသီ၏အမည် နှင့် လက်မှတ် .....

ရက်စွဲ .....

13.3 Dihydroartemisinin-piperaquine and primaquine dosing table

Weight (kg)	DHA+Pip (40+320 mg)		Primaquine (7.5 mg)	
	Tabs	(or mls)1	Tabs	(or mls)1
5		1.3		0.8
6		1.6		1
7		2		1.2
8-12	½		¼	
13-20	1		½	
21-30	1 ½		1	
31-40	2		1 ¼	
41-50	2 ½		1½	
51-60	3		2	
61-70	3 ½		2¼	
71-84	4		2½	
85-100	5		3	

1. If you need to give a suspension of DHA-PPQ or PQ to young children who are unable to swallow tablets, crush the tablet and mix with 5 ml of drinking water and give the volume specified by the table above.

13.4 Ethical approval (OXTREC)

Oxford Tropical Research Ethics Committee

University of Oxford
Room 8, Manor House
The John Radcliffe, Headington, Oxford OX3 9DZ
tel. +44 (0) 1865 743005, fax +44 (0) 1865 743 002
e-mail: Fiona.Goulthorp@admin.ox.ac.uk

Professor N White
Mahidol Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine
Mahidol University
420/6 Rajvithi Road
Bangkok 10400
Thailand

8th March 2011

Dear Nick

Full Title of Study: A multicentre, randomised trial to detect in vivo resistance of Plasmodium falciparum to artesunate in patients with uncomplicated malaria

OXTREC Reference: 06-11

Thank you for your letter 4th March 2011, in which you have responded to the committee's request for further clarification and amendments, and enclosed revised documents:

Documents	Version	Date
Protocol	Version 1.2	04.03.2011
PIS	Version 1.2	04.03.2011

I am therefore happy as Chairman for OXTREC to give approval for this study.

OXTREC are grateful for annual and end of study reports for this study.

Yours sincerely,

Richard Mayon-White

Dr Richard Mayon-White

OXTREC Chair

Oxford Tropical Research Ethics Committee

University of Oxford
Joint Research Office, Block 60
Churchill Hospital, Oxford OX3 7LE
Tel. +44 (0) 1865 (5)72224, Fax +44 (0) 1865 (5)72228
E-mail: fiona.gouthorp@admin.ox.ac.uk

UNIVERSITY OF OXFORD

7th August 2012

Dr E Ashley
Mahidol-Oxford Research Unit (MORU)
Faculty of Tropical Medicine, Mahidol University
3/F, 60th Anniversary Chalermprakiat Bldg
420/6 Rajvithi Road, Rajthevee
Bangkok 1040

Dear Dr Ashley

Full Title of Study: A multicentre, randomised trial to detect in vivo resistance of Plasmodium falciparum to artesunate in patients with uncomplicated malaria

OXTREC Reference: 06-11

Thank you for your email 1st August 2012 in which you have included the annual report. We will update our database accordingly.

This study continues to have OXTREC approval.

Yours sincerely

Dr Richard Mayon-White

OXTREC Chair

Direct Line Tel: +44 (0)1865 (5)72224
Fax: +44 (0)1865 (5)72228 Email: fiona.gouthorp@admin.ox.ac.uk Web: www.admin.ox.ac.uk/rao/

13.5 Ethical Approval (Myanmar)

Ethical Approval from Directorate of Medical Services, Ministry of Defence The Republic of the Union of Myanmar

MEDICAL ETHICS COMMITTEE ☆ OFFICE OF THE COMMANDER-IN-CHIEF (ARMY) ☆
No.................
Date...............

THE REPUBLIC OF THE UNION OF MYANMAR
OFFICE OF THE COMMANDER - IN - CHIEF (ARMY)
DIRECTORATE OF MEDICAL SERVICES
(Phone 95036-30859,95036-30944,Fax 95067 - 416055)

Letter No . 03 / Ethics / 13
Date . August 26 2013

The Medical Ethics Committee, after thoroughly reviewing on medical research involving human subjects approves on the following research project including subject information sheet and informed consent form. Any changes in the protocol should be submitted for revision and further approval.

Protocol title : A study to detect *in vivo* resistance of *Plasmodium falciparum* to artemisinine derivatives in patients with uncomplicated malaria in Myanmar.

Principal Investigator: Maj. Kyaw Myo Tun
Lecture, Preventive and Social Medicine Department
Defence Services Medical Academy, Mingalardon

Chairman
Major. General. Myo Myint Thein
M.B,B.S, M.Med.Sc(Surg)
F.R.C.S(Eng),M.A(Defence Studies)
Director of Medical Services
Chairman of Medical Ethics Committee
Office of the Commander-In-Chief(Army)

LIST OF MEDICAL ETHICS COMMITTEE MEMBERS

(1) Major. Gen. Myo Myint Thein Director,Directorate of Medical Services	M.B,B.S, M.Med.Sc(Surg), F.R.C.S(Eng),M.A(Defence Studies)	CHAIRMAN
(2) Col. Moe Myint Htun Brig. General Staff(Operations) Office of the Commander in Chief(Army)	B.Sc(DSA), M.A(Defence Studies)	MEMBER
(3) Brig. Gen. Kyaw Min Oo Commandant, No.(2)DSGH	M.B,B.S, M.Med.Sc(Otorhinolaryngology), F.R.C.S(Eng),M.A(Defence Studies)	MEMBER
(4) Col. Than Oo Deputy Adjutant General Office of the Adjutant General	B.Sc(Physics), M.A(Defence Studies)	MEMBER
(5) Lt. Col. Kyaw Hlaing Staff Officer Grade(1) Directorate of Medical Services	M.B,B.S, M.Med.Sc(P & TM), Ph.D(Public Health)	MEMBER
(6) Col. Soe Hlaing Deputy Director,Directorate of Medical Services	M.B,B.S, M.Med.Sc(Internal Medicine) M.R.C.P, M.A(Defence Studies)	SECRETARY

13.6 Parasite clearance estimation for individual patient (WWARN PCE)

The limit of detection for this report was 40 parasites/microlitre. The individual patient parasite clearance estimation charts use these colours:

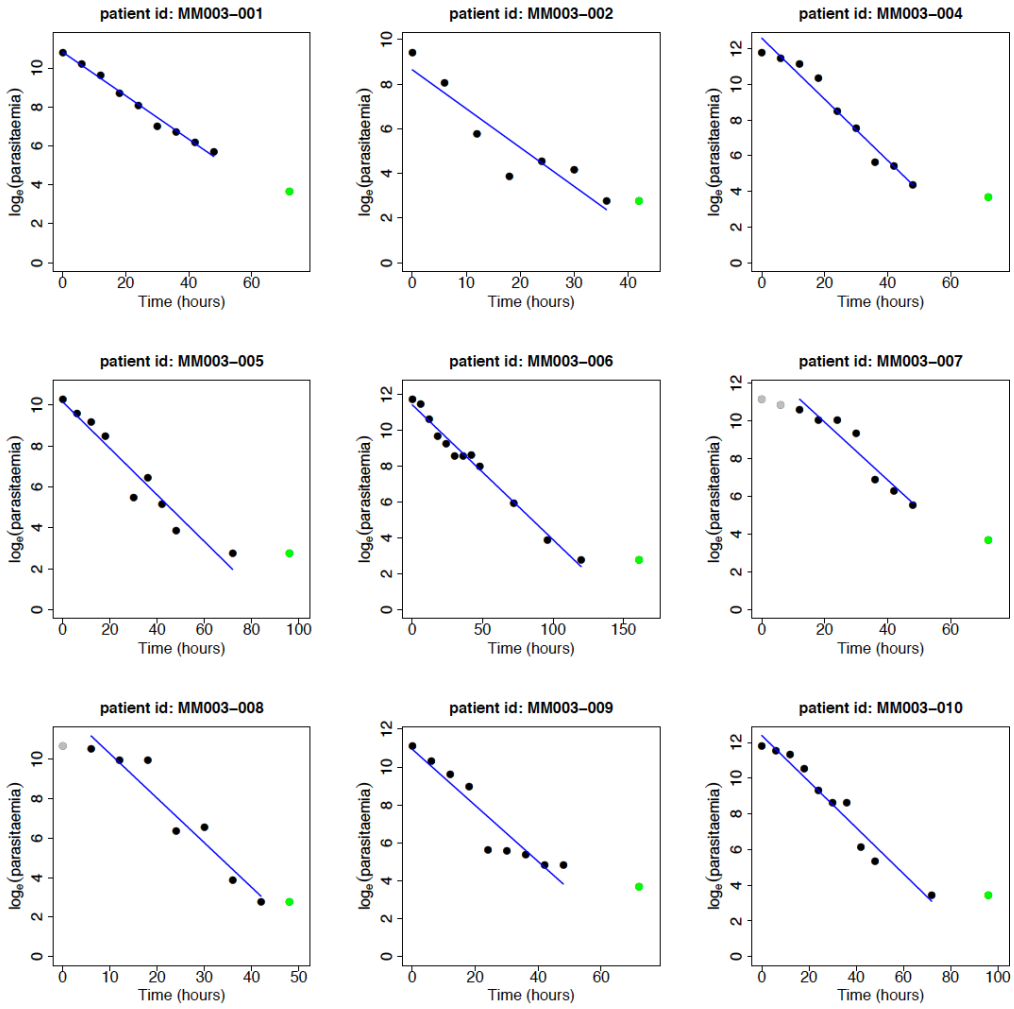
- Gray circle: Measurements during the lag phase

- Black circle: Measurements used in estimation of clearance rate

- Red circle: Outliers excluded from calculations

- Green circle: Measurements below the level of detection

- Blue line: Linear regression model (i.e. predicted log parasitaemia) used to derive clearance rate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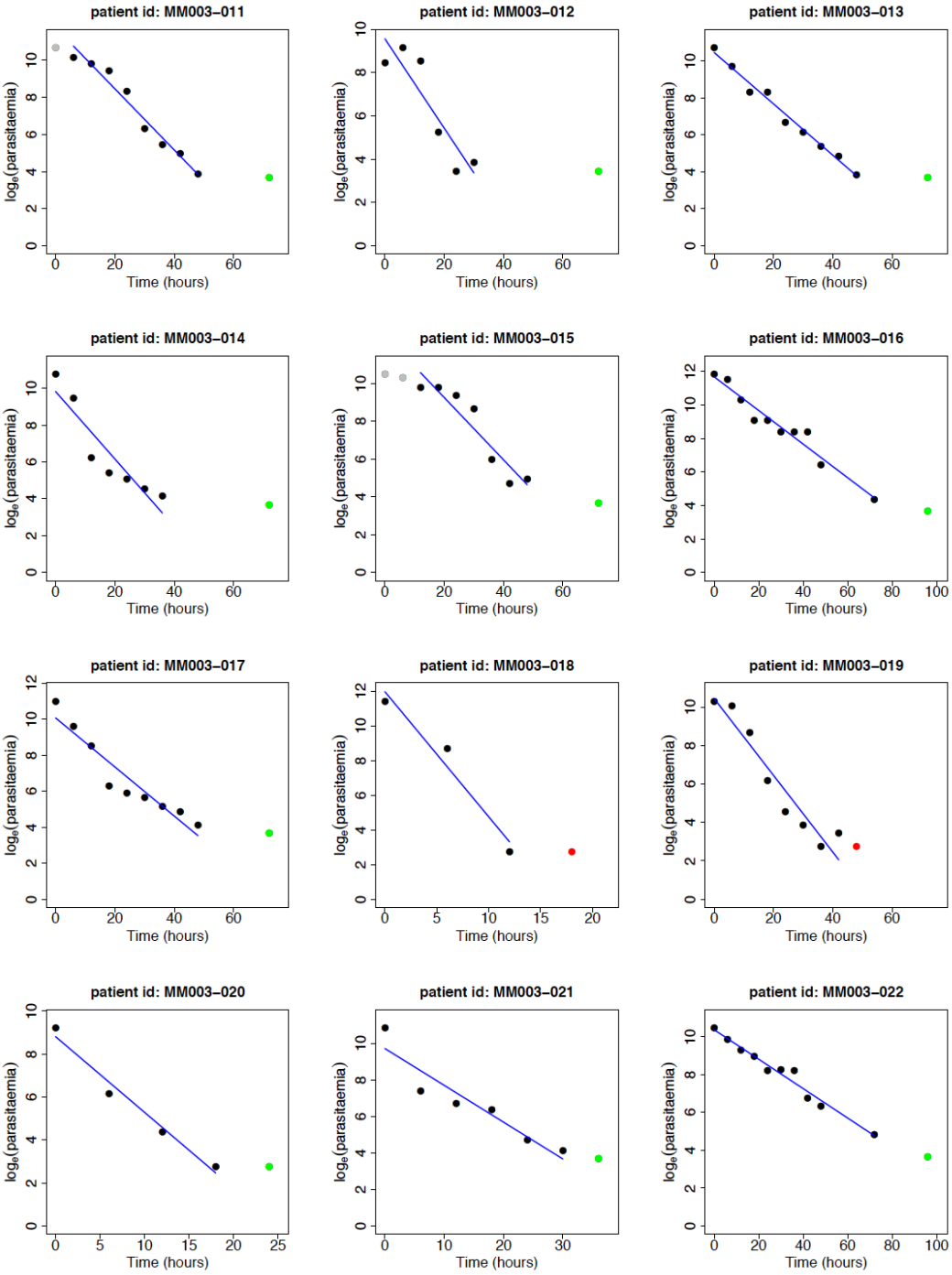
log$_e$(parasitaemia)
Time (hours)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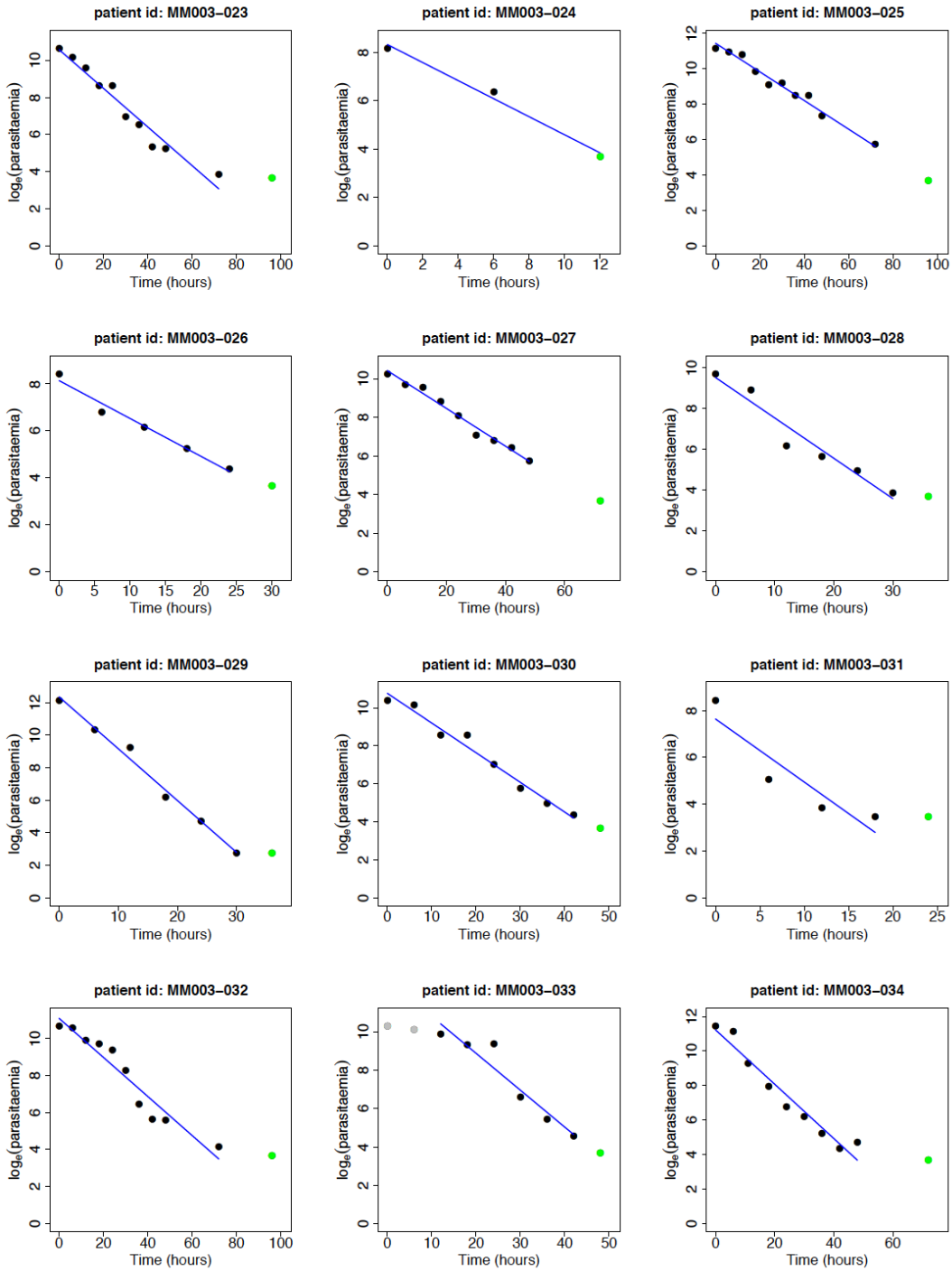
Time (hours)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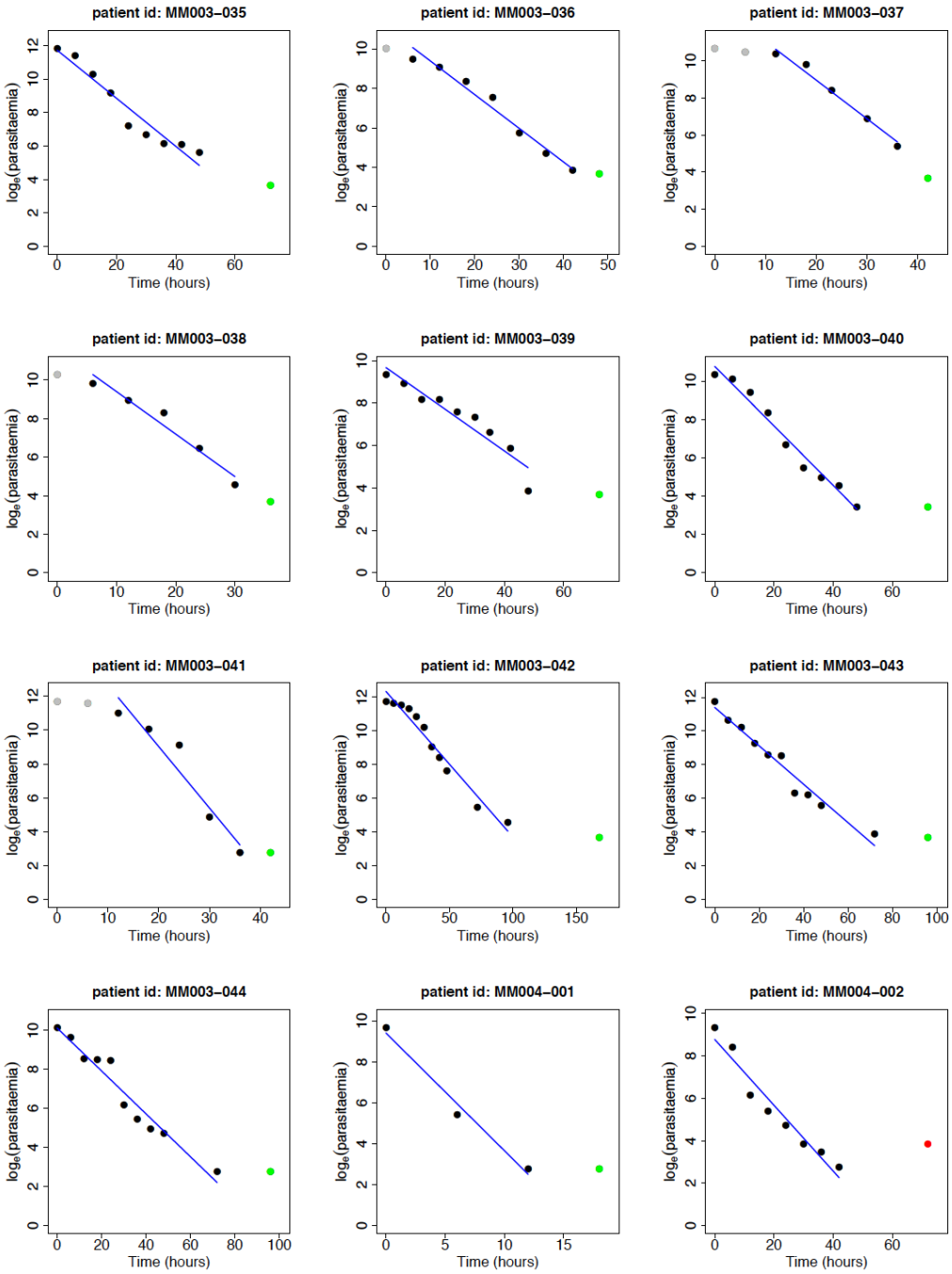
patient id: MM003–034
log$_e$(parasitaemia)
Time (hours)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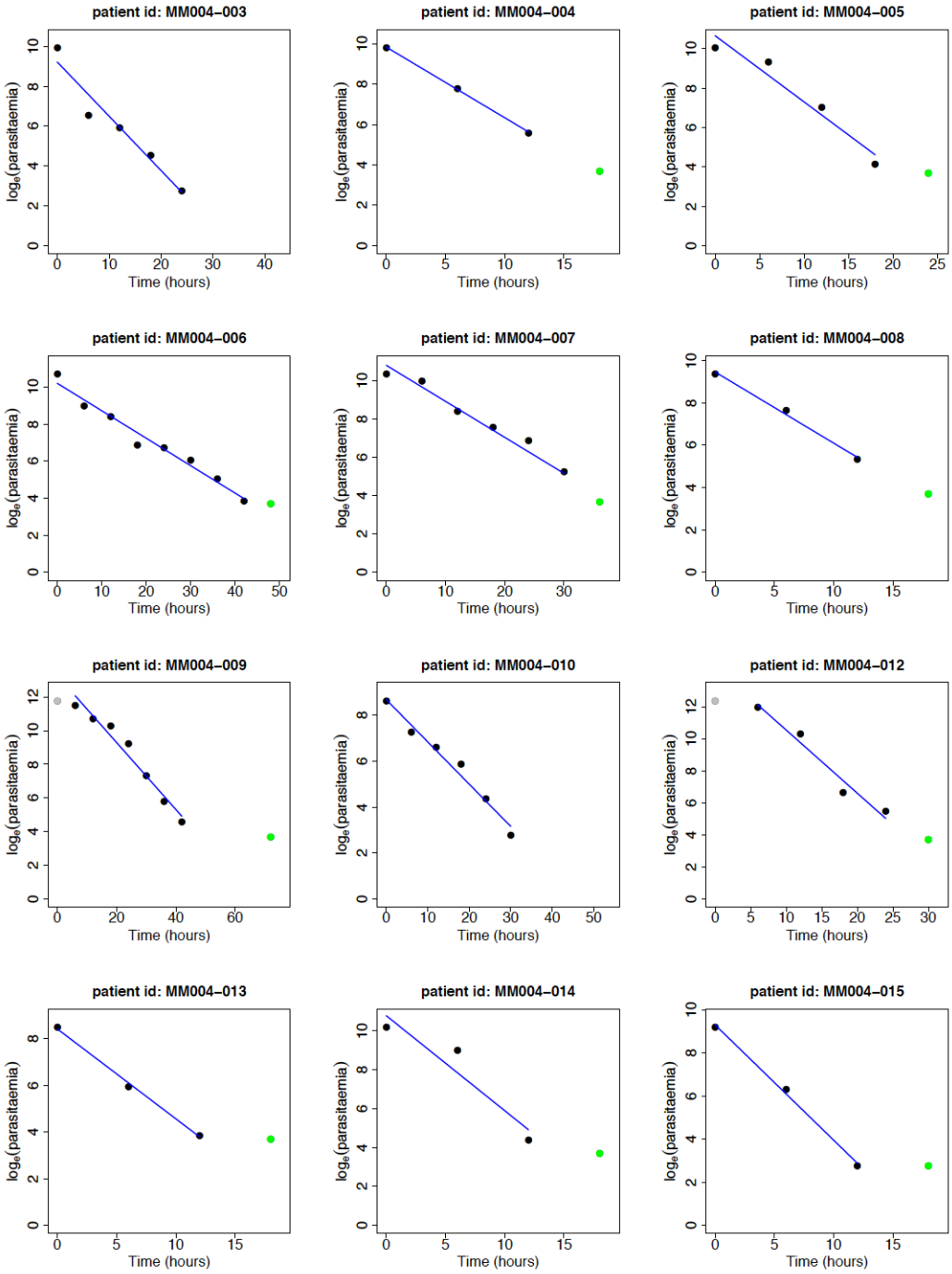
log$_e$(parasitaemia)
Time (hours)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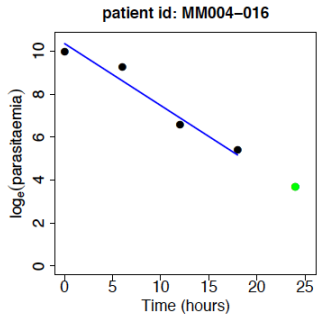
patient id: MM004–015
log$_e$(parasitaemia)
Time (hours)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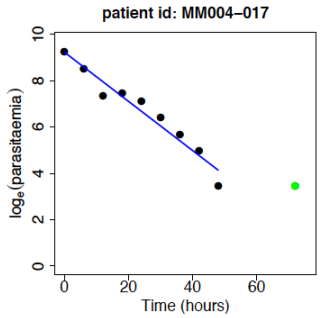
Time (hours)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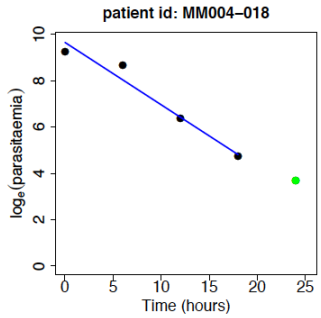
Time (hours)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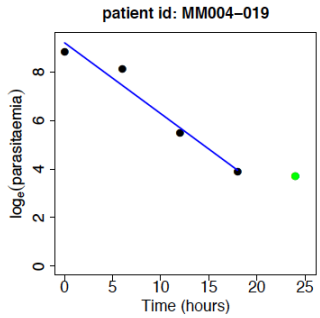
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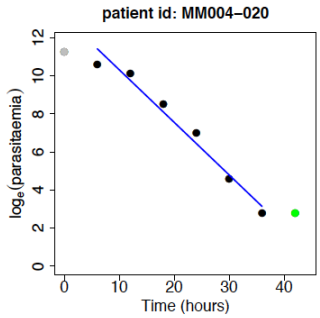
Time (hours)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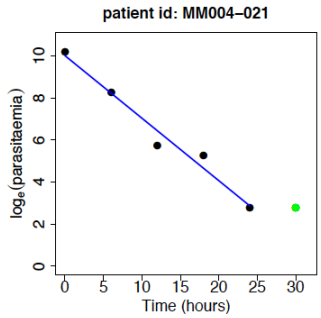
Time (hours)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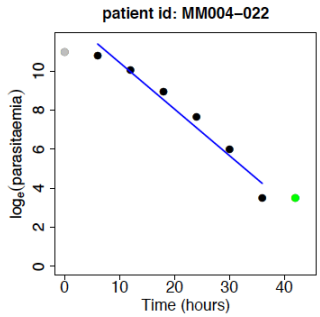
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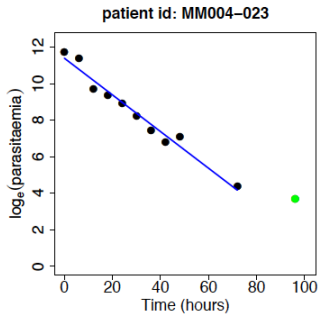
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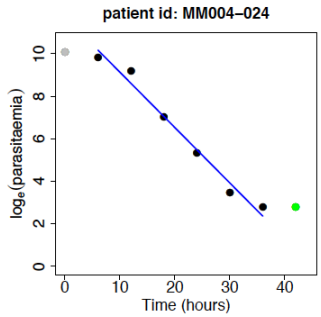
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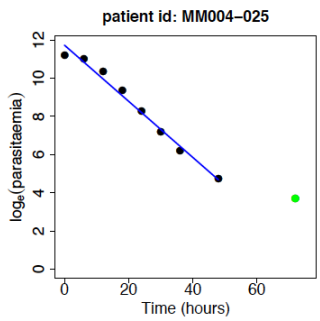
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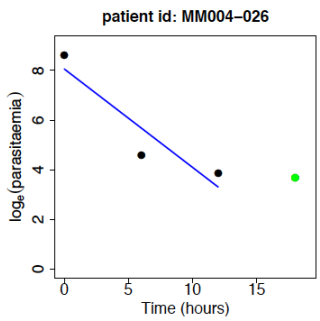
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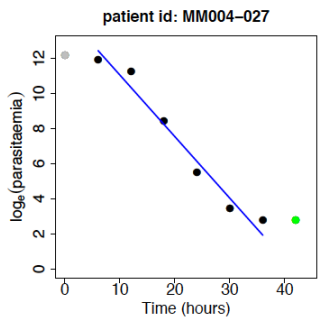
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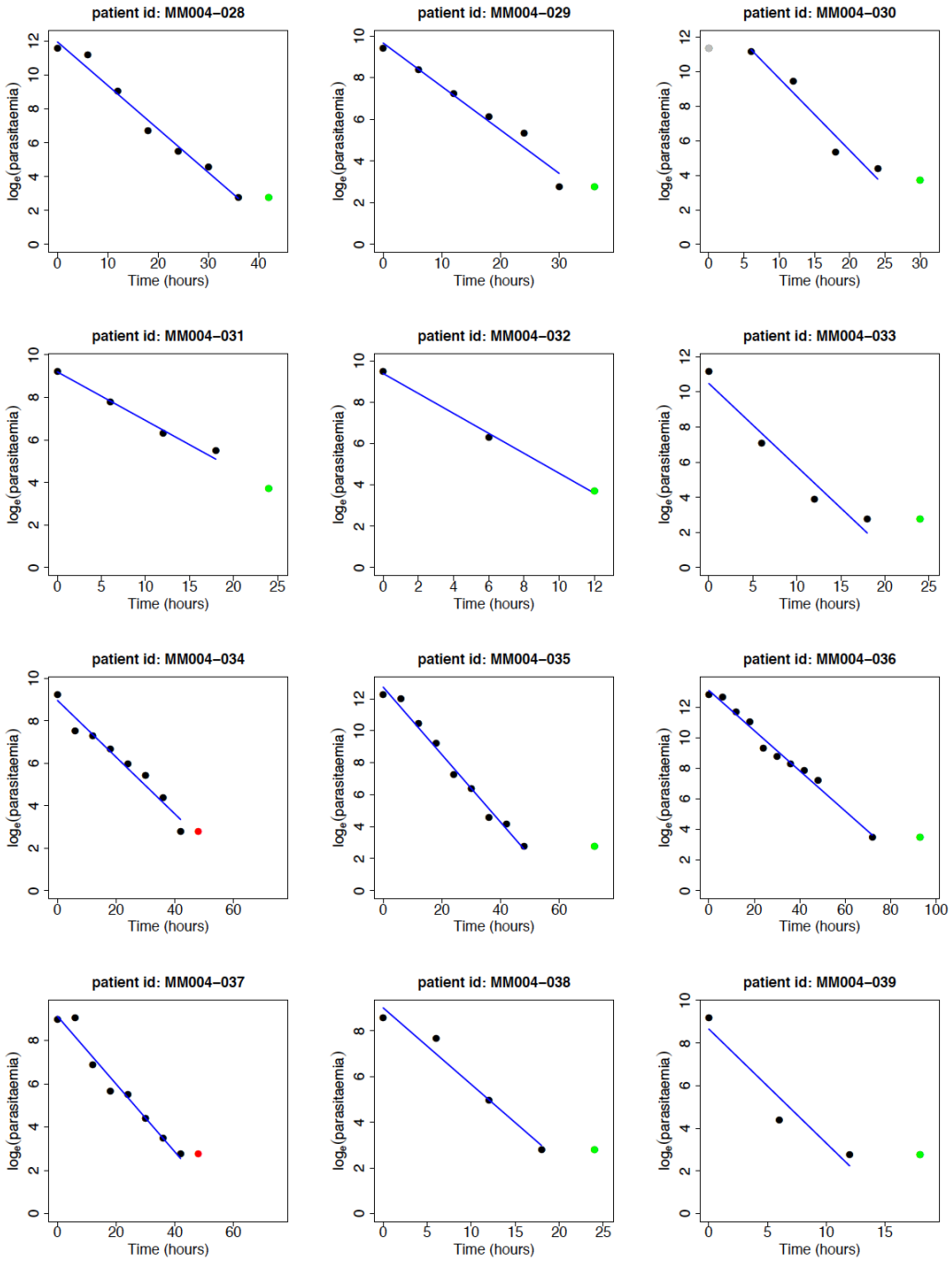
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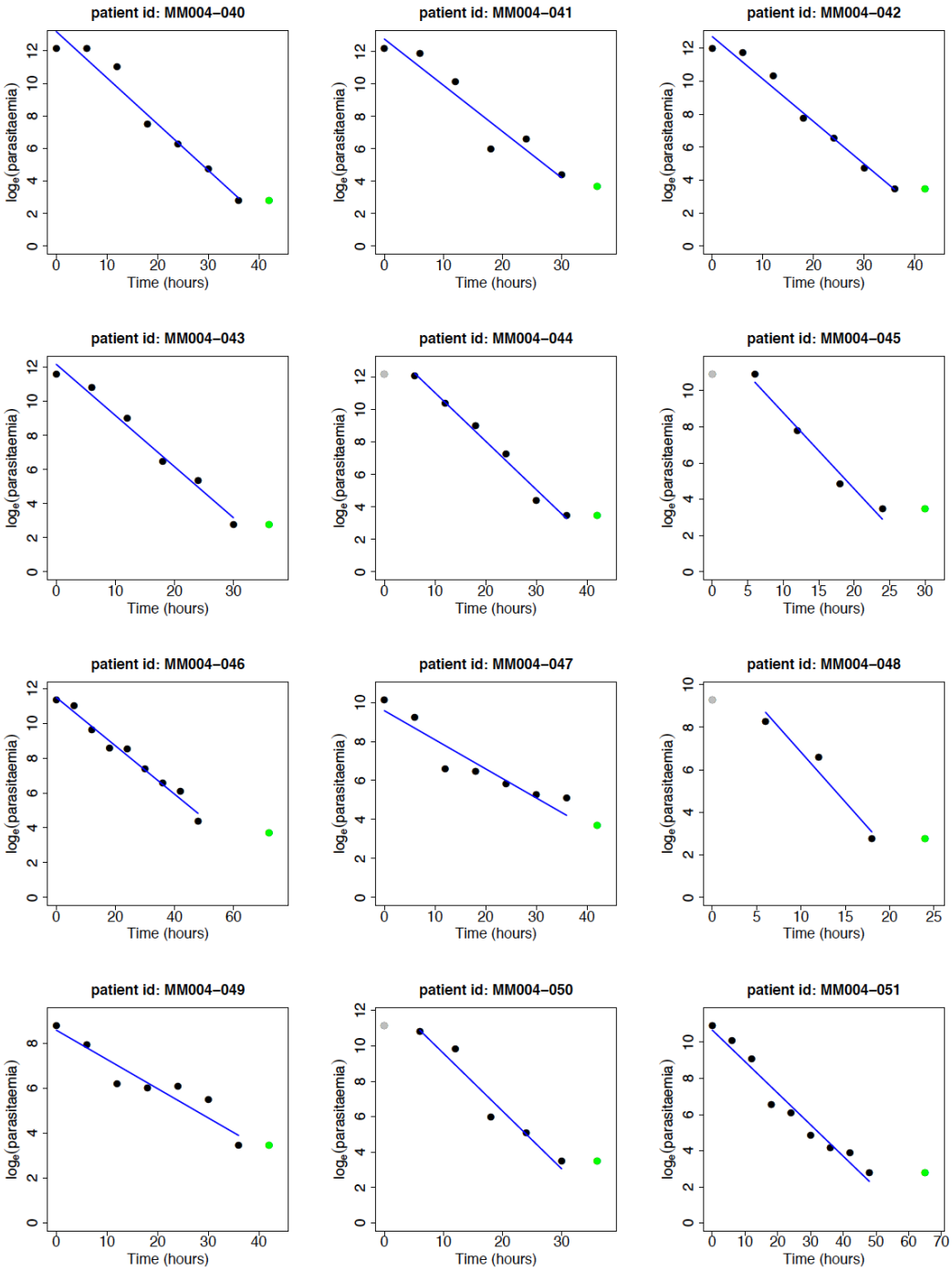
Time (hours)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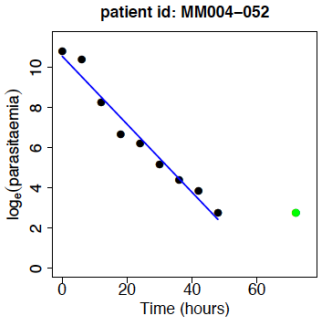
Time (hours)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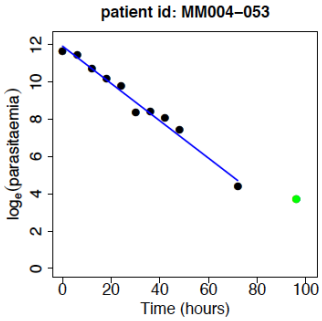
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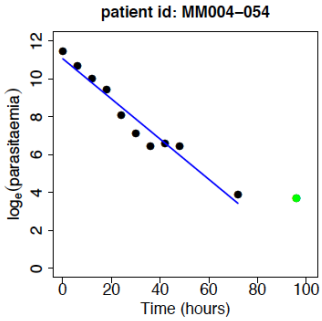
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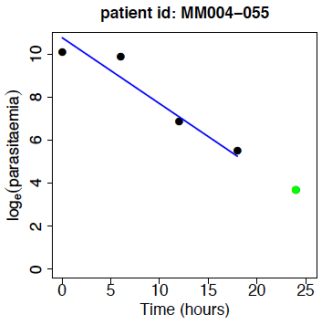
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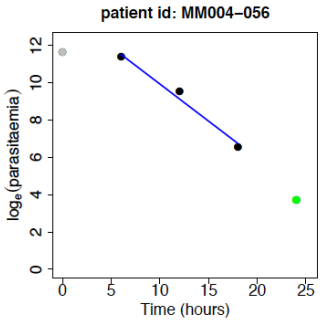
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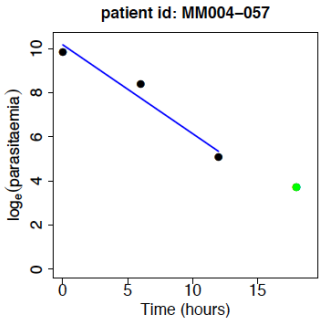
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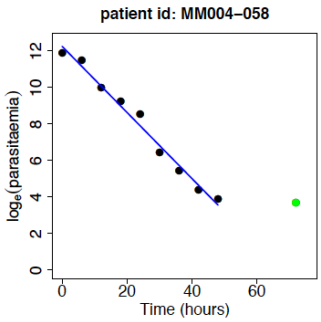
Time (hours)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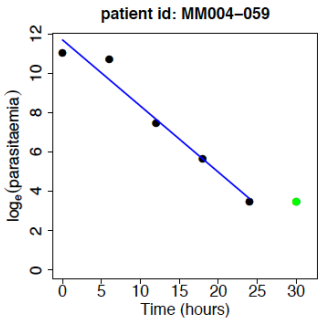
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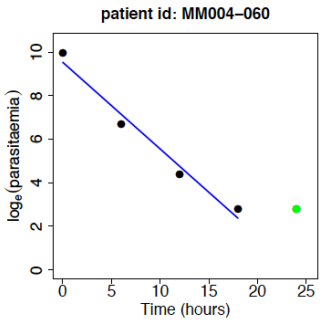
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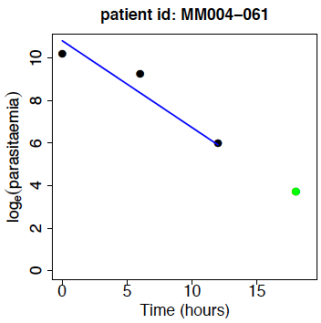
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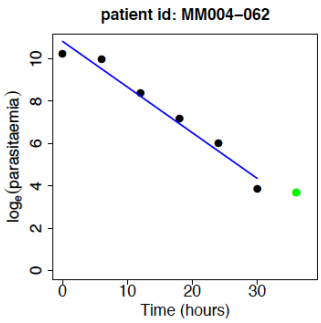
Time (hours)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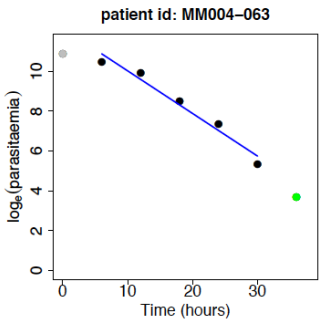
Time (hours)


patient id: MM004–063
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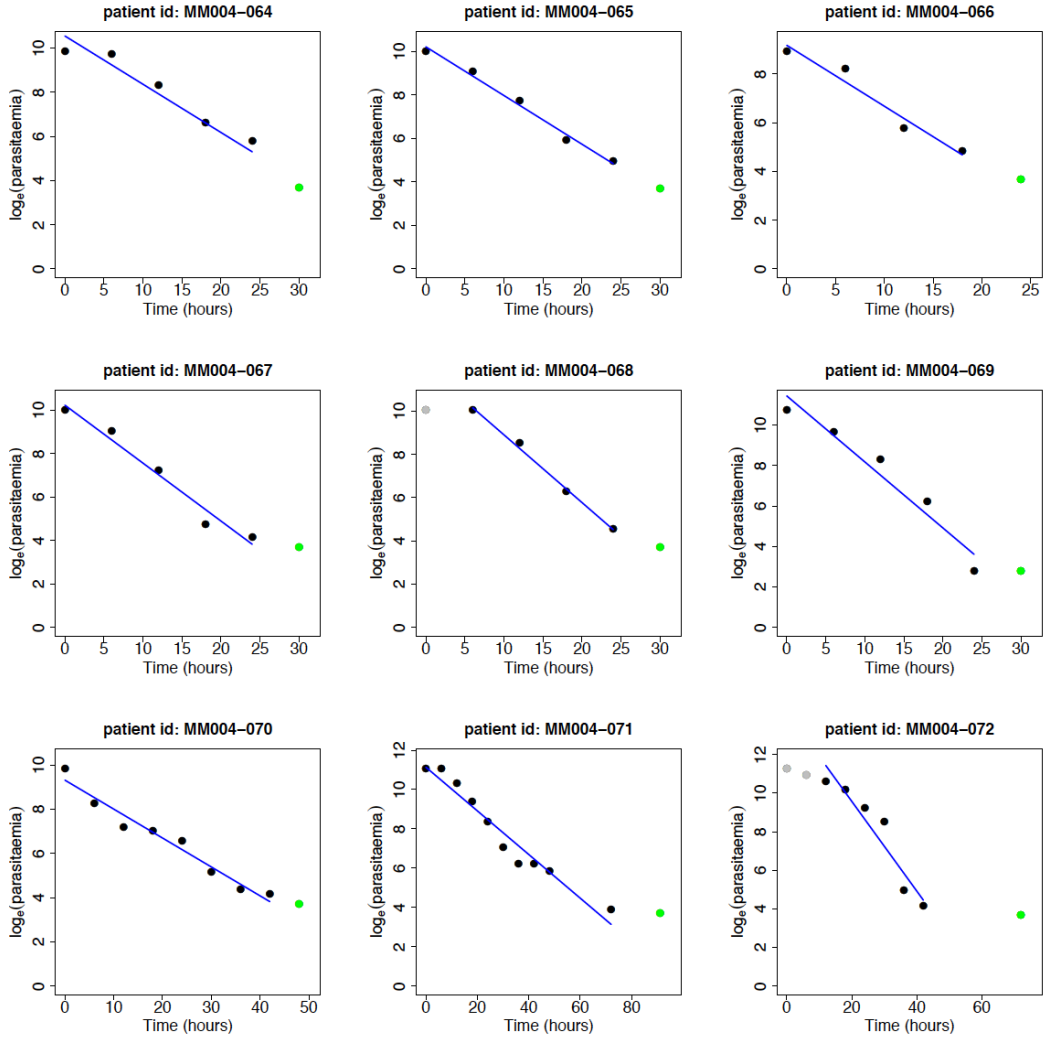
Time (hours)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patient id: MM004–071
patient id: MM004–072
log$_e$(parasitaemia)
Time (hours)

14Appendix D

14.1 Informed consent form (English version)

Institutional Ethical Review Committee

Department of Medical Research (Lower Myanmar)

Yangon, Myanmar

Informed Consent Form for Clinical Study (Adult)

This Informed Consent Form is for adult men and women who we are inviting to participate in the research "Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar".

Name of Principal Investigator – Dr. Frank Smithuis

Name of Organization – Myanmar Oxford Clinical Research Unit (MOCRU)

Name of Funder – 3MDG

Name of proposal- Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar

PART I: Information Sheet

1. Introduction

My name is Dr. ……., and I am working for the Myanmar Oxford Clinical Research Unit. We are doing a research about the treatment of malaria. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask them of me, the study doctor or the staff.

2. Purpose of the research

Malaria is a serious health problem in Mon or Kayin. The main treatment for malaria in Myanmar is called Coartem. This drug is usually given for 3 days; it is effective and has hardly any negative side effects. However, recently there has been malaria that is less sensitive for this treatment in the region. Treatment failures are still very low but we want to study if prolonging the treatment course by a further 2 days (total 5 days treatment) could further improve the treatment result.

We want to follow up patients for 42 days in order to find out if 5 days treatment has a better treatment result and we want to compare the frequency of side effects.

3. Type of research intervention

This research will involve 3 days or 5 days treatment as well as nine follow-up visits to this clinic over the coming 6 weeks. It will involve blood collection from your arm 5 times, half of the teaspoon of blood will collect at each time and you will need to spend 15-30 minutes at each follow up visit.

4. Participant selection

We are inviting all patients with malaria parasites who attend this clinic to participate in this research.

Now we have found malaria parasites in your blood and you need a treatment to cure it. Therefore we invite you to participate in this study.

5. Voluntary Participation

Your (your child's) participation to this study is completely voluntary.

It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this clinic will continue and nothing will change. You may change your mind later and stop participating even if you agreed earlier.

6. Information on the Drugs

The drug we are using in this research is called Coartem. It has been studied extensively, it has very few – if any – side effects and it is being used all over the world. It is currently the official choice of treatment by the National Malaria Control Program in Myanmar.

Coartem is generally considered a very safe drug and reported side effects are generally mild. Possible side effects are GI upset (anorexia, nausea, vomiting, abdominal pain, and diarrhea), headache, dizziness, fatigue and sleep disturbance. Palpitations, myalgia, arthralgia and rash are reported very infrequently. All these complaints are common in malaria and could be disease related (and not related to the medicine).

A single dose of Primaquine (PQ) will also be given (once). It is part of the routine treatment of malaria in Myanmar. Primaquine can cause haemolysis in patients with a rare blood disease (G6PDd), which typically occurs in 2-15% of patients in endemic zones. However, as said, it is part of the normal treatment in Myanmar and severe bleeding has not been reported yet. Other possible side effects are nausea, vomiting, stomach upset, and abdominal cramps.

7. Procedures and protocol

a. Unfamiliar procedures

The study wants to find out if 5 days treatment with Coartem is better than the usual 3 day treatment. Therefore we need to compare these two treatments. To do this, we will put people taking part in this research into two groups. The groups are selected by chance. The participant needs to pick up a treatment card from a plastic box and will be treated accordingly.

Participants in one group will be given the 5 days treatment (twice a day, so 10 doses) while participants in the other group will be given the 3 days treatment (twice a day, so 6 doses) which is the treatment that is currently being used for malaria. We will then compare which of the two has the best results. For female participants (older than 18 years) will be tested with urine pregnancy test and if pregnant she will be excluded from the study.

The healthcare workers will be looking after you and the other participants very carefully during the study. If we are concerned about what the drug is doing, we will find out which treatment (3 or 5 days) you are getting and possibly make changes. If there is anything you are concerned about or that is bothering you about the research please talk to me or one of the other researchers

If we find that the medicine that is being used does not have the desired effect, or not to the extent that we wish it to have, we will use what is called a “rescue medicine.” The medicine that we will use is called DP (Dihydroartemisinin piperaquine). DP is also part of the national protocol for malaria.

b. Description of the Process

If you agree to participate in this study, you (your child) will randomly be assigned to one of the two treatment regimen. We will have a plastic bottle which contains

cards. Each card describes one of the two treatment regimen. You will select one of the cards, the selected card decides the treatment you (your child) will get. You will have to take these drugs every morning and evening until the full treatment course is finished. After today, you will need to visit to the clinic, additional 9 times in total within 42 days of study. Those are on day 3, 5, 6, 7, 14, 21, 28, 35 and 42. 3 ml of blood sample (1ml for your child) will be taken from your arm on day 3, 5, 6 and 7. Finger prick will be done for malaria blood film in other visits. A medical doctor will ask you (your child) for symptoms for malaria disease progress and drug adverse effects. A physical examination will be done in follow up visits. All follow up appointment dates are written on your patient card. This card will record important data and you should keep it carefully.

9. Duration

The total duration of the follow up is 42 days. You need to come to the clinic 10 times, today, and on day 3, 5, 6, 7, 14, 21, 28, 35 and 42. It is important that you (your child) must return to the clinic for the planned visits even if you are (your child is) not sick.

10. Side Effects

Artemether-lumefantrine is generally considered a very safe drug and reported AL side effects have generally been mild. You will be screened for adverse events using a checklist at each follow-up appointment. If serious (adverse) event occurs, the research medical doctor will take an immediate action and will inform to study principle investigator immediately.

11. Risks

By participating in this research it is possible that you will be at greater risk than you would otherwise be. There is, for example, a small risk of getting side effects from the medicines. While the possibility of this happening is very low, you should still be aware of the possibility. We will try to decrease the chances of this event occurring, but if something unexpected happens, we will provide you with proper health care services.

12. Discomforts

By participating in this research it is possible that you may experience some discomfort due to repeated follow ups, repeated finger pricks to check malaria parasite and venipuncture.

13. Benefits

The first obvious benefit you will get is curing your malaria disease. During the follow up, there will be an opportunity to you to check malaria infection and your general health status with a medical doctor. Any interim illnesses will be treated at no charge to you. If you or your children fall sick during this period he/she will be treated free of charge. There may not be any benefit for you but your participation is likely to help us find the answer to the research question. There may not be any benefit to the society at this stage of the research, but future generations are likely to benefit. When the research can find out the significant benefit of 5 day Coartem treatment, it may support to change the current treatment regime to more effective standard anti-malaria treatment regime. This can also improve the malaria situation in your area.

14. Confidentiality

Participants' personal and medical data will be kept confidential and made anonymous by ID instead of name throughout the research process. ID together with names and address are recorded only in one book, study registration book. It will be locked by clinic medical doctor. All the research members will keep all the data in the clinic confidential.

15. Incentives

We will give you 5000 Kyats per follow up visit for your travel cost and the compensation of your lost work time. You will not be given other money or gifts to take part in this research.

16. Sharing the Results

The knowledge that we get from doing this research will be shared with you and/or your local village leader before it is made widely available to the public. Individual's confidential information will not be shared. The results will be described in small community meetings in your areas. After these meetings, we will publish the results in order that other interested people may learn from our research.

17. Right to refuse or withdraw

You do not have to take part in this research if you do not wish to do so. Refusing to participate will not affect your treatment at health centre in any way. You will still have all the benefits that you would otherwise have at health centre. You may stop participating in the research at any time that you wish without losing any of your rights as a participant in our research.

18. Alternatives to participating

If you do not wish to take part in the research, you will receive the standard 3 day Coartem treatment. You will not be asked for follow up but you can come back any time when you are unwell or when you want to consult the medical doctor.

19. Who to Contact

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

Name: Dr. Kyaw Myo Tun

Address: No 32 (A) Kokkine Swimming Pool lane, Sayasan Road, Bahan, Yangon

Contact phone: 01-544537

Email: myo.kyaw.tun@gmail.com

This proposal has been reviewed and approved by the Institutional Ethical Review Committee, Department of Medical Research (Lower Myanmar) which is a committee whose task it is to make sure that research participants are protected from harm. If you wish to find about more about the Committee, contact the secretary of the committee at the Department of Medical Research (Lower Myanmar), No. 5, Ziwaka Road, Dagon PO, Yangon, phone 251508: ext. 150.

PART II: Certificate of Consent

I confirm that Dr._____ has explained me and I have been invited to participate in research of “Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria”. I understand that I will be receiving 3 or 5 day antimalarial drugs and will be taken blood samples from my arm as well as finger pricks to check malaria parasite during 42 day study period. I understand that it will involve taking blood samples for five times about half teaspoonful and eight time finger pricks. I understand that 15 -30 minutes will take for each follow up visit. I have been informed that the risks are minimal. I am aware of my personal benefit and benefit for my community. I have been provided with the name of a researcher who can be contacted using the number and address given.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care.

Name of Participant: _____

Signature of Participant: _____

Date: _____

Day/month/year

If illiterate

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness_____ AND Thumb print of participant

Signature of witness _____

Date _____

Day/month/year

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of Researcher: _____

Signature of Researcher: _____

Date: _____

Day/month/year

A copy of this Informed Consent Form has been provided to participant _____ (initialed by the researcher/assistant)

Institutional Ethical Review Committee

Department of Medical Research (Lower Myanmar)

Yangon, Myanmar

Informed Consent Form for Research Involving Children (Clinical studies)

This is for the parents of children between the ages of 6 and 16 years of age who attend clinic, and who we are asking to participate in research "Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar".

Name of Principal Investigator – Dr. Frank Smithuis

Name of Organization – Myanmar Oxford Clinical Research Unit (MOCRU)

Name of Funder – 3MDG

Name of proposal- Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar

PART I: Information Sheet

1. Introduction

My name is Dr. ……., and I am working for the Myanmar Oxford Clinical Research Unit. We are doing a research about the treatment of malaria. . I am going to give you information and invite you to have your child participate in this research. You do not have to decide today whether or not you agree that your child may participate in the research. Before you decide, you can talk to anyone you feel comfortable with. There may be some words that you do not understand. Please ask me to stop as we go through the information and I

will take time to explain. If you have questions later, you can ask them of me, the study doctor or the staff.

2. Purpose of the research

Malaria is a serious health problem in Mon or Kayin. The main treatment for malaria in Myanmar is called Coartem. This drug is usually given for 3 days; it is effective and has hardly any negative side effects. However, recently there has been malaria that is less sensitive for this treatment in the region. Treatment failures are still very low but we want to study if prolonging the treatment course by a further 2 days (total 5 days treatment) could further improve the treatment result.

We want to follow up patients for 42 days in order to find out if 5 days treatment has a better treatment result and we want to compare the frequency of side effects.

3. Type of research intervention

This research will involve 3 days or 5 days treatment as well as nine follow-up visits to this clinic over the coming 6 weeks. It will involve blood collection from your child arm 3 times, hlaf of the teaspoon of blood will collect at each time and you child will need to spend 15-30 minutes at each follow up visit.

4. Participant selection

We are inviting all patients with malaria parasites who attend this clinic to participate in this research. Now we have found malaria parasites in your child blood and your child need a treatment to cure it. Therefore we invite your child to participate in this study.

5. Voluntary Participation

Your child's participation to this study is completely voluntary.
It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this clinic will continue and nothing will change. You may change your mind later and stop participating even if you agreed earlier.

6. Information on the Drugs

The drug we are using in this research is called Coartem. It has been studied extensively, it has very few – if any – side effects and it is being used all over the world. It is currently the official choice of treatment by the National Malaria Control Program in Myanmar.

Coartem is generally considered a very safe drug and reported side effects are generally mild. Possible side effects are GI upset (anorexia, nausea, vomiting,

abdominal pain, and diarrhea), headache, dizziness, fatigue and sleep disturbance. Palpitations, myalgia, arthralgia and rash are reported very infrequently. All these complaints are common in malaria and could be disease related (and not related to the medicine).

A single dose of Primaquine (PQ) will also be given (once). It is part of the routine treatment of malaria in Myanmar. Primaquine can cause haemolysis in patients with a rare blood disease (G6PDd), which typically occurs in 2-15% of patients in endemic zones. However, as said, it is part of the normal treatment in Myanmar and severe bleeding has not been reported yet. Other possible side effects are nausea, vomiting, stomach upset, and abdominal cramps.

7. Procedures and protocol

a. Unfamiliar procedures

The study wants to find out if 5 days treatment with Coartem is better than the usual 3 day treatment. Therefore we need to compare these two treatments. To do this, we will put people taking part in this research into two groups. The groups are selected by chance. The participant needs to pick up a treatment card from a plastic box and will be treated accordingly.

Participants in one group will be given the 5 days treatment (twice a day, so 10 doses) while participants in the other group will be given the 3 days treatment (twice a day, so 6 doses) which is the treatment that is currently being used for malaria. We will then compare which of the two has the best results.

The healthcare workers will be looking after you and the other participants very carefully during the study. If we are concerned about what the drug is doing, we

will find out which treatment (3 or 5 days) you are getting and possibly make changes. If there is anything you are concerned about or that is bothering you about the research please talk to me or one of the other researchers

If we find that the medicine that is being used does not have the desired effect, or not to the extent that we wish it to have, we will use what is called a “rescue medicine.” The medicine that we will use is called DP (Dihydroartemisinin piperaquine). DP is also part of the national protocol for malaria.

b. Description of the Process

If you agree to participate in this study, your child will randomly be assigned to one of the two treatment regimen. We will have a plastic bottle which contains cards. Each card describes one of the two treatment regimen. You will select one of the cards, the selected card decides the treatment your child will get. You will have to take these drugs every morning and evening until the full treatment course is finished. After today, you will need to visit to the clinic, additional 9 times in total within 42 days of study. Those are on day 3, 5, 6, 7, 14, 21, 28, 35 and 42. About one-fourth of teaspoonful of blood will be taken from your child’s arm on day 3, 5 and 7. Finger prick will be done for malaria blood film in other visits. A medical doctor will ask your child for symptoms for malaria disease progress and drug adverse effects. A physical examination will be done in follow up visits. All follow up appointment dates are written on your child’s patient card. This card will record important data and you should keep it carefully.

9. Duration

The total duration of the follow up is 42 days. Your child need to come to the clinic 10 times, today, and on day 3, 5, 6, 7, 14, 21, 28, 35 and 42. It is important

that your child must return to the clinic for the planned visits even if your child is not sick.

10. Side Effects

Artemether-lumefantrine is generally considered a very safe drug and reported AL side effects have generally been mild. Your child will be screened for adverse events using a checklist at each follow-up appointment. If serious (adverse) event occurs, the research medical doctor will take an immediate action and will inform to study principle investigator immediately.

11. Risks

By participating in this research it is possible that your child will be at greater risk than he/she would otherwise be. There is, for example, a small risk of getting side effects from the medicines. While the possibility of this happening is very low, your child should still be aware of the possibility. We will try to decrease the chances of this event occurring, but if something unexpected happens, we will provide your child with proper health care services.

12. Discomforts

By participating in this research it is possible that your child may experience some discomfort due to repeated follow ups, repeated finger pricks to check malaria parasite and venipuncture.

13. Benefits

The first obvious benefit your child will get is curing his/her malaria disease. During the follow up, there will be an opportunity to your child to check malaria

infection and his/her general health status with a medical doctor. Any interim illnesses will be treated at no charge to you. If your children fall sick during this period he/she will be treated free of charge. There may not be any benefit for your child but his/her participation is likely to help us find the answer to the research question. There may not be any benefit to the society at this stage of the research, but future generations are likely to benefit. When the research can find out the significant benefit of 5 day Coartem treatment, it may support to change the current treatment regime to more effective standard anti-malaria treatment regime. This can also improve the malaria situation in your area.

14. Confidentiality

Participants' personal and medical data will be kept confidential and made anonymous by ID instead of name throughout the research process. ID together with names and address are recorded only in one book, study registration book. It will be locked by clinic medical doctor. All the research members will keep all the data in the clinic confidential.

15. Incentives

We will give you 5000 Kyats per follow up visit for your travel cost and the compensation of your lost work time. You will not be given other money or gifts to take part in this research.

16. Sharing the Results

The knowledge that we get from doing this research will be shared with you and/or your local village leader before it is made widely available to the public. Individual's confidential information will not be shared. The results will be described in small community meetings in your areas. After these meetings, we will publish the results in order that other interested people may learn from our research.

17. Right to refuse or withdraw

Your child do not have to take part in this research if you do not wish to do so. Refusing to participate will not affect your treatment at health centre in any way. Your child will still have all the benefits that you would otherwise have at health centre. Your child may stop participating in the research at any time that you wish without losing any of your rights as a participant in our research.

18. Alternatives to participating

If your child do not wish to take part in the research, he/she will receive the standard 3 day Coartem treatment. Your child will not be asked for follow up but he/she can come back any time when your child is unwell or when you want to consult the medical doctor.

19. Who to Contact

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

Name: Dr. Kyaw Myo Tun

Address: No 32 (A) Kokkine Swimming Pool lane, Sayasan Road, Bahan, Yangon

Contact phone: 01-544537

Email: myo.kyaw.tun@gmail.com

This proposal has been reviewed and approved by the Institutional Ethical Review Committee, Department of Medical Research (Lower Myanmar) which is a committee whose task it is to make sure that research participants are protected from harm. If you wish to find about more about the Committee, contact the secretary of the committee at the Department of Medical Research (Lower Myanmar), No. 5, Ziwaka Road, Dagon PO, Yangon, phone 251508: ext. 150.

PART II: Certificate of Consent

I confirm that Dr._____ has explained me and my child has been invited to participate in research of “Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria”. I understand that my child will be receiving 3 or 5 day antimalarial drugs and will be taken blood samples from his/her arm as well as finger pricks to check malaria parasite during 42 day study period. I understand that it will involve taking blood samples for three times about half teaspoonful and eight time finger pricks. I have been informed that the risks are minimal. I am aware of my personal benefit and benefit for my community. I have been provided with the name of a researcher who can be contacted using the number and address given.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate my child as a participant in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my chlid medical care.

Name of participant’ parents: _____

Signature of one of participant’s parents: _____

Date: _____

Day/month/year

If illiterate

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness_____ AND Thumb print of one of participant's parents

Signature of witness _____

Date _____

Day/month/year

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of Researcher: _____

Signature of Researcher: _____

Date: _____

Day/month/year

A copy of this Informed Consent Form has been provided to participant _____ (initialed by the researcher/assistant)

14.2 Informed consent form (Myanmar translation)

ကျင့်ဝတ်ဆိုင်ရာကော်မတီ

ဆေးသုတေသနဦးစီးဌာန(အောက်မြန်မာပြည်)

ရန်ကုန် ၊ မြန်မာ

ဆေးဘက်ဆိုင်ရာ သုတေသန အသိပေးသဘောတူညီမှုပုံစံ

ဤအသိပေးသဘောတူညီမှုပုံစံသည် အောက်ဖော်ပြပါ ငှက်ဖျားသုတေသနတွင် ပါဝင်မည့် အသက် (၁၆)နှစ်အထက် ကျား၊မ လူကြီးများအတွက်ဖြစ်သည်။

သုတေသနအမည်- မြန်မာပြည်၏သာမာန်ငှက်ဖျားရောဂါကုသမှုတွင် အာတီမီသာ လူမီဖန်ထရင်း ၅ရက် ဆေးကုထုံး၏ အကျိုးအာနိသင်ထိရောက်မှုနှင့် ဘေးကင်းမှု

အဓိကသုတေသီအမည်- ဒေါက်တာ ဖရန့် စမစ်သီးယပ်(စ်)

သုတေသနအဖွဲ့အစည်း- မြန်မာအောက်စ်ဖို့ သုတေသနဌာန

ငွေကြေးထောက်ပံ့သည့်အဖွဲ့အစည်း - ၃ အမ်ဒီဂျီ

အပိုင်း (က) - သုတေသနအကြောင်းအရာ

၁။ နိဒါန်း

ကျွန်တော်၏အမည်မှာ ဒေါက်တာ ..... ဖြစ်ပြီး မြန်မာ အောက်စ်ဖို သုတေသနဌာနတွင်တာဝန် ထမ်းဆောင်နေပါသည်။ ကျွန်တော့်အနေနှင့်

ဌက်ဖျားရောဂါ ကုသမှုနှင့်ပါတ်သက်သော သုတေသနတစ်ခုပြုလုပ်ပါမည်။ ဤသုတေသန အကြောင်းကို ရှင်းပြပြီးနောက် သင့်အား သုတေသနတွင် ပါဝင်ရန်ဖိတ်ခေါ်ပါသည်။ သင့်အနေဖြင့် မရှင်းလင်းသည့်အချက်များ၊ ဆေး၏ဘေးထွက်အာနိသင်များကို ရှင်းလင်းချိန် အတွင်း ကြားဖြတ်မေးမြန်းနိုင်ပါသည်။ သို့မဟုတ် အားလုံးရှင်းလင်းပြီးနောက် နားမလည်တဲ့အချက်များရှိပါက ကျွန်တော့်ကိုသော်လည်းကောင်း၊ သုတေသန အဖွဲ့မှ အခြားသူများကိုသော် လည်းကောင်း မေးမြန်းနိုင်ပါသည်။ သုတေသနတွင်ပါဝင်ရန် ယနေ့မဆုံး ဖြတ်နိုင်သေးပါက နောက်အချိန်တစ်ခုတွင် ဆုံးဖြတ်နိုင်ပါသည်။ သင်နှစ်သက်ရာ လူတစ်ဦးတစ်ယောက် နှင့်လည်း တိုင်ပင်နိုင်ပါသည်။

၂။ သုတေသန၏ရည်ရွယ်ချက်

ဌက်ဖျားရောဂါဟာ မွန်၊ ကရင် ဒေသတွင် အရေးကြီးသော ကျန်းမာရေး ပြဿနာတစ်ခုဖြစ် ပါသည်။ မြန်မာနိုင်ငံ၏ အဓိကဌက်ဖျားဆေးမှာ **ကိုအာတမ်** ဖြစ်ပါသည်။ ထိုဆေးကို သုံးရက်သောက် ရပါသည်။ ယင်းတွင်သိသာထင်ရှားသော ဘေးထွက်ဆိုးကျိုးပြဿနာမရှိပါ။ သို့ရာတွင် ယင်းဆေးကို မတိုးသော ဌက်ဖျားရောဂါများ ပေါ်ပေါက်ခဲ့ပါသည်။ ယင်းဆေးဖြင့်ဆေးကုသမှု မအောင်မြင်မှု နှုန်းမှာများစွာ မရှိသေးသော်လည်း ကျွန်တော်တို့ အနေဖြင့် ဤဆေးကိုနောက်ထပ်နှစ်ရက် ပိုတိုး သောက်ခြင်းဖြင့် ပိုမိုထိ ရောက်သော ဆေးကုသမှု ရနိုင်ခြင်းရှိ/မရှိ သုတေသနလုပ် လိုပါသည်။ ၅ ရက်ကုထုံးအောင်မြင်မှုနှင့် ယင်း၏ ကောင်းကျိုးဆိုးကျိုးများ သုတေသန ပြုရန် ၄၂ ရက်တာ ကာလအထိ စောင့်ကြည့်လေ့လာသွားပါမည်။

၃။ သုတေသနလုပ်ငန်းအဆင့်ဆင့်ဆောင်ရွက်ပုံ

ဤသုတေသနတွင် ၅ ရက် (သို့) ၃ ရက် **ကိုအာတမ်** ၄က်ဖျားဆေးကုထုံးနှင့်အတူ ၄၂ ရက် အတွင်း (၉)ကြိမ် ခေါ်ယူစစ်ဆေးမှုတို့ပါဝင်ပါသည်။ တစ်ကြိမ်လာရောက်လျှင် ၁၅ မိနစ်မှ မိနစ် ၃၀ ခန့် ကြာမြင့် နိုင်ပါသည် ။

၄။ ပါဝင်မည့်သူများရွေးချယ်ခြင်း

ဤဆေးခန်းသို့လာရောက် သွေးစစ်ပြီးသွေးထဲတွင် ၄က်ဖျားပိုး တွေ့ရှိထားသူများ အားလုံးကို ဤသုတေသနတွင်ပါဝင်ရန် ဖိတ်ခေါ်ပါသည်။ သင့်တွင် ၄က်ဖျားပိုးတွေ့ရှိနေသည့်အတွက်ကြောင့်ဆေး သောက်ရန်လိုအပ်ပါသည်။

၅။ မိမိဆန္ဒအလျောက်ပါဝင်ခြင်း

ဤသုတေသနတွင်ပါဝင်ခြင်းသည် သင်၏သဘောဆန္ဒ အလျောက်သာ ဖြစ်ပါသည်။ သင့်အ နေဖြင့် ပါဝင်သည်ဖြစ်စေ မပါဝင်သည်ဖြစ်စေ သင်၏၄က်ဖျားရောဂါ အတွက် ဆေးဝါးကုသမှုကို ရရှိ မည်ဖြစ်ပါသည်။ ပထမပိုင်းတွင်သဘောတူညီပြီး နောင်တွင်မပါဝင်လိုတော့ပါကအချိန်မရွေး ရပ်ဆိုင်း နိုင်ပါသည်။

၆။ အသုံးပြုမည့်ဆေးဝါးများအကြောင်း

ဤသုတေသနတွင် အသုံးပြုမည့်ဆေးအမည်မှာ **ကိုအာတမ်**၄က်ဖျားဆေး ဖြစ်ပါသည်။ ယင်းကိုကမ္ဘာ တဝှမ်းသုံးစွဲနေပြီး သိသာထင်ရှားသော ဘေးထွက်ဆိုးကျိုး အာနိသင်မရှိပါ။ ဤဆေးကိုမြန် မာနိုင်ငံ ၄က်ဖျား တိုက်ဖျက်ရေးအဖွဲ့မှ ယခုလက်ရှိ အသိအမှတ်ပြု သုံးစွဲနေပါသည်။

ဤဆေးသည် ဘေးကင်းမှု ရှိပြီး ဘေးထွက်အာနိသင်များသည် ယေဘူယျ အားဖြင့် နည်းပါး ပြီး မပြင်းထန်ပါ။ အဓိကဘေးထွက်ဆိုးကျိုးများမှာ အစားပျက်ခြင်း၊ ပျို့ခြင်း၊

အန်ခြင်း၊ ဗိုက်နာခြင်း၊ ဝမ်းသွားခြင်းတို့ဖြစ်နိုင်ပါသည်။ တခြားဆိုးကျိုးများမှာ ခေါင်းမူးခြင်း၊ ကိုက်ခြင်း၊ မောပန်းခြင်းနှင့် အိပ်မ ပျော်ခြင်းတို့ဖြစ်ပါသည်။ နှလုံးတုန်ခြင်း၊ ကြွက်သားများကိုက်ခဲခြင်း၊ အဆစ်အမြစ်ကိုက်ခြင်း၊ အင်ပျင် ထခြင်းတို့ ရံဖန်ရံခါရှိနိုင်သော်လည်းယင်းတို့မှာ ဌက်ဖျားရောဂါ၏ လက္ခဏာများလည်းဖြစ်နိုင်ပါသည်။

ကိုအာတမ်ဌက်ဖျားဆေး နှင့်အတူ **ပရိုင်မာကွင်း**ဌက်ဖျားဆေးကို ပထမဆုံး ဆေးတိုက်ချိန်တွင် တစ်ကြိမ်ထည့်သွင်းတိုက်ကျွေးပါမည်။ ယင်းသို့ပေးခြင်းမှာ မြန်မာနိုင်ငံ၏ ဌက်ဖျား တိုက်ဖျက်ရေးစံကုထုံးတွင်ပါ ဝင်ပါသည်။ **ပရိုင်မာကွင်း**ဌက်ဖျားဆေးသည် အချို့သောသွေးရောဂါရှိသူများ တွင် သွေးနီဥပျက်စီးမှုဖြစ်စေနိုင်သော်လည်း ဖြစ်နိုင်ချေမှာ လူတစ်ရာလျှင် ၂ ယောက်မှ ၁၅ ယောက် အထိဖြစ်နိုင်ချေရှိပါသည်။ အခြားဖြစ်နိုင်သော ဘေးထွက်ဆိုးကျိုးများမှာ အစားပျက်ခြင်း၊ ပျို့ခြင်း၊ အန်ခြင်း၊ ဗိုက်နာခြင်းတို့ဖြစ်နိုင် ပါသည်။

၇။ လုပ်ငန်းအဆင့်ဆင့်

(က) ဤသုတေသနတွင် ဌက်ဖျားကုသဆေးကို ၅ ရက် သောက်သုံးခြင်းသည် ၃ ရက် သောက် သုံးခြင်းထက်ပိုမို ထိရောက်ကြောင်းစမ်းသပ်ရန်ဖြစ်ပါသည်။ ထို့ကြောင့် ကျွန်တော်တို့သည် ဆေးကုသမှုကို အုပ်စု ၂ စုခွဲရန် လိုအပ်ပါသည်။ ကျဘန်းရွေးချယ်မှုဖြင့် အုပ်စုခွဲပါမည်။ သုတေသနတွင်ပါဝင်သူတို့သည် ပလပ်စတစ်သေတ္တာထဲမှ ကဒ်တစ်ခုကို နှိုက်ယူရပါမည်။ သုတေသနတွင်ပါဝင်ပါက ဌက်ဖျားဆေးကို ၃ ရက် သို့မဟုတ် ၅ ရက်ခန့် သောက်သုံးရမည်ဖြစ်ပါသည်။ ဆေး ၅ ရက် သောက်ရမည့်အုပ်စုသည် တစ်နေ့ နှစ်ကြိမ် စုစုပေါင်း ၁၀ ကြိမ်၊ ၃ ရက်အုပ်စုသည် တစ်နေ့နှစ်ကြိမ် စုစုပေါင်း ၆ ကြိမ် သောက်ရန် လိုပါသည်။ အမျိုးသမီးလူနာဖြစ်ခဲ့ပါက ကိုယ်ဝန်ရှိမရှိကိုစမ်းသပ်စစ်ဆေးပြီး ကိုယ်ဝန်မရှိမှ သာ သုတေသနတွင် ဆက် လက်ပါဝင်နိုင်ပါသည်။ ကျန်းမာရေး ဝန်ထမ်းများ

သည် သုတေသနတွင် ပါဝင်သူ များကို အထူးစောင့် ကြည့်နေပါမည်။ လိုအပ်ပါက ကုထုံး ပြုပြင် ပြောင်းလဲမှုများ ကို ပြုလုပ်ပါမည်။ သင့်တွင်လည်း တစ်စုံတစ်ရာ အနှောက် အယှက် ရှိပါက သုတေသနအဖွဲ့ဝင်များကို ပြောပြနိုင်ပါ သည်။ လက်ရှိကုထုံးသည် မထိရောက် ဟုယူဆခဲ့ပါက သင့်လျော်သောအခြား ဌက်ဖျားကုသဆေးဖြင့် ပြောင်းလဲ ကုသပေးပါမည်။ ဤဆေးသည် လည်း မြန်မာနိုင်ငံ ၏ စံဌက်ဖျား ကုထုံး တွင်ပါဝင်ပါ သည်။

(ခ) သုတေသနတွင် ပါဝင်ရန် ဆုံးဖြတ်ပြီးပါက သင့်ကို ကုထုံး နှစ်မျိုးအနက် မှတစ်မျိုး ဖြင့်ကုသ ပါမည်။ ပလပ်စတစ်သေတ္တာထဲမှ ကဒ်တစ်ခုကို နှိုက်ယူရမည်ဖြစ်ပြီး ကဒ်ပေါ်တွင်ရေးထားသည့် ကုထုံး အတိုင်း ဆေးကုသမှုခံယူရပါမည်။ တစ်နေ့နှစ်ကြိမ် မနက်၊ ည သင်၏ဆေးမှတ်တမ်းကဒ်ပါ အချိန်ဇယား အတိုင်း သောက်ရပါမည်။ သုတေသန ၄၂ ရက်အတွင်း နောက်ထပ် ၉ ကြိမ်ဆေးခန်းသို့ လာရန် လိုပါသည်။ လာရမည့်ရက်များမှာ ၃၊၅၊၆၊၇၊၁၄၊၂၁၊၂၈၊၃၅ နှင့် ၄၂ ရက်များတွင်ဖြစ်ပါသည်။ ၃၊ ၅၊ ၆ ရက် နှင့် ၇ ရက်မြောက်နေ့များတွင် လက်ဘက်ရည်ဖွန်း တစ်ဝက်ပမာဏရှိ သွေးနမူနာကို သင့်၏ လက်မောင်းမှ ရယူပါမည်။ တခြားသောဆေးခန်းလာရက်များတွင် သင့်ကိုလက်ချောင်းထိပ်မှ ဌက်ဖျားပိုးသွေးဖောက် စမ်းသပ်ခြင်းကို တစ်ကြိမ်စီပြုလုပ်ပါမည်။ ဆရာဝန်မှ စမ်းသပ်စစ်ဆေးမှုပြုလုပ်ပြီး ဌက်ဖျားရောဂါ လက္ခဏာနှင့် ဆေး၏ဘေးထွက် လက္ခဏာများ ရှိ/မရှိကို မေးခွန်းများ မေးပါမည်။ သင်လာရောက်ရ မည့်ရက်များကို သင်၏ လူနာမှတ်တမ်းကဒ်တွင် အသင့်ရေးထားပေးပါမည်။ ဤမှတ်တမ်းကဒ်သည် သင့်အတွက် အရေးကြီး ပါသဖြင့် စနစ်တကျ သိမ်းထားရန်လိုပါသည်။

၈။ သုတေသနကာလ

စုစုပေါင်းကြာမြင့်မည့်ကာလမှာ ဆေးသောက်ပြီး ၄၂ ရက် ဖြစ်ပါသည်။ နေကောင်းသည် ဖြစ်စေ နေမကောင်းသည်ဖြစ်စေ ဆေးခန်းသို့စုစုပေါင်း ၁၀ ကြိမ်အနည်းဆုံး လာရောက်ရမည်ဖြစ်ပြီး ကြားကာလအတွင်း နေမကောင်းပါက ဆေးခန်းသို့အချိန်မရွေး လာရောက်တိုင်ပင်ကုသနိုင်ပါသည်။

၉။ ဆေး၏ဘေးထွက်ဆိုးကျိုးအာနိသင်များ

ကိုအာတမ် ဌက်ဖျားဆေးသည် ဘေးကင်းသောဆေးဖြစ်ပြီး အစီရင်ခံစာများအရ ဘေးထွက် အာနိသင်များမှာ ယေဘုယျအားဖြင့် မပြင်းထန်ပါ။ အကယ်၍ ပြင်းထန်သော အရေးကြီးသော ဘေးထွက်ဆိုး ကျိုးများပေါ်ခဲ့ပါက ဆေးကုသသည့် ဆရာဝန်မှချက်ချင်းတုန့်ပြန်မှုပြုပြီး အဓိကသုတေသီထံအကြောင်း ကြားပါမည်။

၁၀။ ဖြစ်ပေါ်နိုင်သောဆိုးကျိုးများ

ဤသုတေသနတွင်ပါဝင်ခြင်းဖြင့် ဆိုးကျိုးတစ်ချို့ဖြစ်ပေါ်နိုင်ပါသည်။ ဥပမာ - ဆေးဝါးတစ် ချို့၏ ဘေးထွက်အာနိသင်များရရှိနိုင်ပါသည် ။ ဤသို့ဆိုးကျိုးများမှာ ဖြစ်နိုင်ချေနည်းပါးလှသော်လည်း သင့် အနေဖြင့်သိရှိထားရန်လိုအပ်ပါသည်။ ကျွန်တော်တို့သည် ဤသို့ဆိုးကျိုးများကို နည်းနိုင်အောင်ကြိုးစား ရပါမည်။ မမျှော်လင့်သော ဆိုးကျိုးများပေါ်ပေါက်ပါက ကျွန်တော်တို့ဖက်မှ သင့်တော်သော ကျန်းမာရေး စောင့်ရှောက်မှုများကို ပေးပါမည်။

၁၁။ ကိုယ်စိတ်အနှောင့်အယှက်

ဤသုတေသနတွင်ပါဝင်ခြင်းဖြင့်သင့်တွင် ကိုယ်စိတ်အနှောင့်အယှက်များ ရှိနိုင်ပါသည်။ ဥပမာ အကြိမ်များစွာ ဆေးခန်းသို့လာရောက်ရန်လိုအပ်ခြင်း၊ လက်ထိပ် သွေးဖောက်ခံရခြင်း၊ လက်မောင်း သွေးဖောက်ခံရခြင်းစသည်တို့ ဖြစ်ပါသည် ။

၁၂။ အကျိုးကျေးဇူး

အသိသာဆုံးအကျိုးကျေးဇူးမှာ သင်၏ဝှက်ဖျားရောဂါကို ကုသနိုင်ခြင်း ဖြစ်ပါသည်။ ဆေးခန်း သို့ အကြိမ်များစွာ လာရောက်ပြီး သွေးစစ်နိုင်မှုကြောင့် သင်၏ ဝှက်ဖျားရောဂါကို စဉ်ဆက်မပြတ် စစ်ဆေး နိုင်ပါသည်။ ထို့အပြင်တခြားသင်၏ အထွေထွေကျန်းမာရေး အခြေအနေကိုလည်း ဆရာဝန်နှင့် အခမဲ့စစ် ဆေးခွင့်ရရှိပါမည်။ အကယ်၍သုတေသနမှ **ကိုအာတမ်** ဝှက်ဖျားဆေး (၅) ရက်ကုထုံး၏ အကျိုးကျေးဇူး များကို ဖော်ထုတ်နိုင်ပါက နိုင်ငံတဝန်းပိုမိုထိရောက်သော ကုထုံး ပေါ်ထွက်ရေးတွင် အထောက်ကူပြုနိုင် ပါသည်။

၁၃။ လျှို့ဝှက်ထားရှိခြင်း

သုတေသနတွင်ပါဝင်သူများ၏အမည်နာမကို မသုံးဘဲ ကုဒ် နံပါတ်ကိုသာသုံး၍ ပုဂ္ဂိုလ်ရေး ရာနှင့် ဆေးဘက်ဆိုင်ရာအချက်အလက်များကို လျှို့ဝှက်ထားရှိပါမည်။ ကုဒ်နှင့် အမည်ကို မှတ်တမ်းစာအုပ် တစ်ခုတွင်သာတွဲမှတ်ထားပြီး ယင်းကိုဆရာဝန်မှ သေချာစွာသော့ခတ်ထားရှိပါမည်။ သုတေသနအဖွဲ့ဝင် အားလုံးသည် ဆေးခန်းတွင်းရှိ မှတ်တမ်းအားလုံးကို လျှို့ဝှက်ထားရှိရမည် ဖြစ်ပါသည်။

၁၄။ သုတေသနပါဝင်မှု အတွက် ထောက်ပံ့ကြေး

ဆေးခန်းသို့တစ်ကြိမ်လာတိုင်း သင်၏ ခရီးစရိတ်နှင့်အလုပ်ချိန် ဆုံးရှုံးမှုအတွက် ကျပ်ငွေ ၅၀၀၀ ထောက်ပံ့ပေး ပါမည်။

၁၅။ သုတေသနရလဒ်များကိုမျှဝေခြင်း

သုတေသနရလဒ်များကို လူထုတွင်းမဖြန့်ဝေမီ ဤဒေသမှ သင်နှင့်သင်၏ ရွာလူကြီးများကို ဦးစွာသိနှင့်စေမည်ဖြစ်ပါသည်။ လူတစ်ဦးချင်းစီ၏ လျှို့ဝှက်ချက်များကိုမျှဝေမည် မဟုတ်ပါ။ ဤရလဒ်များကို သင်၏ဒေသလူထုအစည်းဝေးများအတွင်း ရှင်းလင်းတင်ပြမည်ဖြစ်ပါသည်။ ထိုလူထုအစည်းအဝေးပြီးမှသာ ဤရလဒ်များကို ပုံနှိပ် ထုတ်ဝေမည်ဖြစ်ပါသည်။

၁၆။ သုတေသနမှနှုတ်ထွက်ပိုင်ခွင့်၊ ငြင်းပိုင်ခွင့်

သင့်အနေဖြင့်ပါဝင်ရန် ဆန္ဒမရှိတော့ပါက ငြင်းဆိုနိုင်ပါသည်။ ဤသိုငြင်းဆိုမှုသည် သင်၏ ဆေးကုသမှုကို သက်ရောက်မည်မဟုတ်ပါ။ သင်ရမည့် ကျန်းမာရေးစောင့်ရှောက် ခံရမှုအခွင့်အရေးကို ရရှိရ ပါမည်။ သင့်အနေဖြင့် သုတေသနတွင်ပါဝင်ခြင်းကို သင့်ဆန္ဒဖြင့် အချိန်မရွေး ရပ်ဆိုင်းနိုင်ပါသည်။

၁၇။ သုတေသနမှ နှုတ်ထွက်ခဲ့ပါက (ပါဝင်ရန်ငြင်းဆိုခဲ့ပါက)

သင့်အနေဖြင့် သုံးရက် **ကိုအာတမ်** ၄ က်ဖျားဆေးကုထုံးကိုရရှိပါမည်။ သင့်ကိုပြန်လာရမည့် ရက်ချိန်းများ ထားရှိမည်မဟုတ်ပါ။ သို့ရာတွင်သင်နေမကောင်းဖြစ်ပါက၊ သို့မဟုတ် ဆရာဝန်နှင့်တိုင်ပင် လိုပါက၊ အချိန်မရွေးဆေးခန်းသို့ လာရောက်နိုင်ပါသည်။

၁၈။ ဆက်သွယ်ရန်

သင့်တွင်မေးစရာမေးခွန်းများရှိပါက ယခု သို့မဟုတ် သုတေသနစပြီး နောက်မှဖြစ်စေ မေးမြန်းနိုင်ပါသည်။ အောက်လိပ်စာသို့ဆက်သွယ်ပြီးလည်း အသေးစိတ် မေးမြန်းနိုင်ပါသည်။

(က) ဆေးခန်း ရှိ သုတေသနဆရာဝန်

ဒေါက်တာ.....

(ခ) မြန်မာအောက်စ်ဖို့ သုတေသနဌာန ရုံးချုပ်၊ရန်ကုန်။

ဒေါက်တာကျော်မျိုးထွန်း

လိပ်စာ။ ၃၂ (A) ကုက္ကိုင်းရေကူးကန်လမ်း၊ ဆရာစံလမ်း ၊ ဗဟန်း၊ ရန်ကုန်။

ဖုန်း။ ၀၁ - ၅၄၄၅၃၇

ဤသုတေသန အဆိုပြုလွှာကို ဆေးသုတေသနဦးစီးဌာန(အောက်မြန်မာပြည်) ကျင့်ဝတ်ဆိုင်ရာကော်မတီမှ စစ်ဆေးသုံးသပ်ကာ အတည်ပြုချက်ရယူပြီးဖြစ်ပါသည် ။ ယင်းကော်မတီ၏တာဝန်မှာ သုတေသနတွင် ပါဝင်သူများကို ထိခိုက်နစ်နာမှုမှ ကာကွယ်ပေးရန်ဖြစ်ပါသည် ။ ကော်မတီအကြောင်းပိုမိုသိရှိလိုပါက " **အတွင်းရေးမှူး ဆေးသုတေသနဦးစီးဌာန(အောက်မြန်မာပြည်) အမှတ် (၅) ၊ ဇီဝကလမ်း ၊ဒဂုံမြို့နယ် ၊ ရန်ကုန်မြို့ ၊ ဖုန်း ၂၅၁၅၀၈ လိုင်းခွဲ ၁၅၀** " သို့ ဆက်သွယ်မေးမြန်းနိုင်ပါသည် ။

အပိုင်း (ခ) သုတေသနတွင်ပါဝင်ရန် သဘောတူညီမှု

ကျွန်ုပ်အား "မြန်မာပြည်တွင်း သာမန်ငှက်ဖျားရောဂါကုသမှုတွင် အာတီမီသာလူမီဖန်ထရင်း ၅ ရက်ကုထုံး၏အကျိုးအာနိသင်ထိရောက်မှုနှင့် ဘေးကင်းမှု" သုတေသနတွင်ပါဝင်ရန် ဖိတ်ခေါ်ထား ပါသည်။ ကျွန်ုပ်အနေနှင့်ငှက်ဖျားဆေးကို ၃ ရက် သို့မဟုတ် ၅ ရက်သောက်ရန်လိုပြီး သုတေသနကာလ ၄၂ ရက်အတွင်း လက်မောင်းမှသွေးကို လက်ဘက်ရည်ဇွန်းတစ်ဝက်ခန် . ၅ ကြိမ်နှင့် လက်ထိပ်များမှ သွေးဖောက်စစ်ဆေးခြင်းကို ၈ ကြိမ်ခန် .ပြုလုပ်ရမည်ကိုသိရှိထားပါသည်။ ဖြစ်နိုင်သော အန္တရယ်မှာ နည်းပါးကြောင်းသိရှိထားပါသည်။ဆေးခန်းသို့ တစ်ကြိမ်လာရောက်တိုင်း ၁၅ - ၃၀ မိနစ် အထိကြာမြင့် မည်ဖြစ်ကြောင်းကိုလည်း သိရှိထားပါသည်။ ကျွန်ုပ်၏အကျိုးလူထုအတွက်အကျိုးများကို သိရှိပြီးဖြစ် ပါသည်။ သုတေသီ၏အမည် ဖုန်းနှင့် ဆက်သွယ်ရန် လိပ်စာတို့ကို ရရှိပြီးဖြစ်ပါသည်။

ကျွန်ုပ် အနေဖြင့် ဒေါက်တာ ..... မှ သုတေသန ၏ ရည်ရွယ်ချက် နှင့် လုပ်ဆောင်မှု အဆင့်ဆင့်ကို ရှင်းပြပြီး ဖြစ်ကြောင်း အတည်ပြုပါသည်။

သုတေသန၏အချက်အလက်များကို ကျွန်ုပ်ကိုယ်တိုင်ဖတ်ရှု / ကျွန်ုပ်အားဖတ်ပြပြီး ကျွန်ုပ် အနေဖြင့် မေးခွန်းများမေးခွင့်ရှိခဲ့ပြီး ကျေနပ်ဖွယ်ရာအဖြေများကို ရခဲ့ပြီးဖြစ်ပါသည်။ ကျွန်ုပ်၏သဘော ဆန္ဒအလျောက် ဤသုတေသနတွင်ပါဝင်ရန်သဘောတူညီပါသည်။ ထို့အပြင်ကျွန်ုပ်ရရှိမည့် ကျန်းမာရေး စောင့်ရှောက်မှုကိုမထိခိုက်စေဘဲ ဤသုတေသနတွင်ပါဝင်ခြင်းမှ အချိန်မရွေးနှုတ် ထွက်ခွင့်ရှိကြောင်းကိုလည်း နားလည်ထားပါသည်။

အကယ်၍စာမတတ်ခဲ့ပါက သဘောတူခြင်းကို စာတတ်သည့်သက်သေမှ လက်မှတ်ထိုးရမည် ဖြစ်ပါသည်။ (သက်သေကို ကာယကံရှင်က ရွေးချယ်ရမည်ဖြစ်ပြီး သက်သေ သည် သုတေသနအဖွဲ့နှင့် မသက် ဆိုင်သော ဆက်သွယ်မှုမရှိသောသူဖြစ်ရပါမည်။) စာမတတ်သော၊ သုတေသနတွင်ပါဝင်သူ၏ လက်မလက် ဗွေရာကို နှိပ်ယူရပါမည်။

ကျွန်ုပ်သက်သေအနေဖြင့် သုတေသန အချက်အလက်များကို သေချာစွာဖတ်ရှုပြီး မေးခွန်းများမေးခြင်း အခွင့်အလမ်း ကိုလည်းနားလည်ပြီးဖြစ်ပါသည်။ သုတေသနတွင် ပါဝင်သူသည် ယင်း၏ဆန္ဒအလျောက်ပါ ဝင်ခြင်းဖြစ်ကြောင်း အတည်ပြုပါသည်။

သက်သေအမည် ..... သုတေသနတွင်

သက်သေလက်မှတ် ..... ပါဝင်သူ၏လက်ဗွေရာ

ရက်စွဲ .....

ကျွန်ုပ်အနေဖြင့် သုတေသနအကြောင်းအရာများကို တိကျစွာဖတ်ပြခြင်း (သို့မဟုတ်)ပါဝင်သူမှ တိကျစွာ ဖတ်ရှုခြင်း၊ ပါဝင်သူမှ မေးခွန်းမေးမြန်းခွင့်ရှိခြင်း ၊ မေးခွန်းမေးမြန်းခြင်းများကိုသက်သေအတည်ပြုပါသည်။ သုတေသနတွင်ပါဝင်ရန်ယင်း၏ ဆန္ဒအလျောက် သဘောတူကြောင်းကိုလည်း အတည်ပြု ပါသည်။

သုတေသနပြုလုပ်သူ၏အမည် .....

လက်မှတ် .....

ရက်စွဲ .....

ဤအသိပေးသဘောတူညီမှုပုံစံမိတ္တူကို သုတေသနတွင်ပါဝင်သူအားပေးအပ်ရန်

14.3 Drugs and dosages

Artemether-lumefantrine (Coartem®, Novartis, Switzerland). One tablet contains 20mg artemether and 120mg lumefantrine. The standard regimen is twice daily for 3 days with a delay of at least 8 hours between the first and second dose. It is dosed by weight categories. Participants will be advised to take the drug with milk / food.

One tablet of AL contains 20mg artemether and 120mg lumefantrine.

	No. of tablets recommended at approximate timing of dosing[a]					
Body weight in kg (age in years)	0 h	8 h	24 h	36 h	48 h	60 h
5–14 (<3)	1	1	1	1	1	1
15–24 (3–9)	2	2	2	2	2	2
25–34 (9–14)	3	3	3	3	3	3
>34 (>14)	4	4	4	4	4	4

Patients receiving the 5 day course will continue the same daily dose for 2 additional days.

14.4 Ethical Approval (OXTREC)

Oxford Tropical Research Ethics Committee

University of Oxford
Joint Research Office, Block 60
Churchill Hospital, Oxford OX3 7LE
Tel. +44 (0) 1865 (5)72346, fax +44 (0) 1865 (5)72224
E-mail: jacqueline.gerencser@admin.ox.ac.uk

Dr Frank Smithuis
Mahidol Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine, Mahidol University,
Bangkok, Thailand

22 October 2013

Dear Dr Frank Smithuis

Full Title of Study: Optimising operational use of artemether-lumefantrine: An open-label randomized controlled trial to evaluate the effectiveness and safety of a 3 day vs 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar

OXTREC Reference: 1046-13

Thank you for your letter of the 16 October 2013, in which you have responded to the committees request for further clarifications and amendments, and included revised documents:

Documents:	Version:	Date:
Protocol	V2.0	16/10/13
PIS/ICF	V2.0	16/10/13
Protocol Review form		29/08/13
PI Signatory Page		29/08/13

I am therefore pleased as Chairman for OXTREC to give approval for this study,

Approval is given for the first five years and subject to receiving the local ethical approval.

We look forward to receiving your annual report of this study.

Yours sincerely,

Dr Mary Warrell
OXTREC Chairman

Direct Line Tel: +44 (0)1865 (5)72224
Fax: +44 (0)1865 (5)72228 Email: jacqueline.gerencser@admin.ox.ac.uk
Web: www.admin.ox.ac.uk/rso/

Oxford Tropical Research Ethics Committee

University of Oxford
Joint Research Office, Block 60
Churchill Hospital, Oxford OX3 7LE
Tel. +44 (0) 1865 (5)72346, Fax +44 (0) 1865 (5)72228
E-mail: jacqueline.perenciser@admin.ox.ac.uk

Dr Frank Smithuis
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine, Mahidol University,
420/6 Rajvithi Road
Bangkok 10400,
Thailand

08 May 2015

Dear Dr Smithuis

Full Title of Study: Optimising operational use of artemether-lumefantrine: An open-label randomized controlled trial to evaluate the effectiveness and safety of a 3 day versus 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar.

OxTREC Reference: 1046-13

Thank you for your letter of 28 April 2015 and email of the 30 April 2015, in which you have included the End of Study Report.

We will update our database accordingly.

Yours sincerely

pp

Dr Mary Warrell

OxTREC Chairman

Direct Line Tel: +44 (0)1865 (5)72346
Fax: +44 (0)1865 (5)72228 Email: jacqueline.perenciser@admin.ox.ac.uk Web: www.admin.ox.ac.uk/tsc/

14.5 Ethical Approval (Myanmar)

The Government of The Republic of The Union of Myanmar

Ministry of Health

Department of Medical Research (Lower Myanmar)

No. 5, Ziwaka Road, Dagon Township, Yangon 11191
Tel : 95-1-375447, 95-1-375457, 95-1-375459 Fax : 95-1-251514

Letter No. 50/ Ethics 2013
Dated: 31.10.13

The Ethical Review Committee on Medical Research Involving Human Subjects, Department of Medical Research (Lower Myanmar), approves to conduct the following proposed research project.

Effectiveness and safety of a 5 day course of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Myanmar

Principal Investigator: Dr Frank Smithius

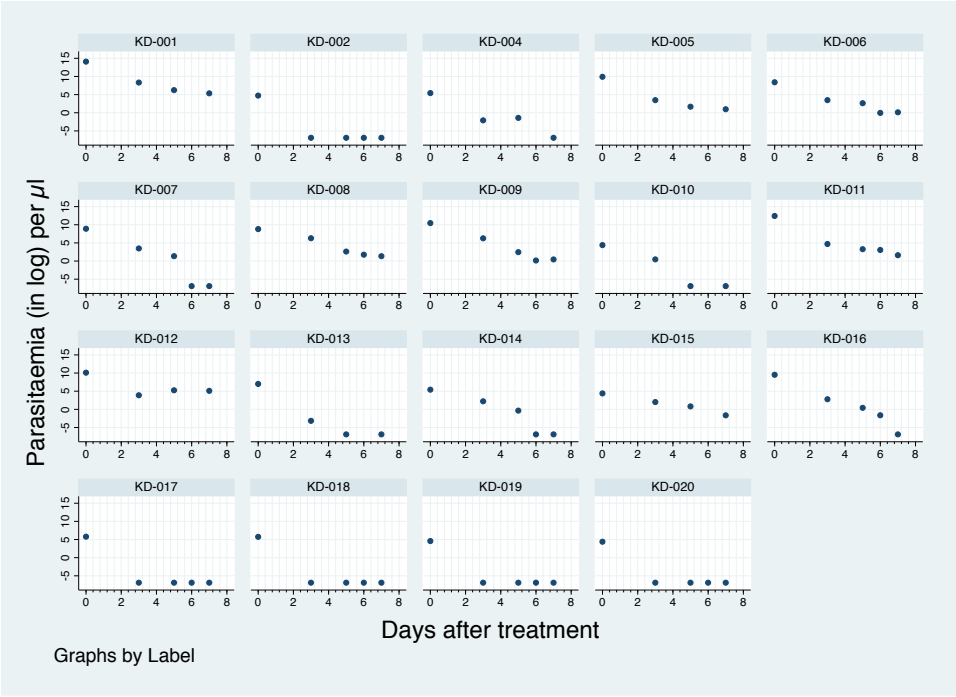
31.10.13

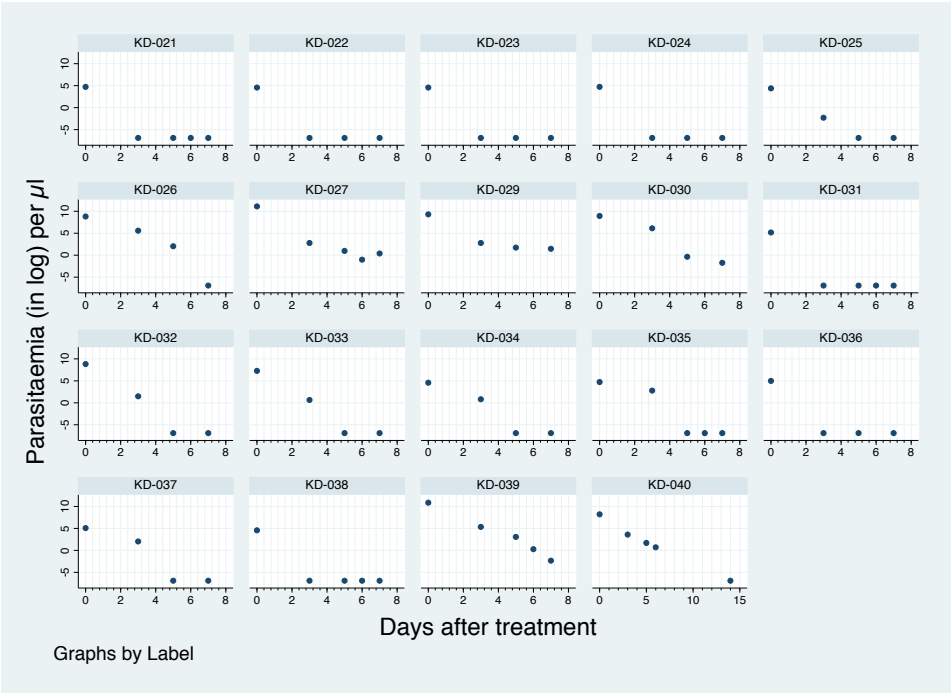
Dr. Myint Htwe
Chairperson
Ethical Review Committee
Department of Medical Research
(Lower Myanmar)

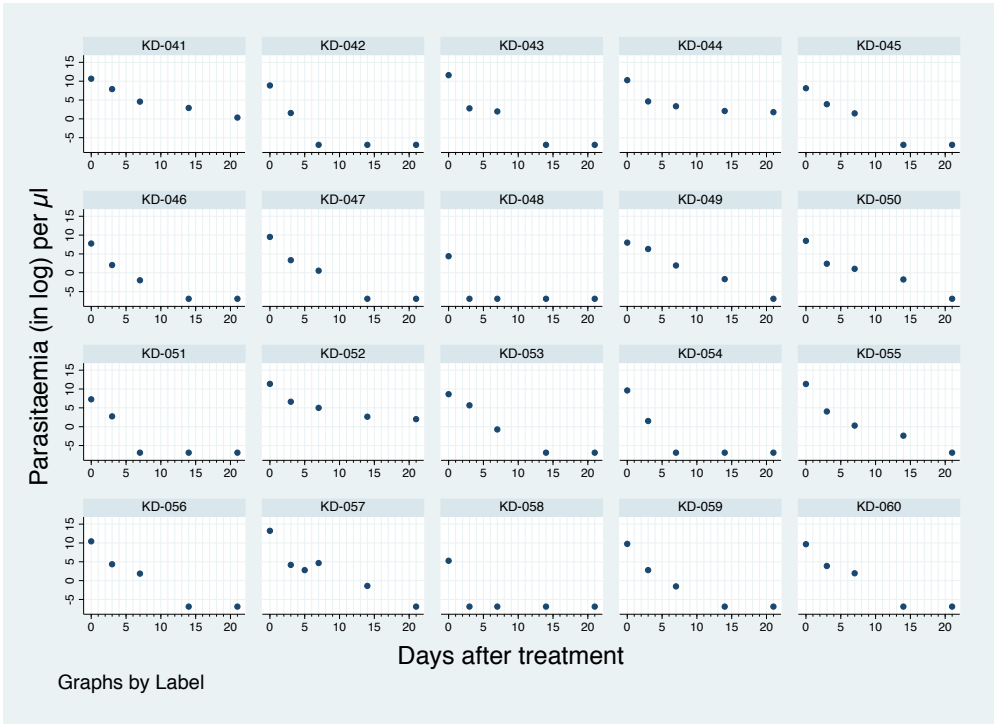
(*** Approval of the research is for the period of one year from the date mentioned)

50

14.6 Individual plot of parasitaemia using uqPCR by days after treatment



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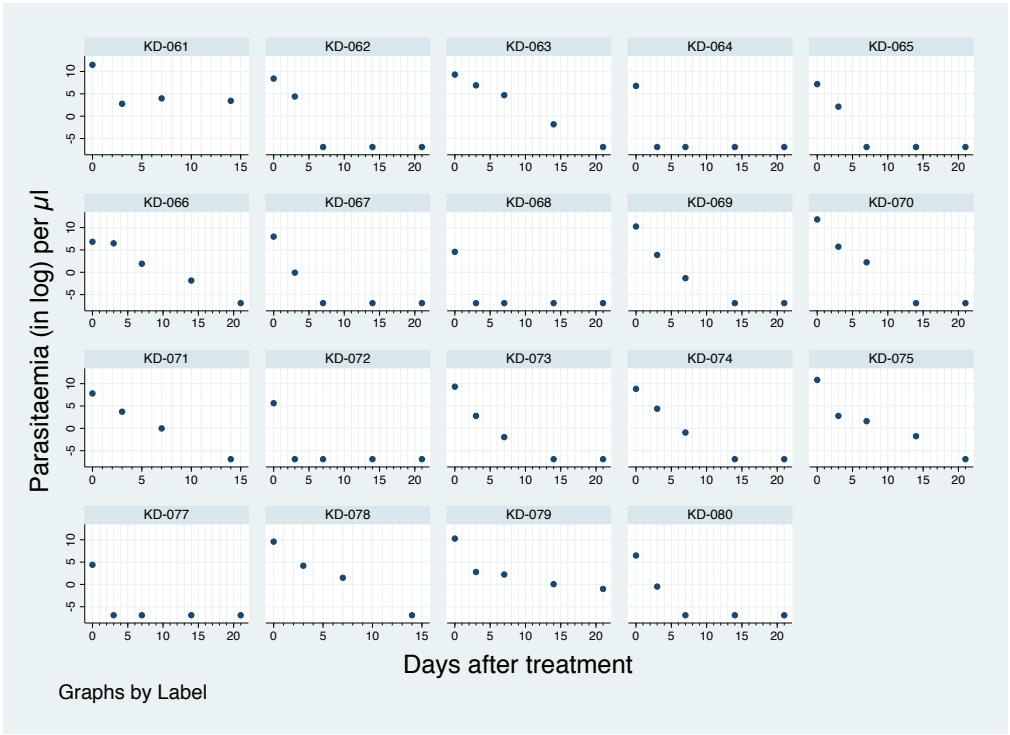
Parasitaemia (in log) per µl
Days after treatment
Graphs by Label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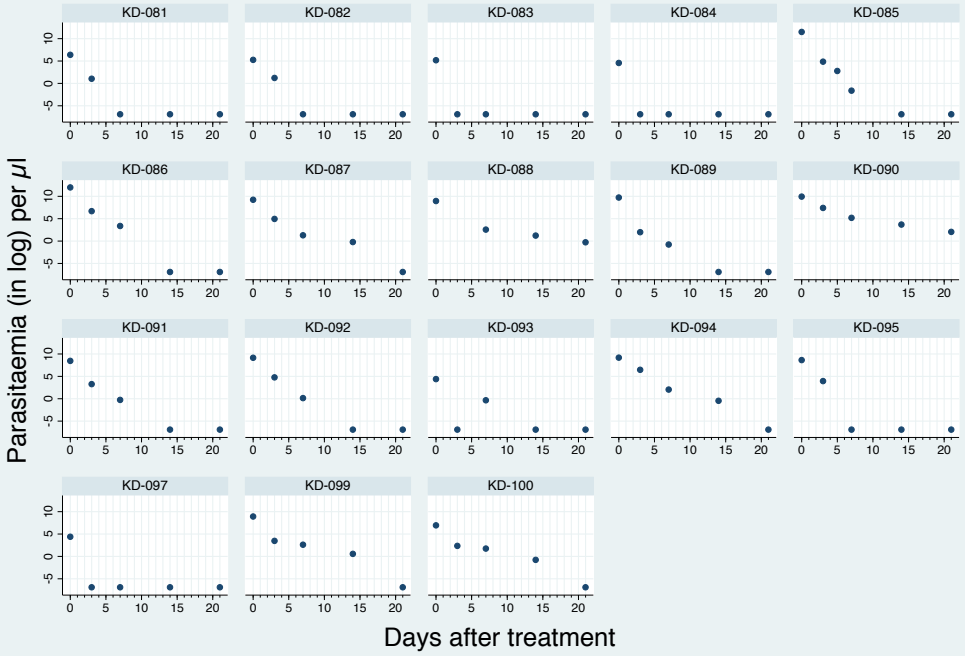
KD-080
Parasitaemia (in log) per µl
Days after treatment
Graphs by Label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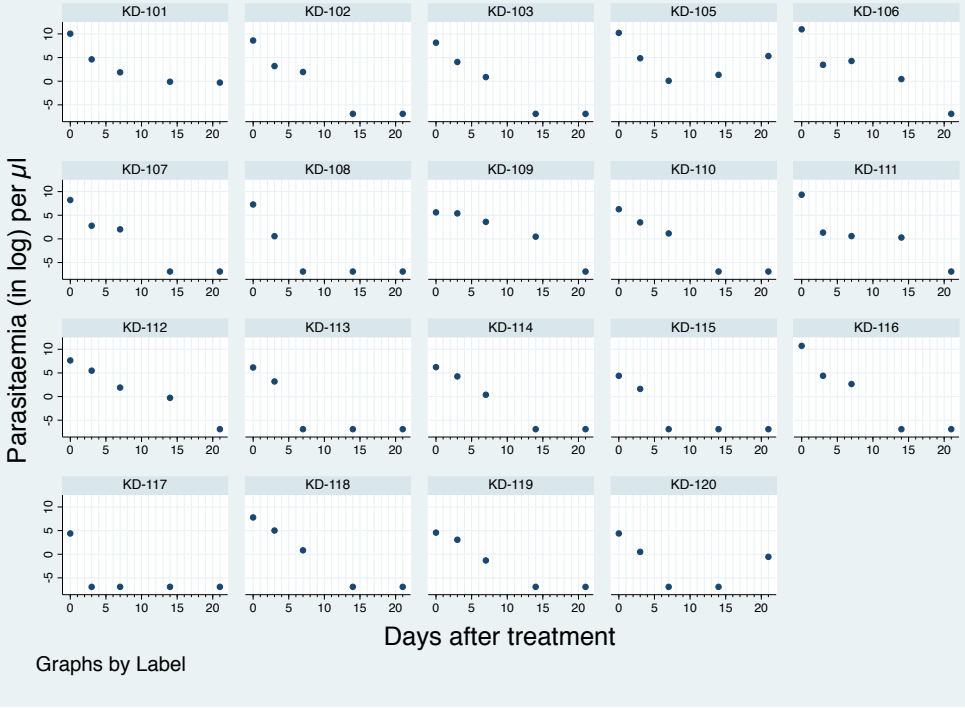
Parasitaemia (in log) per µl
Days after treatment
Graphs by Label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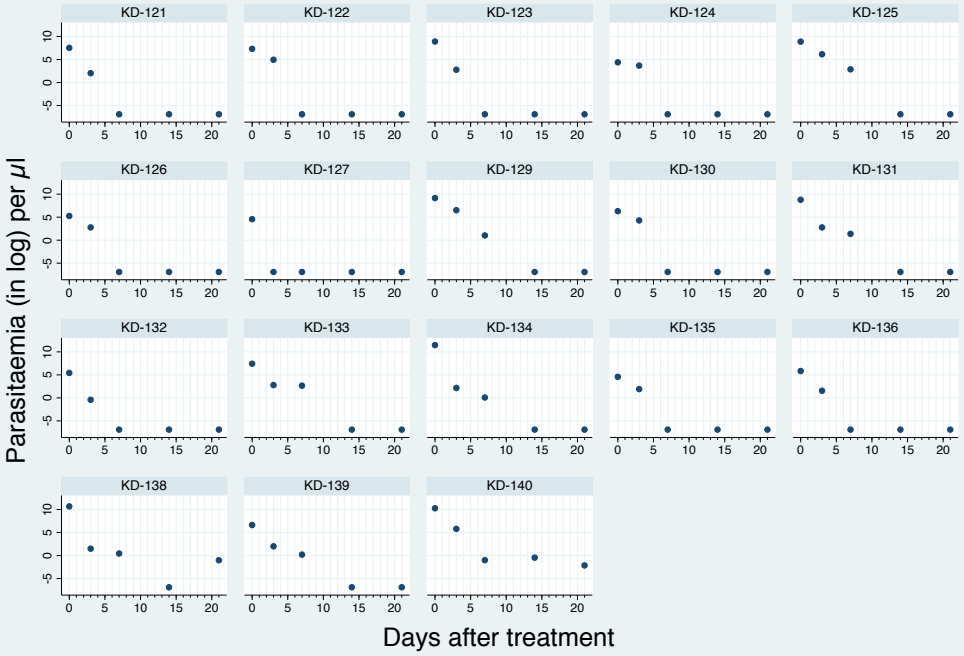
KD-118
KD-119
KD-120
Parasitaemia (in log) per µl
Days after treatment
Graphs by Label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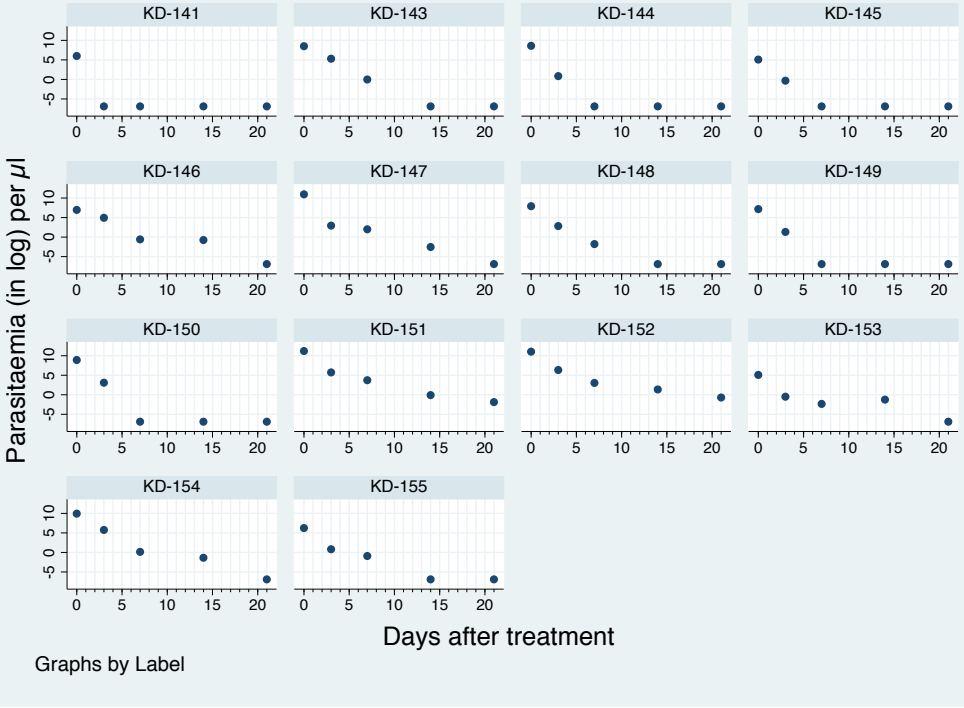
KD-136
KD-138
KD-139
KD-140
Parasitaemia (in log) per µl
Days after treatment
Graphs by Label


KD-141
KD-143
KD-144
KD-145
KD-146
KD-147
KD-148
KD-149
KD-150
KD-151
KD-152
KD-153
KD-154
KD-155
Parasitaemia (in log) per µl
Days after treatment
Graphs by Label