Clinical and Molecular evaluation of the therapeutic efficacy of the antimalarial drug artemether-lumefantrine

Balikagala Betty

Submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy

March 2017

Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Ehime, Japan

EHIME UNIVERSITY

Abstract

Individual drug treatment may select resistant parasites in the human body, a process termed *in vivo* selection. Some single nucleotide polymorphisms (SNPs) in *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) and multidrug resistance gene 1 (*pfmdr1*) genes have been reportedly selected after artemether-lumefantrine treatment. However, there is a paucity of data regarding *in vivo* selection of *Plasmodium falciparum* Kelch propeller domain (*pfkelch13*) polymorphisms, responsible for artemisinin-resistance in Asia, and six putative background mutations for artemisinin resistance; D193Y in *ferredoxin*, T484I in *multiple resistance protein 2*, V127M in *apicoplast ribosomal protein S10*, I356T in *pfcrt*, V1157L in *protein phosphatase* and C1484F in *phosphoinositide-binding protein*.

Artemether-lumefantrine efficacy study with a follow-up period of 28 days was conducted in northern Uganda in 2014. The above-mentioned genotypes were comparatively analysed before drug administration and on days; 3, 7, and 28 days after treatment.

In 61 individuals with successful follow-up, artemether-lumefantrine treatment regimen was very effective with PCR adjusted efficacy of 95.2%. Among 146 isolates obtained before treatment, wild-type alleles were observed in 98.6% of isolates in *pfkelch13* and in all isolates in the six putative background genes except I356T in *pfcrt*, which had 2.4% of isolates as mixed infections. *In vivo* selection study revealed that all isolates detected in the follow-up period harboured wild type alleles in *pfkelch13* and the six background genes.

Mutations in *pfkelch13* and the six background genes may not play an important role in the *in vivo* selection after artemether-lumefantrine treatment in Uganda. Different mechanisms might rather be associated with the existence of parasites after treatment.

Summary

Malaria is a life threatening tropical disease, caused by protozoan parasites of the genus *Plasmodium*. Five species of plasmodia cause malaria in humans via the bite of female mosquitoes of the genus *Anopheles* during a blood meal. These include; *Plasmodium falciparum*, *P. vivax*, *P. malariae, P. ovale* and *P. knowlesi*. Of the five species, *P.falciparum* poses the greatest threat. This thesis focuses on *P.falciparum*. Nearly half of the world's population is at risk of malaria infection and 91 countries have ongoing malaria transmission. According to the latest 2016 WHO estimates, 212 million cases of malaria and 429 000 deaths were reported worldwide, Africa disproportionately hosting 90% of the malaria cases and 92% of malaria deaths. Several malaria control strategies involving vector control (i.e. the use of insecticide-treated mosquito nets (ITNs), indoor residual spraying (IRS) and early diagnosis and treatment with artemisinin-based combination therapy (ACT) have resulted in significant decline in malaria infections and death. Indeed between 2010 and 2015, the number of new malaria cases fell by 21% globally and malaria death rates fell by 29% globally and by 31% in the African Region.

The recent gain in malaria control is partly attributed to the wide-scale use of ACTs for the treatment of malaria in all endemic regions. Unfortunately these fragile gains are threatened by the emergence of artemisinin resistance. Currently artemisinin resistance has been confirmed in 5 countries in South East Asia (SEA). These include; Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Vietnam. Current studies in Africa show no evidence of artemisinin resistance. However, there is a possibility that the established resistance in South East Asia may invade the African continent, which faces the greatest burden of the disease, as

previously observed in chloroquine and sulfadoxine/pyrimethamine resistance. Yet there are no treatment alternatives to the current ACT in the drug development pipeline.

In 2014, *Pfkelch13* gene (PF3D7_1343700) was identified as the molecular marker for tracking artemisinin resistance. Some mutations in this gene are associated with artemisinin resistance in South East Asia. In addition, genome wide association studies identified several single nucleotide polymorphisms (SNPs); D193Y in *ferredoxin* (*fd*), T484I in *multidrug resistance protein 2*+ (*mdr2*), V127M in the *apicoplast ribosomal protein S10* (*arps10*), I356T in *chloroquine-resistance transporter* (*crt*), V1157L in *protein phosphatase* (*pph*) and C1484F in *phosphoinositide-binding protein* (*pibp*) assumed to be background genetic changes for artemisinin resistance in South East Asia. In Africa, polymorphisms in these genes have been occasionally observed, but they are different from those reported in South East Asia. Making it unclear whether the existence of these mutations in Africa is a consequence of selection induced by the use of antimalarial drugs.

During treatment with a malaria drug, less susceptible parasites can be selected in the human body, a process termed *in vivo* selection. This is because the treatment creates drug concentration circumstances that are sufficient to kill susceptible, but not less susceptible parasites. Previous investigations revealed that artemether-lumefantrine (AL) treatment (an ACT widely used in the treatment of uncomplicated malaria selected for parasites harbouring alleles with K76 in the *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) gene and N86, 184F and D1246 in *Plasmodium falciparum* multidrug resistance gene 1 (*pfmdr1*). However, the possibility of similar *in vivo* selection has not been fully investigated in *pfkelch13* and the putative background genes.

Therefore, this study evaluated the therapeutic efficacy of AL, (the current first line treatment for uncomplicated malaria in Uganda), in Gulu Northern Uganda, a region of intense malaria transmission in May and October 2014. Individuals suspected to have malaria were consecutively enrolled from the outpatient department in St. Mary's hospital Lacor, one of the hospitals offering healthcare to the people in Gulu Northern Uganda. Before enrolling participants in the study, permission for participation (informed consent) was sought from each individual. Thereafter, the presence of malaria was confirmed by microscopy and species-specific polymerase chain reaction (PCR). Blood for parasite genotyping was also collected. Supervised administration of oral AL was performed for all recruited individuals and followed up on days 1, 2, 3, 7 and 28. On each follow up day, as at enrollment, the presence of malaria was confirmed microscopically and by species-specific PCR. Blood for parasite genotyping was also collected. At the end of the follow-up period, participants were assigned treatment outcomes according to WHO guidelines as: adequate clinical and parasitological response (ACPR), early treatment failure (ETF), late clinical failure (LCF) and late parasitological failure (LPF). Genotypes in the above mentioned genes were quantified at baseline and then comparatively analysed before drug administration and on days; 3, 7, and 28 days after treatment to determine evidence of *in vivo* selection for polymorphisms associated with AL resistance.

Excellent early response to AL treatment was observed in almost all patients. Only one (1.6%) child showed microscopically residual parasites (delayed parasite clearance) on day 3 after treatment. However, this prevalence of day 3 parasite positive individuals in the present study was less than 5% or 10%, which are the benchmarks for artemisinin-resistance. Confirming the absence of artemisinin resistance in Africa. PCR-confirmed day 3 parasite positivity after AL treatment was much higher than microscopically confirmed positivity; 22.9%, versus 1.6%. Also,

individuals that were parasite positive by PCR on day 3 had significantly higher enrollment parasitaemia compared to the PCR negative group, suggesting that parasite biomass before treatment may be associated with treatment success and PCR parasite-positive outcome on day 3 after treatment. Similar to the excellent early treatment response, artemether-lumefantrine treatment was very effective with PCR adjusted efficacy of 95.2%. Only three individual failed on treatment by developing new malaria infections within 28 days after initial treatment.

The molecular analysis revealed that among 146 isolates obtained before treatment, wild-type alleles were observed in 98.6% of isolates in *pfkelch13* and in all isolates in the six putative background genes except at position I356T in *pfcrt*, which had 2.4% of isolates as mixed infections. *In vivo* selection analysis revealed that *pfkelch13* mutations were not observed in the parasite positive samples on day 3, 7 and 28, consistent with the recent observations from Kenyan children. Also, no selection of putative six non-synonymous polymorphisms was observed, suggesting that these genetic changes may not be responsible for parasite persistence in the present study. In contrast, *Pfcrt* K76 and *Pfmdr1* N86/D1246 were observed in all recurrent parasites. The prevalence of *Pfmdr1* Y184F (33.3%) in the recurrent parasites was higher than baseline (14%), although not statistically significant. These observations support the potential selection of *Pfcrt* K76 and *Pfmdr1* N86/Y184F/D1246 haplotype after AL treatment. *In vivo* selection of these mutations would increase these allele prevalence in the parasite population.

In conclusion, this study demonstrated that AL treatment remains of high efficacy for the treatment of *P. falciparum* malaria after 8 years of use in a region of high malaria transmission in Uganda. Mutations in *pfkelch13* and the six background genes may not play an important role in the *in vivo* selection after artemether-lumefantrine treatment in Uganda. Different mechanisms might rather be associated with the existence of parasites after treatment.

審査結果の要旨

マラリアは、熱帯・亜熱帯地域に分布し、マラリア原虫の感染によって引き起こされハマダラカによって媒介される疾患である。2016 年現在、年間２億人以上が罹患し約 43 万人が死亡している。世界保健機関は、1950 年代からマラリア治療薬および殺虫剤を用いて世界規模でマラリア対策を進めてきた。しかし、特効薬クロロキンに対する耐性熱帯熱マラリア原虫や殺虫剤耐性のハマダラカが蔓延したため、1970 年代以降マラリア死亡者数は増加に転じた。その後、薬剤耐性熱帯熱マラリア原虫にも有効な新薬アルテミシニンが実用化され、2005 年以降再びマラリアによる死亡者数は減少に転じた。しかしその頃から、カンボジアにおいてアルテミシニン耐性熱帯熱マラリア患者が報告され、その後現在までに近隣の４ヶ国からも報告される事態となっている。また、アルテミシニン耐性原虫から、Pfkelch13（K13）と呼ばれる原虫遺伝子の多型が同定された。さらに、耐性に関連するその他 6 種類の原虫遺伝子多型として、*ferredoxin* (*fd*) の D193Y、*multidrug resistance protein 2* (*mdr2*)の T484I、*apicoplast ribosomal protein S10* (*arps10*)の V127M、*chloroquine-resistance transporter* (*crt*)の I356T、*protein phosphatase* (*pph*)の V1157L、 *phosphoinositide-binding protein* (*pibp*)の C1484F が報告された。しかし、治療中の患者体内における上記遺伝子多型の変化と臨床的耐性の関連性を解析した研究はこれまで無い。また、現在までアフリカにおけるアルテミシニン耐性熱帯熱マラリアの報告は無いが、これまでの薬剤耐性熱帯熱マラリアの分布拡大の歴史から、アフリカにおけるアルテミシニン耐性熱帯熱マラリアの出現は予断を許さない状況と考えられている。そこで申請者は、アフリカにおけるアルテミシニン耐性熱帯熱マラリアの現状

と、耐性に関連する原虫遺伝子多型の変化を把握し、アフリカにおける今後のマラリア対策に資することを目的に本研究を実施した。

研究方法は、ウガンダ共和国北部の高度マラリア流行地であるグルにおいて、2014年 5 月および 10 月からそれぞれ 1 ヶ月間の患者追跡研究として実施した。同地域の中核病院であるラチョー病院において、熱帯熱マラリアと診断された患者の中でインフォームドコンセントが得られた 146 名を対象とした。その内 61 名に対しては、ウガンダ共和国で使用が許可されているアルテミシニン合剤 artemether-lumefantrine（AL）を用いた治療前、治療後 3、7 及び 28 日目に治療効果判定および薬剤耐性遺伝子マーカーの検査に用いるため、少量の血液試料を採取した。残りの 85 名は、薬剤耐性遺伝子マーカーの検査に用いる血液試料を AL 投与前にのみ採取した。また熱帯熱マラリア原虫の感染の診断は、顕微鏡観察および Polymerase Chain Reaction（PCR）により行った。

AL に対する治療後 3 日目でも顕微鏡観察により熱帯熱マラリア原虫が残存していた患者は、61 名中 1 名（1.6%）のみであり、WHO の耐性マラリア出現基準である 10%に達していなかった。また顕微鏡観察より高感度の PCR 診断を用いても、AL の治療効果は 95.2%と非常に高かった。次に、本研究で得られた熱帯熱マラリア原虫フィールド分離株中の治療前のアルテミシニン耐性関連遺伝子の多型を解析した。その結果、Pfkelch13 ならびに *fd*、*mdr2*、*arps10*、*crt*、*pph*、*pibp* のいずれも、98.6%は野生型の遺伝子型であり、アルテミシニン耐性と関連する遺伝子型は認められなかった。さらに、61 名の治療後の上記遺伝子型の変化を追跡したが、いずれも治療期間中の患者体内での遺伝子型の変化は認められなかった。したがって、ウガンダ共和国においては AL 治療の臨床的有効性が再確認され、またアルテミシニン耐性に関与する原虫遺伝子型は未

だ存在しておらず、治療期間中にも変化していないことが明らかとなった。これらの結果は、今後も継続的に AL の有効性と遺伝子マーカーを流行地でモニターすることが、アルテミシニン耐性熱帯熱マラリア原虫の出現を早期に発見し、その拡散の防止に有用であることを示している。

以上のように、本研究は流行地におけるマラリア対策に資するのみならず、分子生物学、生命科学においても高い学術的価値があると考えられる。

Declaration

I satify that this dissertation is the result of my personal work towards the degree of Doctor of Philosophy and further state that it has not been submitted, or being submitted for any degree, or qualification at any other University.

Name: Balikagala Betty

Date: March 2017

Acknowledgements

I would like to thank my academic supervisors Prof. Takafumi Tsuboi and Prof. Toshihiro Mita for the opportunity to learn from them and for the unwavering mentorship through out the program.

To Prof. Takafumi Tsuboi, You personally warmly welcomed me to Matsuyama and your laboratory with trust and faith. You ensured that I was confortable to stay and study. Such leadership I have never experienced throughout my years of study in Africa. In the laboratory, you bridged the gap between student and professor. It was free conversation and exchange of ideas, free laughter. I sincerely appreciate your great leadership, support and guidance, your never ending ideas and enthusiasm for science. I was privileged to belong to your laboratory.

To Prof. Toshihiro Mita, I am very grateful for the unwavering mentorship, wisdom and supervisory role in all the different perspectives on my research project. I greatly appreciate your encouragement and vision at the time when patient enrollment was low. At that time, I thought that the research project wouldn't be successful. However, with your wisdom, valor and perseverance we made it. We published.

I am greatly indebted to Prof. Toshihiro Horii for believing in me and offering me a great opportunity to train as a PhD student in Japan. If I hadn't worked under your great leadership and mentorship, this PhD dream would have remained on the shelves. Your passion for science and to fight malaria is a true inspiration.

I appreciate Assoc. Prof. Eizo Takashima for accepting to train me in Prof. Tsuboi's laboratory. Thank you very much for keeping the laboratory alive and making science fun. It was a great learning experience.

I also greatly appreciate Assoc. Prof. Nirianne Palacpac for all and every assistance, guidance and advise in all academic and social aspects. Prof. Palacpac you are a very special. Thank you being selfless and always being available to provide valuable advice and guidance at all steps before and during this project. You were never too busy to assist. Your door was always open. Thank you for the motherly love. You will always be dear to us.

I am indebted to Prof. Thomas Gordon Egwang for introducing me to scientific research world. It was your devoted selfless mentorship that stirred a great desire in me to pursue research. Thank you very much for opening great doors in our lives.

I am grateful for the kindness and support from the administration of the Proteo-Science center, the past and present members of Prof. Tsuboi laboratory who made my experience at the Proteo-Science center better and memorable.

More appreciation goes to all staff in international office Ehime University who have in one way or another contributed to the success of this PhD program. Prof. Ruth Vergin, I sincerely appreciate every arrangement for the comfort of the international community within Ehime University. I also appreciate Yamamoto-san and Iwata Go-San for all the assistance for a smooth stay in Matsuyama.

Further acknowledgements go to children that participated in this study as well as their guardians. You have made a great contribution to science. Thank you very much.

I whole-heartedly thank my family and friends. You have always been my biggest fans. I specially thank my daughters Nissi Atuwadde Mawagali and Ninette Mulungi Mawagali for patiently waiting for three years to allow mummy accomplish this greatest achievement in life. Your constant love turned good days great. My two sons Muwereza Noah Mawagali and Muwereza Nahum Mawagali, thank you too for the patience and daily encouragement that enabled me reach this achievement. You are my heroes. To Noah Mawagali thank you this may not have been possible without you. I greatly appreciate my parents Mr. C.J. Balikagala and Mrs. Mary Balikagala for always encouraging me to aim highest. Through your encouragement I have achieved the highest and aiming for more. I specially thank my sisters Mrs. Phoebe Balikagala, Mrs. Robinah Kasule, Mrs. Maggie Balikagala and Joanita Balikagala. In every way you have taken care of my daughters Nissi and Ninette, who stayed behind in Uganda as I pursued this project. You cared for my daughters as your very own. Through thick and thin you have been there for them. Thank you very much. I sincerely appreciate the love amongst us. Joanita thank you very much for enduring all my stress. I am very grateful to all my dear friends for the support that enabled us smoothly sail through the program. Indeed this achievement wouldn't be without you. Thank you very much.

Contents

List of Figures

List of Tables

List of Abbreviations

ABC	ATP binding cassette
ACT	Artemisinin based combination therapy
AL	Artemether lumefantrine
DBB	Desbutylbenflumetol
DHA	Dihydroartemisinin
EC	Effective Concentration
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment for pregnant women
IRS	Indoor residual spraying
ITN	Insecticide treated net
PCR	Polymerase chain reaction
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
Pfcrt	*Plasmodium falciparum chloroquine resistance transporter gene*
Pfkelch13	*Plasmodium falciparum Kelch 13*
Pfmdr1	*Plasmodium falciparum multidrug resistance gene 1*
Pgh1	P-glycoprotein homologue 1
SNP	Single nucleotide polymorphism
WHO	World Health Organization

1.0 BACKGROUND

1.1 Malaria and its global burden

Malaria is a life threatening tropical disease caused by protozoan parasites of the genus *Plasmodium*. Five species of plasmodium parasites cause malaria in humans. Four of these are: *Plasmodium. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* which are spread from one person to another via the bite of female anopheles mosquitoes (malaria vectors) during a blood meal. The fifth is *P. knowlesi* that causes malaria in macaque monkeys. *P. knowlesi* malaria is transmitted to people when an *Anopheles* mosquito infected by a monkey bites and infects humans (zoonotic transmission) (Cox-Singh J et al 2008). Of the five species, *P. falciparum* and *P. vivax* are the most prevalent and pose the greatest threat to humans. *P. falciparum* is most prevalent on the African continent, and is responsible for most malaria-related morbidity and deaths globally, while *P. vivax* is the dominant parasite in most countries outside Sub-Saharan Africa.

Nearly half of the world's population is at risk of malaria infection and at least 91 countries have ongoing malaria transmission. According to the latest WHO estimates, 212 million cases of malaria and 429 000 deaths were reported worldwide (World Malaria Report 2016). Sub-Saharan Africa disproportionately carries the highest share of this global malaria burden. In 2015, the African region was home to 90% of malaria cases and 92% of malaria deaths. However, South-East Asia, Latin America and the Middle East, are also at risk (World Malaria Report 2016). Populations at greatest risk of contracting malaria include infants, children under 5 years of age, pregnant women, people living with HIV/AIDS, non-immune migrants, mobile populations and

travellers. These high-risk groups require, National Malaria Control Programmes to take special measures to protect them from malaria infection, taking into consideration their specific circumstances.

The burden of malaria extends far beyond morbidity and mortality, as malaria strongly impacts on the social and economic development of both individuals and governments (Malaney *et al*., 2004). Individuals and their families incur costs in purchase of drugs for treating malaria at home; expenses for travel to, and treatment at, clinics; lost days of work; absence from school; expenses for preventive measures; expenses for burial in case of deaths. Similarly, governments are burdened with maintenance, supply and staffing of health facilities; purchase of drugs and supplies; public health interventions against malaria, such as insecticide spraying or distribution of insecticide-treated bed nets; lost days of work with resulting loss of income; and lost opportunities for joint economic ventures and tourism. Direct costs (like, illness treatment, premature death) have been estimated to be at least US$ 12 billion per year (CDC).

This thesis focuses on *P. falciparum*.

1.2 Life cycle of *p.falciparum*

Malaria parasites have a complex life cycle alternating between the female *Anopheles* mosquito and the human host as illustrated in Figure 1. When an infected *Anopheles* mosquito bites to feed, saliva containing sporozoites is injected from its salivary gland into the human host. After entering the human bloodstream, sporozoites reach the liver and invade the hepatocytes where

they undergo asexual replication, which takes about 1-2 weeks. Each sporozoite may result in tens of thousands of merozoites. On release from the liver, the merozoites are delivered into the bloodstream, where they rapidly infect erythrocytes. Inside the erythrocyte the asexual division starts. The merozoite matures from ring-stage trophozoite to pigmented trophozoite and multinuclear schizont. Finally the rupture of the erythrocyte releases several merozoites into the bloodstream to continue the erythrocytic cycle, which is about 48 hours. The erythrocytic cycle is followed by the malaria characteristic fever. Some merozoites in the erythocytes develop into micro- and macrogametocytes (male and female). These are necessary for carrying on the infection to a new host. When an anopheline mosquito takes a blood meal, the gametocytes may be taken up. They are transported to the mosquito midgut, where the sexual cycle starts. Macrogametes and microgametes fuse and develop into a zygote and then an ookinete, which goes through the wall of the midgut and develops into an oocyst. The rupture of the oocyst will result in many sporozoites that migrate to the salivary glands for transmission to a new host during the next blood meal of the mosquito. The development in the mosquito takes about 10-18 days, although the mosquito can be infective for another 1-2 months.

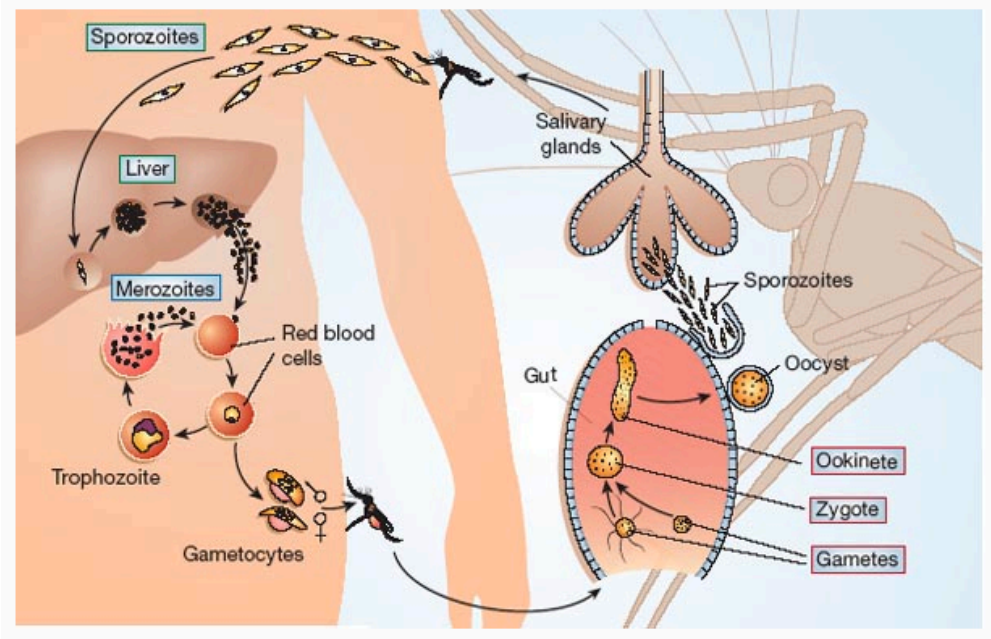


Figure 1: *P.falciparum* life cycle (Ménard R. Nature Medicine 2005)

1.3 Malaria transmission

Malaria is transmitted through the bite of adult female *Anopheles* mosquitoes at the time of taking a blood meal to nurture their eggs. More than 400 species of *Anopheles* mosquitoes are reported but only 30 are of major importance. The major vector in Sub-Saharan Africa is Anopheles gambiae, which like other vectors, bites between dusk and dawn. *Anopheles* mosquitoes lay their eggs in water, which hatch into larvae, eventually emerging as adult mosquitoes ready to feed. Each species of *Anopheles* mosquito has its own preferred aquatic habitat; for example, some prefer small, shallow collections of fresh water, such as puddles and hoof prints, which are abundant during the rainy season in tropical countries.

Malaria transmission and epidemiology depends on factors related to the parasite, vector, human host, and the environment. Transmission is more intense in places where the mosquito lifespan is longer (so that the parasite has time to complete its development inside the mosquito) and where it prefers to bite humans rather than other animals. The long lifespan and strong human-biting habit of the African vector species is the main reason why nearly 90% of the world's malaria cases occur in Africa. Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity.

In many places, transmission is seasonal, with the peak during and just after the rainy season. Malaria epidemics can occur when the climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission. Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Partial immunity is developed over years of exposure to malaria, and while it never provides complete protection, it does reduce the risk of severe disease. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk. Figure 2 presents the distribution of malaria endemic regions worldwide.

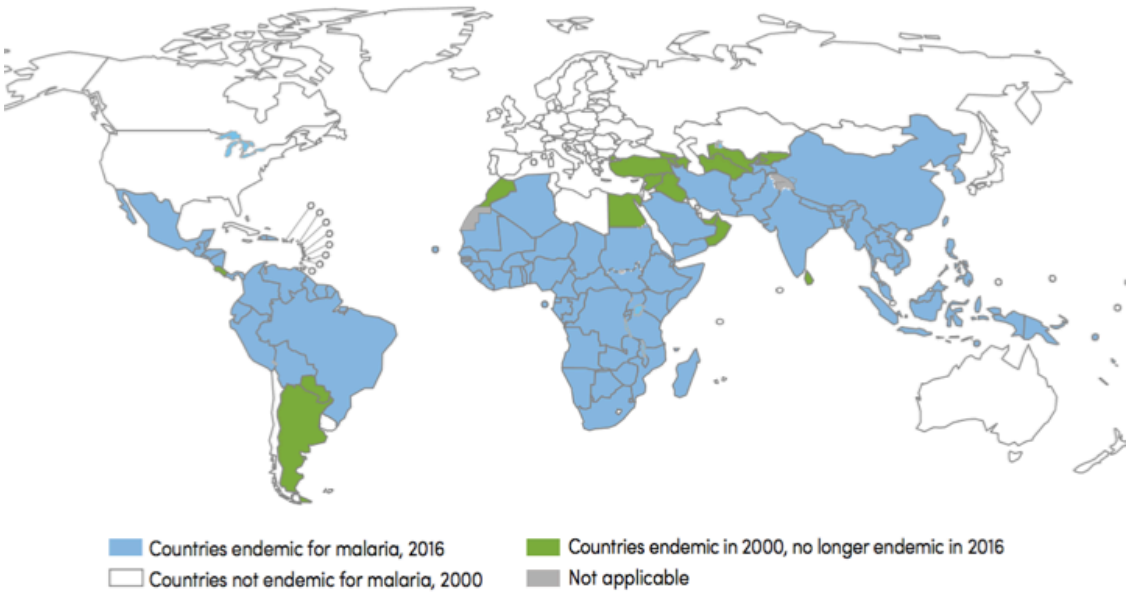


Figure 2 Countries endemic for malaria in 2000 and 2016. Source: WHO database

1.4 Clinical manifestations of *P. falciparum* malaria

Malaria is an acute febrile illness, but its outcome is influenced by host and parasite factors. In high transmission areas, individuals progressively develop partial immunity to malaria, after repeated infections (premunition). This process of premunition is protective against clinical malaria (uncomplicated and severe), but especially severe malaria. The malaria infections are usually suppressed however asymptomatic infections occur. The process of premunition is absent in areas of low malaria transmission (Langhorne *et al*., 2008). In fact non-immune individuals become ill at the first exposure. Malaria is not symptomatic during the liver stage of the infection, with clinical symptoms only being associated with the erythrocytic stage. In non-immune individuals, malaria symptoms appear 7 days or more (usually 10–15 days) after the infective mosquito bite. Malaria symptoms usually begin as non-specific like presence of headaches,

fatigue, abdominal discomfort, muscle and joint aches, followed by specific symptoms of fever, chills, sweating, anorexia and vomiting. If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness, often leading to death (World Malaria Report 2016). Co-infections like HIV/AIDs, may affect the outcome of a malaria infection by interfering with malaria immunity (Renia, L. & S. M. Potter. 2006).

1.5 Malaria control

An integrated approach comprised of prevention (vector control and chemoprophylaxis) and early diagnosis with prompt effective treatment is utilized in malaria control. Vector control measures include; the use of insecticide treated nets (ITNs), and Indoor residual spraying (IRS) which have majorly contributed to the decline in malaria morbidity and mortality (Lengeler, 2004, Kigozi R et al 2012). Regarding the other arm of control measures, a number of antimalarial drugs are currently under clinical use for treating malaria. These include the 4-aminoquinolines (amodiaquine), the arylaminoalcohols (mefloquine, halofantrine, lumefantrine), antifolates (sulfadoxine-pyrimethamine (SP)), atovaquone, artemisinin and its derivatives (artesunate, artemether) (WHO: Guidelines for treating malaria). Different treatment regimens are suited for different malaria manifestations i.e. complicated, uncomplicated or asymptomatic malaria. The main objective of treating severe malaria is to prevent death. Secondary objectives are prevention of disability and recrudescent infections. Currently WHO recommends that adults and children with severe malaria (including infants, pregnant women in all trimesters and lactating mothers) be treated with intravenous or intramuscular artesunate for at least 24 hours or until when they can tolerate oral medication. There after, complete 3 days treatment with of an ACT (single dose

of primaquine is add in low transmission areas).

For uncomplicated malaria the objective of treatment is to cure, i.e. eradicate the infection to prevent progression to severe disease. Furthermore, from the public health perspective the objective is also to reduce transmission of the infection to others (WHO: Guidelines for treating malaria). Uncomplicated *P.falciparum* malaria in children and adults (except pregnant women in their first trimester) is treated with one of the following recommended artemisinin-based combination therapies (ACT); (a) artemether + lumefantrine (b) artesunate + amodiaquine (c) artesunate + mefloquine (d) dihydroartemisinin + piperaquine and (d) artesunate + sulfadoxine-pyrimethamine (SP). Antimalarial drugs are also used in malaria prevention, both as chemoprophylaxis for travellers to malaria endemic areas or for intermittent preventive treatment in pregnant women (IPTp) and infants (IPTi). IPTp, involving the administration of a full dose of sulfadoxine-pyrimethamine (SP), is recommended in high endemic areas. In the future, other malaria control measures such as vaccines or genetically modified mosquitos can become useful.

1.6 Antimalarial drug resistance

1.6.1 Definition

Antimalarial drug resistance is defined as the ability of a parasite to survive and/or multiply, despite the administration and absorption of the drug given in doses equal to or higher than those recommended.

Key drivers of antimalarial drug resistance include the different genetic structure of malaria parasites especially in regions known for antimalarial drug resistance, substandard treatment, unregulated or poorly administered antimalarial drugs and artemisinin monotherapy (World wide Antimalarial Resistance Network), or changes in the drug exposure due to for example poor absorption.

1.6.2 Emergence of antimalarial drug resistance

Antimalarial drug resistance may result by two events: (i) the initial event; i.e. genetic changes resulting into resistant parasites and (ii) selection of resistant parasites that survive treatment within an individual. The genetic events (mutations) that confer antimalarial drug resistance are usually spontaneous, rare and are independent of the drug used (White NJ. 2004). They may be mutations in or changes in copy numbers of genes or related to the drug's parasite target or influx/efflux pumps that affect intra-parasitic drug concentrations. A single genetic change may be all that is required to effect drug resistance or multiple independent events (White NJ. 2004).

The rate and frequency of antimalarial drug resistance is directly influenced by a number of factors. Firstly, high mutation rates, which promote faster emergence of resistance but also enable the parasite, to quickly adapt to its changing environments (Sniegowski PD *et al*., 2000), which is the situation when parasites are exposed to changing drug selection pressures. An "accelerated resistance to multiple drugs" (ARMD) phenotype has been suggested to be present in parasites from Southeast Asia. The parasites putatively acquire drug resistance at a much higher rate than strains from other regions, explaining why resistance to new drugs often arises first in Southeast

Asia (Rathod PK *et al*., 1997).

Secondly, drug pressure influences selection of mutations. Selection occurs when parasites in the primary infection survive treatment, and recur (recrudescence), while the sensitive parasites are killed. Effective treatment is important to maintain adequate drug levels within circulation. In the case that parasites are exposed to sub-therapeutic drug concentrations like the use of sub-standard drugs, improper dosing, poor drug pharmacokinetics and pharmacodynamics or occurrence of infections during the terminal elimination phase of the prior antimalarial drug, less susceptible parasites may be selected (Müller O 2011). These parasites may be subsequently transmitted to mosquitoes and to other hosts. In high transmission settings selection usually occurs, when new infections (re-infections) emerge after treatment specifically when large parasite populations are exposed to sub-therapeutic drug concentrations of slowly eliminated drugs (Peters, W 1987). At lower levels of resistance, the sub-therapeutic drug concentrations may however be sufficient to eliminate the sensitive parasites. Unlike high transmission settings, in low transmission regions e.g. South East Asia, most malaria infections result in clinical disease. Therefore almost all parasites are under drug pressure leading to the selection of even rare parasite subpopulations.

1.6.3 Impact of antimalarial drug resistance

Antimalarial drug resistance poses considerable threat to malaria control efforts. At the moment, resistance has emerged to all classes of antimalarial drugs, artemisinins inclusive (Noedl H *et al*., 2009). For high transmission settings like the Africa, which is home to over 90% of malaria infections, antimalarial resistance poses devastating effects like frequent malaria morbidity,

increased risk of severe malaria, high mortality as was evidenced with chloroquine resistance (Trape, J. F 2001). Drug resistance has also been associated with the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. It has also significantly increased the occurrence of epidemics in low transmission areas (Björkman A, Bhattarai A, 2005).

1.7 Artemisinin based combination therapy (ACT)

Identification of artemisinins as antimalarial components of the ubiquitous annual wormwood *Artemisia annua* was to stop the spread of chloroquine-resistant *P. falciparum*. Artemisinin derivatives specifically possess important pharmacological characteristics, which include rapid onset of action, a short half-life, broad range of activity against all stages of the parasite's life cycle, and are very safe (White NJ, 2008). Most importantly, artemisinin derivatives are active against gametocytes, the sexual stage of parasites (Pukrittayakamee S, 2004). A decrease in gametocytes potentially reduces malaria transmission especially in endemic areas (Okell LC, *et al.,* 2008). However, the excellent pharmacological properties of artemisinins adduce the possibility of inadequate treatment. Mainly because malaria patients quickly get symptom relief after the initial dose, that they stop the treatment without completing the fully prescribed dose. This incomplete cessation of treatment creates situations of sub-therapeutic drug concentrations that are associated with the emergence of artemisinin resistance (White NJ, *et al*., 2009). Failure to complete treatment is even more likely to occur if patients are treated with longer treatments like artemisinin-based monotherapy, which requires 7-days of treatment. In fact, the recurrence of parasites, which might have escaped the artemisinin treatment, is occasionally observed in

artemisinin monotherapy as recrudescence (Price R, *et al*., 1998). This is why drug combinations have been proposed (*e.g*. artemisinin combination therapy, ACT) to reduce the duration of therapy and, more importantly, to clear residual parasites that may have escaped artemisinin treatment (White NJ. 2004).

Currently, the fixed-dose ACTs including artemether-lumefantrine, artesunate-mefloquine, and artemether-amodiaquine have been implemented as a first-line treatment in most malaria endemic countries. Piperaquine only being recently added among ACTs and implemented as first-line treatment for uncomplicated malaria in Cambodia, China, Myanmar, and Vietnam (World Malaria Report 2011). The rationale of ACTs can be explained from the pharmacokinetic profile of artemisinins and partner drugs. After administration, artemisinins are converted to the more potent form of dihydroartemisinin *in vivo* and rapidly eliminated with half-lives of ~1 hour. In case of a 3-day regimen, it covers only two asexual parasite cycles. Although this treatment results in a 100 million reduction in parasite numbers, it is not enough to completely clear all parasites in the human body. Requiring additional effect from partner drugs. All partner drugs in ACT combinations, have long blood concentration half-lives sufficient to kill residual parasites after 3 days of treatment with artemisinins (White NJ. 1997). The use of ACTs has undoubtedly contributed to the substantial reduction in the malaria burden (Enserink M. 2007). However, clinical artemisinin resistance is a major threat especially in South East Asia. Schema of an ACT is presented in figure 3.

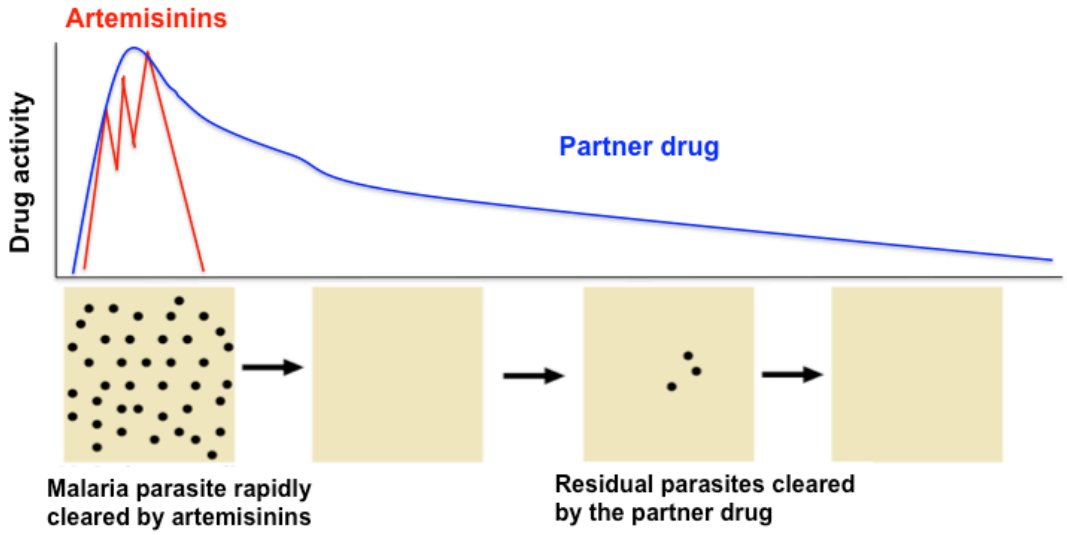
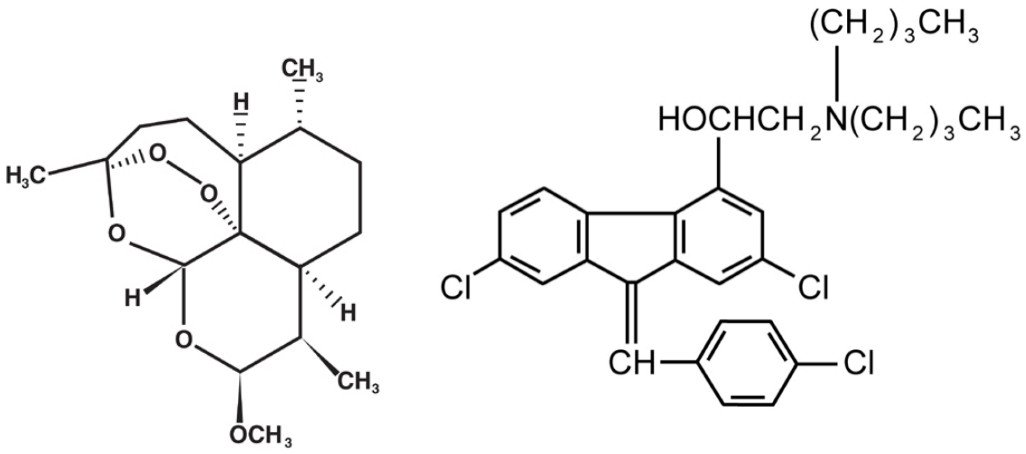


Figure 3. Activity of artemisinins and their partner drugs in the ACT drug combination

1.7.1 Artemether-lumefantrine combination (Coartem®)

Artemether-lumefantrine combination (Coartem®) is a safe and well tolerated 6-dose regimen of artemether (20 mg) co-formulated with lumefantrine (120 mg). Lumefantrine formerly known as benflumetol, is a blood schizontocide that belongs to the arylaminoalcohol group of antimalarials, which also includes mefloquine and halofantrine. It has a half-life of about 3–5 days and offers post treatment antimalarial prophylaxis of up to 4 weeks (Ezzet *et al*., 1998). It is absorbed and cleared more slowly, to eliminate residual parasites that may remain after artemether or DHA have been cleared from the body and thus prevent recrudescence. Lumefantrine is a highly lipophilic drug and its bioavailability is markedly increased if the drug is administered with a fatty meal (Ashley E.A. *et al*., 2007).



Artemether Lumefantrine

Figure 4. The chemical structures of artemether and lumefantrine.

Artemether on the other hand is a semisynthetic artemisinin derivative. Like lumefantrine it's a lipophilic compound. The main active metabolite, of artemether is dihydroartemisinin, which is more potent. Desbutylbenflumetol (DBB), (Ntale *et al*., 2008) the active metabolite of lumefantrine, shows to a certain extent *in vitro* cross-resistance with both artemether and lumefantrine (Noedl *et al*., 2001) warning of a risk for a common mechanism of reduced susceptibility to both drugs (Wernsdorfer, 2004).

1.8 *P. falciparum* in-vivo drug resistance

The clinical treatment failure following appropriate administration of drugs is considered the golden standard for monitoring antimalarial drug resistance, because it is the real world experience. Therefore clinical trials involving treating patients under direct supervision and then following them up for certain duration of time best reflects response to treatment within an area. In the circumstances that less susceptible or resistant parasites exist in an area, during the period of antimalarial treatment, less susceptible parasites may be selected in the human body, a process

termed in vivo selection. This is because the treatment creates drug concentration circumstances that are sufficient to kill susceptible, but not less susceptible parasites. Although clinical trials, may be disadvantaged with logistic demands, and the possibility of detecting parasites resistant to only one of the drugs in the combination, they best reflect the real drug resistance level within a region.

Currently in vivo artemisinin resistance is a major threat to malaria control. In August 2014, WHO developed a working definition for *in vivo* artemisinin resistance based on findings from routine therapeutic efficacy studies of ACTs, clinical trials of artesunate monotherapy, and *pfkelch13* gene sequencing. Suspected partial artemisinin resistance was defined as: presence of 5% or more patients with *pfkelch13* resistance associated mutations; or ≥ 10% of patients with persistent parasitemia by microscopy on day 3 after treatment with ACT or artesunate monotherapy; or ≥ 10% of patients with a parasite clearance half-life of ≥ 5 hours after treatment with ACT or artesunate monotherapy. Confirmed partial artemisinin resistance was defined as: ≥ 5% of patients carrying *pfkelch13* resistance associated mutations, all of whom have been found, after treatment with ACT or artesunate monotherapy, to have either persistent parasitaemia by microscopy on day 3, or a parasite clearance half-life of ≥ 5 hours (WHO Status report on artemisinin resistance). Such criteria were evaluated in this project.

1.9 Drug resistance mechanisms

Drug resistance may result following different mechanisms for instance: (i) Genetic mutations

that alter drug metabolism or change drug targets with eventual loss of the drug's activity; (ii) Decreased access of the drug to its target, especially when the drugs are removed from their cellular compartment by mutations in transporters.

1.9.1 Genes influencing artemether- lumefantrine resistance

1.9.1.1. The Kelch13 propeller gene

In 2014, Ariey et al identified PF3D7_1343700 (*Pfkelch13*), as the gene responsible for artemisinin resistance (Ariey F *et al*., 2014) by establishing an artemisinin-resistant parasite line from the artemisinin-susceptible F32 Tanzania clone following a 125-cycles of stepwise artemisinin selection for 5 years. Whole-genome sequencing of the resistant parasite line together with its parental line identified at least eight SNPs in seven genes of the resistant parasites. The M476I Pfkelch13 mutation was identified as the mutation responsible for artemisinin resistance because: (i) It was the first mutation to be acquired by the resistant parasites during the stepwise drug selection and (ii) it appeared concurrently with a steep increase in parasite survival (Ariey F *et al*., 2014). However, using RSA in 49 culture-adapted parasite isolates obtained from Cambodia, the M476I mutation acquired in the stepwise selection experiment was not observed, but four other mutations (Y493H, R539T, I543T and C580Y) were identified and closely associated with high RSA survival rates (Ariey F, *et al*., 2014). In 2015, direct evidence associating the *Pfkelch13* mutations M476I, Y493H, R539T, I543T or C580Y to artemisinin resistance were reported by Straimer J et al.

1.9.1.1.2 Pfkelch13 protein structure and function

The *Pfkelch13* gene a single exon gene, with orthologues in rodent and simian malaria parasites and is highly conserved among them. It encodes a 726 amino acid protein consisting of three domains. The N-terminal: a *Plasmodium*- specific sequence, a broad-complex, tramtrack, bric-a-brac/poxvirus and zincfinger (BTB/POZ) domain and a C-terminal 6-blade propeller domain of the kelch motif (Ariey F et al 2014). The function of the N-terminal plasmodium-specific sequence is unknown, because it lacks functional motifs. The *Pfkelch13* BTB/POZ domain consists of about 120 amino acids and functions as a mediator for homo-dimerization and in some cases hetero-dimerization with other proteins (Bardwell VJ & Treisman R, 1994). The kelch motif consists of approximately 50 amino acids and its four-stranded anti-parallel beta sheet, forms a blade structure (Adams J *et al*., 2000). This motif is observed as 6–8 repeats in a protein, forming a large toroidal shape termed the beta-propeller domain (Mita *et al* 2016).

According to PlasmoDB data, the Pfkelch13 protein is expressed at the asexual, sexual and transmission stages. As reviewed in Mita *et al*., 2015, Pfkelch13 performs several functions, like; regulation of gene expression (e.g., RAG2) and intracellular transport (e.g., p40). Pfkelch 13 like other proteins with kelch motifs conducts its function via protein–protein interactions through a beta-propeller domain. For instance, in *P. falciparum*, Pfkelch13 exerts its function through interactions between beta-propeller or BTB/POZ domains and other proteins.

As reviewed in Mita et al 2015, *Pfkelch13* has high sequence similarity to the human kelch-like ECH-associated protein 1 (KEAP1), which is a negative regulator of the antioxidant response. Under normal conditions, KEAP1 binds to nuclear factor erythroid 2-related factor 2 (NRF2), a

transcription factor that activates a set of antioxidant responsive genes. The Formation of the KEAP1–NRF2 complex facilitates ubiquitination and subsequent proteasomal degradation of NRF2 in the cytosol, whereby expression of NRF2-regulated antioxidant genes is suppressed. However, the KEAP1–NRF2 complex is disrupted with oxidative stress and NRF2 translocates to the nucleus where it activates a set of antioxidant-responsive genes. Therefore, since malaria parasites are exposed to oxidative stress by artemisinin treatment, it is possible that *Pfkelch13* may function similar to KEAP1, although no NRF2 orthologue has been identified in *Plasmodium* spp.

1.9.1.1.3 Population genetics of pfkelch13

Previously studied antimalarial drugs, showed either one or a few mutations conferred antimalarial resistance, for example the *pfcrt*-K76T mutation for chloroquine resistance and dihydrofolate reductase (*dhfr*)-S108N mutation in pyrimethamine resistance (Mita T *et al*., 2009, Fidock DA *et al*., 2000). In *Pfkelch13* however, several mutations have been associated with artemisinin resistance (Ashley EA *et al*., 2014, Straimer J., 2015). As reviewed by Mita et al, approximately 100 non-synonymous mutations with substantial geographical difference have been reported in Pfkelch13 (Mita *et al*., 2015). The five *Pfkelch13* mutations M476I, Y493H, R539T, I543T and C580Y associated with artemisinin resistance are exclusively distributed in GMSR. Mainly because, ACT was introduced earlier in the GMSR than in other regions and also, the stronger artemisinin pressure in the region may have selected multiple *Pfkelch13* mutations, leading to a considerably higher prevalence of mutant parasites.

Pfkelch13 mutations M476I, Y493H, R539T, I543T and C580Y confer high level artemisinin resistance as determined by RSA survival rate (Straimer J *et al*., 2015), but C580Y is predominantly observed in the field. The wide spread distribution of the C580Y mutation in GMSR suggests the likelihood of this mutation being the most adapted for survival. The wide repertoire of *Pfkelch13* mutations suggests multiple origins of artemisinin resistance (Miotto O *et al*., 2015), contrasting observations of chloroquine and pyrimethamine resistance (Roper C *et al*., 2004).

1.9.1.1.4 Clinical implications of pfkelch13 mutations

Associations between *Pfkelch13* mutations and treatment outcomes have been demonstrated. First, Ashley et al using artemisinin monotherapy (Ashley *et al*., 2014) observed that parasites with non-synonymous *Pfkelch13* mutations were associated with parasite-clearance half-life longer than 5 h (Ashley *et al*., 2014). Another study in China showed that the most prevalent mutation in the area, F446I was significantly associated with much longer parasites clearance half-lives and day 3 parasitemia after artemisinin monotherapy (Huang F et al 2015). One study in Uganda also suggested the potential association between A578S mutation and prolonged parasite-clearance time in children with severe malaria (Hawkes M, *et al*., 2013). In Cambodia, using combination therapy, with follow up duration of 42-day *Pfkelch13* polymorphisms were the most significant risk factors for treatment failure (Leang R *et al*., 2015). Two studies conducted in Africa however showed high ACT efficacy with no significant association between clinical outcomes and *Pfkelch13* polymorphisms (Ouattara A *et al*., 2015, Plucinski MM *et al*., 2015).

1.9.1.1.5 *P.f kelch* 13's role in artemisinin resistance

Different molecular mechanisms and targets have been suggested regarding the role of *Pf kelch* 13 in artemisinin resistance.

1.9.1.1.5.1 Increased production of PI3P

Mbengue et al (Mbengue A et al 2015) reported that the active metabolite of artemisinins (dihydroartemisinin) inhibited the enzymatic activity of *P. falciparum* phosphatidylinositol 3-kinase (PfPI3K), which is required to phosphorylate *P. falciparum* phosphatidylinositol to produce phosphatidylinositol-3-phosphate (PI3P). PI3P reportedly mediates cell signaling and survival in other organisms (Davis WJ *et al.,* 2015). Therefore, in artemisinin-susceptible parasites a decrease in PI3P concentrations induced by artemisinin treatment would impair activation of cell survival signaling pathways, which would be fatal to parasites (Mbengue A *et al*., 2015). In contrast, PI3P concentrations were higher in artemisinin-resistant parasites, suggesting that PI3P-mediated signaling may be important in artemisinin resistance. In more experiments using transgenic parasite lines overexpressed the human VSP34, PI3K, and increased PI3P concentration, the authors observed that the transgenic parasites exhibited higher RSA survival than the parent 3D7 strain, suggesting that PI3P levels would strongly affect artemisinin resistance (Mbengue A *et al*., 2015).

Relating to the role of Pfkelch13 in artemisinin resistance, the authors demonstrated that wild type *Pfkelch13* binds to PfPI3K facilitating K48-linked ubiquitination and subsequent proteasome degradation of PfPI3K. In contrast, *Pfkelch13* mutation C580Y bound less to PfPI3K, causing less degradation of PfPI3K and consequently high PI3P concentrations in the resistant parasites.

Highlighting the role of this mutation in artemisinin resistance.

1.9.1.1.5.2 Up-regulation of ubiquitin/proteasome system

Oxidative stress inhibits protein translation and induces protein degradation by the ubiquitin/proteasome system (Amm I *et al.,* 2014). In the artemisinin-resistant parasites, the ubiquitin/proteasome system is up regulated compared to artemisinin-susceptible parasites (Dogovski C *et al*., 2015). They established that the parasites mount a stress response, which may manifest as growth retardation with engagement of the proteasome-ubiquitin pathway. The stress response in parasites with K13 mutations is enhanced with up-regulation of the ubiquitin/proteasome system, which induces prompt degradation of the damaged proteins. In contrast, Epoxomicin a clinically used proteasome inhibitor inhibits the stress response, promoting parasite death, synergizing the effect of artemisinins. This mechanism is presented in figure 5.

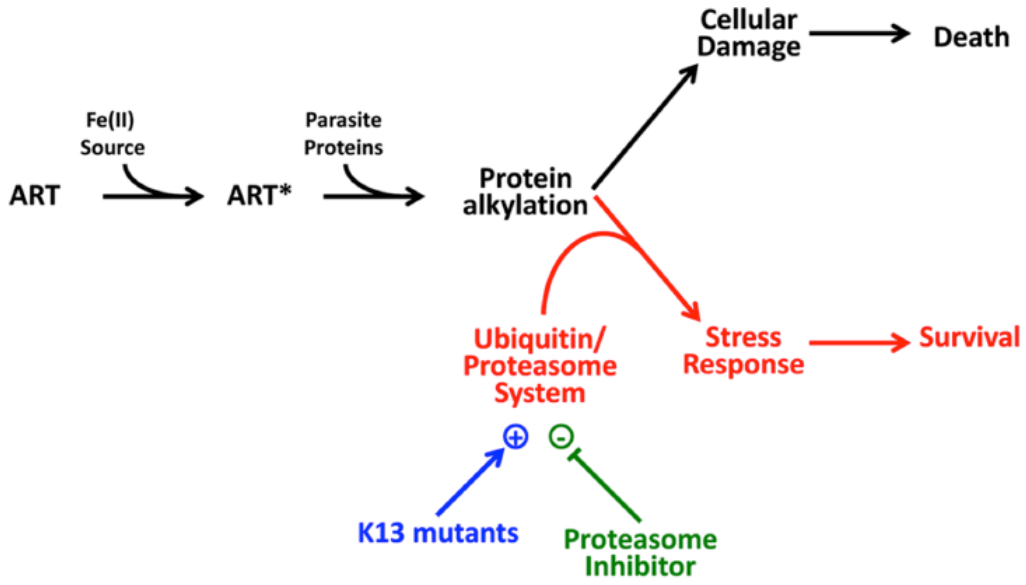


Figure 5 Model of killing and survival-promoting events following treatment with ART, (Dogovski C *et al*., 2015)

1.9.1.1.5.3 Activation of unfolded protein response pathway

Experiments evaluating artemisinin resistance using transcriptomic profiling, showed that most up-regulated genes in the resistant parasites were exported proteins (Witkowski B *et al*., 2010). Mok S et al, in another study conducted in Southeast Asia, observed that artemisinin-resistant parasites displayed modifications of the transcriptional profiles in erythrocytic asexual stages (Mok S *et al*., 2011). Genes related to basic metabolic and cellular pathways in the ring stage were delayed in artemisinin-resistant parasites compared with artemisinin- susceptible parasites. This is partially explained by the observation that artemisinin-resistant parasites express growth retardation at this stage (Dogovski C *et al*., 2015). Functional pathways of the up-regulated genes were largely associated with the unfolded protein response that is also known to be a coping

mechanism in response to endoplasmic reticulum stress, (Hetz C *et al*., 2015) implying that artemisinin resistance weakens potential damage of parasite proteins by artemisinin. Such mechanisms have been observed in parasites with Pfkelch13 resistance associated mutations.

1.9.1.2 Markers of a genetic background for artemisinin resistance

Mutations in Pf kelch13 have been reported to arise on a particular genetic background that is common in Southeast Asia. The strongest markers of this genetic background are non-synonymous mutations in *apicoplast ribosomal protein S10* (arps10) gene on chromosome 14 and *ferredoxin* (fd) on chromosome 13. Other background markers include non-synonymous mutations in *multidrug resistance transporter 2* (mdr2) on chromosome 14, *chloroquine resistance transporter* (crt) on chromosome 7, *protein phosphatase* (*pph*) on chromosome 10 and *phosphoinositide-binding protein* (*pibp*) on chromosome 7 (Miotto O *et al*., 2015). Associations between background markers and Pfkelch13 mutations are multiple and the proportion of samples with Pfkelch13 mutations correlates with the background marker frequency (Miotto O *et al*., 2015). Background markers have also been strongly associated with slow parasite clearance rates highlighting the possibility that they can be regarded as markers of the risk that an artemisinin-sensitive parasite in Southeast Asia will acquire a Pfkelch13 mutation that makes it artemisinin resistant.

1.9.1.3 Pfcrt

The *P.falciparum chloroquine resistance transporter* (*pfcrt*) gene is the main determinant of chloroquine resistance, with K76T as the key mutation mediating resistance (Fidock *et al*., 2000) (Figure 6). Chloroquine acts inside the digestive vacuole where it binds to hematin, preventing the detoxification process of the byproducts produced during haemoglobin digestion (Bray *et al*., 1998). The PfCRT protein is located in the digestive vacuole membrane. The mutated form is believed to function as an exporter, transporting chloroquine out from the digestive vacuole (Valderramos & Fidock, 2006). In addition to its recent involvement in chloroquine resistance**,** PfCRT has also been seen to affect the response to arylaminoalcohols and artemisinins. Sidhu et al observed in a transfection experiment, that acquisition of the *pfcrt* K76T mutation increased the susceptibility to mefloquine and artemisinin (Sidhu *et al*., 2002). Moreover, when 76T is exchanged to K76, the wild type form shows decreased susceptibility to mefloquine and to some extent also to artemisinin (Lakshmanan *et al*., 2005).

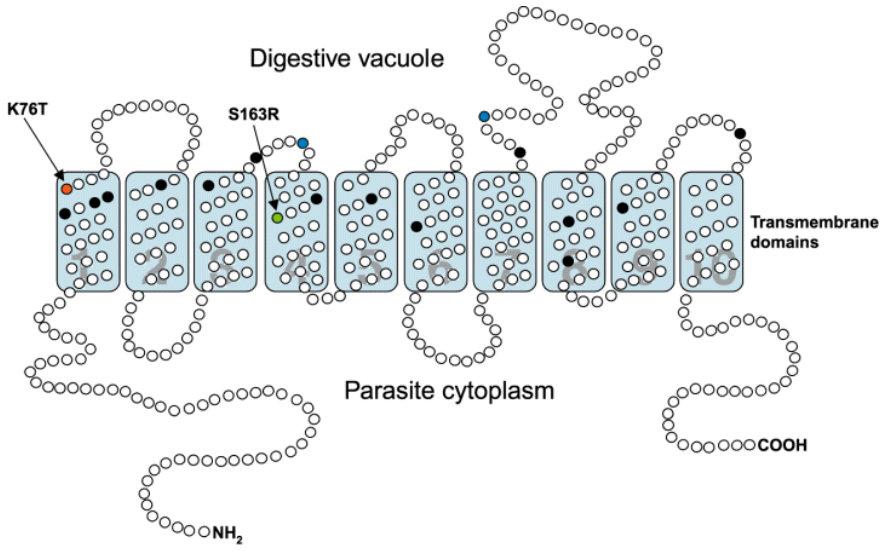


Figure 6 The PfCRT protein, showing the predicted ten trans-membrane domains. Adapted from (Valderramos & Fidock, 2006).

1.9.1.4 Pfmdr1

The *P. falciparum* homologue, P- glycoprotein homologue 1 (Pgh1), was discovered through the cloning of its coding gene, named *P. falciparum multidrug resistance gene 1* (*pfmdr1*) (Foote *et al*., 1989). Pgh1 belongs to the ATP-binding cassette (ABC) transporter superfamily and is made of two domains of six trans-membrane regions, two nucleotide binding domains (NBDs) and Walker A and B conserved sequences, which are typical for ABC transporters (Peel, 2001). The protein is expressed during the asexual erythrocytic stages and is essentially located in the membrane of the digestive vacuole (Cowman *et al*., 1991). Sequencing of *pfmdr1* using laboratory-adapted strains from different geographical areas resulted in the identification of SNPs N86Y, Y184F, S1034C, N1042D and D1246Y, which modulate multi drug susceptibility (Foote *et al*., 1990) (Figure 7).

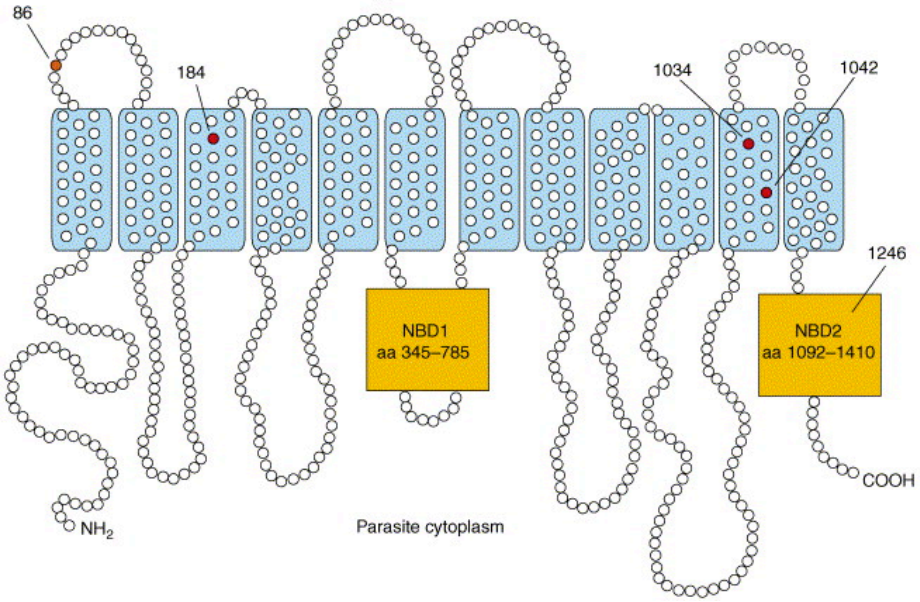


Figure 7 The Pgh1 protein, encoded by *pfmdr1*, with two domains, consisting of six trans-membrane regions. The N86Y, Y184F, S1034C, N1042D and D1246Y SNPs are marked at their respective amino acid position. Adapted from (Valderramos & Fidock, 2006).

Field isolates have shown associations between *pfmdr1* SNPs and susceptibility to mefloquine, lumefantrine and halofantrine, as well as to quinine and artemisinins (Reed *et al*., 2000, Price *et al*., 2006,). Furthermore, gene amplification of *pfmdr1* may have an important role in the mechanism of resistance to several antimalarials. Increased *pfmdr1* copy number has been associated with decreased *in vitro* susceptibility to mefloquine, halofantrine and quinine in field isolates (Price *et al*., 1999). Supporting the available *in vitro* data, *pfmdr1* amplification has been associated with treatment failures after mefloquine, artesunate-mefloquine and artemether-lumefantrine treatment in Southeast Asia (Price *et al*., 2006).

2.0. EVALUATION OF THE THERAPEUTIC EFFICACY OF ARTEMETHER LUMEFANTRINE

2.1 Study Rationale

Since the mid-2000s, artemisinin-based combination therapy (ACT) has been deployed as first-line treatment for uncomplicated *Plasmodium falciparum* malaria in nearly all malaria endemic countries (World Malaria Report 2014). The wide-scale deployment has been regarded as one of the central causes of the recent decline in malaria related morbidity and mortality rates (Eastman RT & Fidock DA., 2009). However, since the first report of artemisinin-resistant *P. falciparum* malaria in Western Cambodia (Noedl H *et al*., 2009), geographical areas of artemisinin resistance have steadily spread into the Greater Mekong sub-region (Ashley EA., et al 2014, WHO Status report on artemisinin resistance). In Africa, although previous clinical trials have demonstrated rapid parasite-clearance after ACT treatment (4ABC Study Group 2011), there is a global concern that artemisinin resistance may invade this region following the path previously observed in chloroquine and sulfadoxine/pyrimethamine resistance (Roper C *et al*., 2004, Mita T *et al*., 2011, Mita T *et al*., 2009, Mita T *et al*., 2009).

In 2014, *pfkelch13* (PF3D7_1343700) was identified as a useful molecular marker for tracking the emergence and spread of artemisinin resistant *P. falciparum*. *Pfkelch13* encodes a 726 amino acid protein with a broad-complex, tramtrack, bric-a-brac/poxvirus and zincfinger (BTB/POZ) domain and a C-terminal 6-blade propeller domain (Ariey F *et al*., 2014). Some mutations in these two domains are associated with delayed parasite-clearance time following artemisinin treatment in Southeast Asia (Ashley EA *et al*., 2014, Ariey F *et al*., 2014). In addition, a recent

genome-wide association study also identified several single nucleotide polymorphisms (SNPs) that are assumed to be background genetic changes for artemisinin resistance, these include; D193Y in *ferredoxin* (*fd*), T484I in *multidrug resistance protein 2*+ (*mdr2*), V127M in the *apicoplast ribosomal protein S10* (*arps10*), I356T in *chloroquine-resistance transporter* (*crt*), V1157L in *protein phosphatase* (*pph*) and C1484F in *phosphoinositide-binding protein* (*pibp*) (Miotto O *et al*., 2015). In Africa, polymorphisms in these genes have been occasionally observed, but mostly different from those reported in Southeast Asia (MalariaGen 2015, Ménard D *et al*., 2016). Making it unclear whether the existence of these mutations in Africa is a consequence of selection induced by anti-malarial use.

During antimalarial treatment, less susceptible parasites can be selected in the human body, a process termed *in vivo* selection. This is because the treatment creates drug concentration circumstances that are sufficient to kill susceptible, but not less susceptible parasites. Previous investigations revealed that artemether-lumefantrine (AL) treatment selected for parasites harbouring alleles with K76 in *pfcrt* and N86, 184F and D1246 in *pfmdr1* (Conrad MD *et al*., 2014, Sisowath C *et al*., 2009, Happi CT *et al*., 2009). However, the possibility of similar *in vivo* selection has not been fully investigated in *pfkelch13* and the putative background genes.

Uganda adopted AL as the first line treatment for uncomplicated malaria in 2004, although actual implementation was in 2006. Since then, marked clinical efficacy has been reported; 0-0.5% of cases with residual parasites by day 3 (Yeka A *et al*., 2016, Yeka A *et al*., 2014, Yeka A *et al*., 2013, Muhindo MK *et al*., 2014, Kapisi J *et al*., 2015, Arinaitwe E *et al*., 2009, Kamya MR *et al*.,

2007, Bukirwa H *et al*., 2006, Yeka A *et al*., 2008, Dorsey G *et al*., 2007) and 1.0 - 6.0% of recrudescence (Yeka A *et al*., 2014, Yeka A *et al*., 2013, Arinaitwe E *et al*., 2009, Bukirwa H *et al*., 2006). All these values were determined microscopically. Molecular assessments using high-sensitive PCR are however able to detect sub-microscopic infection of parasites during follow-up after anti-malarial treatment, some of which might possess resistant phenotypes (McNamara DT *et al*., 2006).

In the absence of a vaccine, malaria control and treatment relies heavily on the use of antimalarial drugs, particularly dramatic for the African continent where the burden is high and cheap alternative drugs are sought. Drug pressure has been identified as one key factor in the emergence of resistance, especially when coupled with drug misuse or when used as monotherapy. Thus, evaluating drug resistance, looking for molecular markers that reliably detect resistant parasites, would support malaria control efforts. Therefore, in this study, we evaluated the therapeutic efficacy of AL in Gulu, Northern Uganda. Recruited individuals were followed up for a period of 28 days after treatment and the presence of parasites was determined using polymerase chain reaction (PCR) to detect sub-microscopic infections. Genotyping of *pfkelch13* polymorphisms, and putative background SNPs for artemisinin resistance in *P. falciparum* recurrent infections was done to evaluate whether AL treatment selected for polymorphisms in a region where it has been used for a long time.

2.2. Study objectives

2.2.1. General objective

To monitor the emergence of *P. falciparum* parasites showing resistance to artemether lumefantrine the currently used antimalarial drug for treating uncomplicated malaria in Uganda, and assessing the existence of parasitemia after treatment.

2.2.2. Specific objectives

1. To evaluate artemether lumefantrine clinical efficacy in Gulu Northern Uganda.
2. To determine whether artemether-lumefantrine treatment selects for polymorphisms in a region where it has been used for a long time.

2.3. Materials and Methods

2.3.1 Study site and study population

The study was conducted at the peak of malaria transmission between May – July and October – November 2014 at St Mary's Hospital Lacor in Gulu district, Northern Uganda. Malaria transmission in the study region is perennial with an estimated prevalence of > 60%, and entomological inoculation rate (EIR) of 100 or more infective mosquito bites per person per year (Uganda Malaria Indicator Survey 2009). *Anopheles funestus* is the major mosquito vector and a few infections are due to *Anopheles gambiae* (Okello PE. *et al*., 2006). Malaria control measures in the region include, indoor residual spraying (IRS), long-lasting insecticide-treated nets (LLINs), malaria case management with ACT and intermittent preventive treatment during pregnancy (IPTp). In particular, IRS has been scaled-up since 2009 to cover 10 high-malaria

burden districts (Apac, Kole, Gulu, Amuru, Nwoya, Pader, Agago, Kitgum, Oyam and Lamwo) in the mid-northern region (Uganda Malaria Indicator Survey 2014-15). Symptomatic individuals with *P. falciparum* positive results by rapid diagnostic test (RDT) were referred to the study physicians. The criteria for recruitment are shown in Supplementary Table 1. Individuals who had received anti-malarial treatment within two weeks prior to enrollment were excluded from AL efficacy study but recruited in the *in vivo* selection study.

2.3.2 Ethics, consent and permissions

Before enrollment, written informed consent was obtained from the participants' parents or guardians, and children aged ≥7 years were assented. The study was reviewed and approved by Lacor Hospital Institutional Research and Ethics Committee (LHIREC) (Study protocol number LHIREC 008/05/2013 and 021/09/13) and regulatory approval was obtained from the Uganda National Council for Science and Technology (UNCST) (HS 1395).

2.3.3. Artemether-lumefantrine treatment and follow-up assessment

For the efficacy study, AL (Coartem®, Novartis 20 mg artemether/120 mg lumefantrine tablets) was orally administered twice daily for 3 days and follow-up assessments were performed on days 1, 2, 3, 7, and 28 after initial drug treatment. Dosage of oral Coartem® was adjusted according to the participant's body weight: one (5–14 kg), two (15–24 kg), or three (25–34 kg) tablets. The drug was given as directly observed treatment (DOTS) for all patients by study nurses and physicians. After each treatment, patients were carefully observed for 30 minutes, and the same dose was re-administered if vomiting occurred. Rescue treatment regimen

(dihydroartemisinin-piperaquine) was administered daily for 3 days to any individuals who failed on the initial AL therapy. If the recruited patients developed severe malaria during follow-up, they were referred to the hospital for parenteral artesunate.

At enrollment, venous blood samples (1 mL) were obtained from the cubital vein before initial treatment except for children < 2 years where finger prick sampling was performed. A finger-prick blood sample of 100µL was obtained at each follow-up visit. Blood was spotted on chromatography filter paper (ET31CHR; Whatman Limited, Kent, UK). Haemoglobin (Hb) concentration was measured using a portable spectrophotometer Hemocue Hb 201 (HemoCue, Ängelholm, Sweden) on days 0 and 28 or on the day of late clinical failure. Anaemic patients with Hb level <10.0 g/dL were treated with Ferrous sulphate tablets for 14 days. Plasma concentrations of artemether and lumefantrine were not measured. Treatment outcomes were classified according to WHO guidelines for areas of intense malaria transmission as: adequate clinical and parasitological response (ACPR), early treatment failure (ETF), late clinical failure (LCF) and late parasitological failure (LPF) (WHO Methods for surveillance of antimalarial drug efficacy).

2.3.4. Microscopic and molecular diagnosis of malaria parasites

Thick and thin blood smears were stained with 2% Giemsa for 30 minutes. The number of parasites was counted per 200 white blood cells (WBCs), assuming 8,000 WBC/μL. Parasite density was calculated by averaging independent counts made by two microscopists. Discordant results (difference in parasite density of >50%) were re-examined by a third microscopist and,

parasite density calculated by averaging two closest counts. Slides were considered negative if after examination of thick smears, no parasite was detected in 100 high power fields. Parasite DNA was extracted from a quarter of dried blood spot (25μL) using a QIAamp DNA Kit (Qiagen, Hilden, Germany) (Sakihama N *et al*., 2001). In all first visit and follow-up cases, *P. falciparum* infections were assessed by species-specific PCR as previously described (Rubio JM *et al*., 1999). Genotyping of *merozoite surface protein 1* (*msp1*), *merozoite surface protein 2* (*msp-2*) and *glutamate rich protein* (*glurp*), was also performed to differentiate between recrudescence and new infections on day 0 and the day of positive infection (Tanabe K *et al*., 1999, WWARN: Tools and resources/procedures). Nested PCR products were analysed by electrophoresis using 2% agarose for *msp-1* and *msp-2* and 1.5% agarose for *glurp*. Patient samples were run side by side. Gel images were digitised and molecular weights determined using imagej software, an open platform for scientific image analysis. Densitometric curves were generated for each gel lane, and dominant bands in each lane were assigned molecular weights. Using reference strains, alleles were considered the same if molecular weights were within 10 bp for *msp 2* and 20 bp for *glurp*.

2.3.5. Genotyping of drug-resistant genes

Polymorphisms in *pfcrt* (K76T) and *pfmdr1* (N86Y, Y184F, S1034C, N1042D and D1246Y) were determined as reported (Duraisingh MT *et al*., 2000, Takahashi N *et al*., 2012). K13-propeller domain was amplified by nested PCR, covering almost all the six propeller domain sequences, as described (Ariey F et al., 2014). The sequences were aligned using MUSCLE in MEGA software, version 6.06 (Tamura K et al., 2013) with *P. falciparum* 3D7 full-length sequence of K13-propeller domain (PF3D7_1343700) from PlasmoDB (PlasmoDB database) as

reference.

Six background mutations for artemisinin resistance (D193Y in *fd*, T484I in *mdr2*, V127M in *arps10*, I356T in *crt*, V1157L in *pph* and C1484F in *pibp*) were amplified by multiplex PCR using gene specific primers (Table 2). In brief, 10 μL reaction mixture consisted of 1 μL of DNA template, 0.5 μM of 6-primer sets, and PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Otsu, Japan). Cycling conditions were: denaturation at 98ºC for 10 s, followed by 40 cycles of amplification (98ºC for 10 s, 60ºC for 10 s, and 72ºC for 90 s), with a final elongation period of 90 s at 72ºC. ExoSAP-IT Kit was used for the purification of PCR products. SNP typing was performed on amplified products with 5 μL of reaction mixture consisting of 2.5 μL of Premix Ex Taq (Probe qPCR) (Takara Bio Inc.), 0.2 μM of each primer, 0.1 μM of LNA probe set, 0.05 μL of ROX Reference Dye II, and 0.5 μL of template DNA using the 7500 Real-Time PCR System (Applied Biosystems). Primers and probes for SNP assay are also shown (Supplementary Table 2). The probes for detecting wild type and mutant SNPs were labeled with HEX and 6-FAM (6-carboxyfluorescein) as reporters at 5' end, respectively. All probes contained Iowa Black® FQ (IBFQ) as a dark quencher at 3' end.

To evaluate performance of the SNP assay system in all 6 genes, two *P. falciparum* strains, wild type (3D7) and a mutant-type (a strain from Thailand identified during preliminary experiments) were used as positive controls. Nucleotide sequence data are available in the GenBank™, EMBL, and DDBJ databases under the accession numbers: LC193525 - LC193693.

2.3.6. Statistical analysis

Kaplan–Meier product limit formula was used for the estimation of day 28-PCR-adjusted cure rates, which was the primary efficacy endpoint. The uncorrected and PCR-corrected Kaplan–Meier cumulative treatment success rates up to day 28 were calculated for all participants. Patients were censored when either lost to follow-up or withdrawn from the study. Either Fisher's exact test or Pearson's chi-square test (χ^2) test were used for comparison of categorical variables, and Wilcoxon rank sum test was used for the continuous variables. *P* values < 0.05 were considered statistically significant. R statistical software (version 3.2.0; R Foundation for Statistical Computing) was used for all the statistical analyses.

2.4 Results

2.4.1 Studied individuals

A total of 672 patients were screened, out of which 169 microscopically confirmed *P. falciparum* mono-infection were recruited. Sixty four of the 169 patients met the inclusion criteria for the artemether-lumefantrine therapeutic efficacy study (Figure. 8). The remaining 105 patients were excluded due to: antimalarial-drug usage within two weeks (n = 46), living outside the study catchment area (n = 18), severe malaria (n = 17) and for other reasons (n = 24). Of the 64 enrolled individuals, two were lost to follow-up and one was withdrawn for taking antimalarial drugs outside the study protocol. This resulted in 61successfully followed-up cases (retention rate = 95.3%). Mean age and parasitemia of the studied individuals were 3.3 years and 11,579/μL (parasite density = 0.26%), respectively (Table 1). Older children (≥ 5 years) tended to have higher parasitemia as compared to the younger children < 5 years though not statistically significant ($p = 0.31$). Other characteristics remained comparable between age groups.

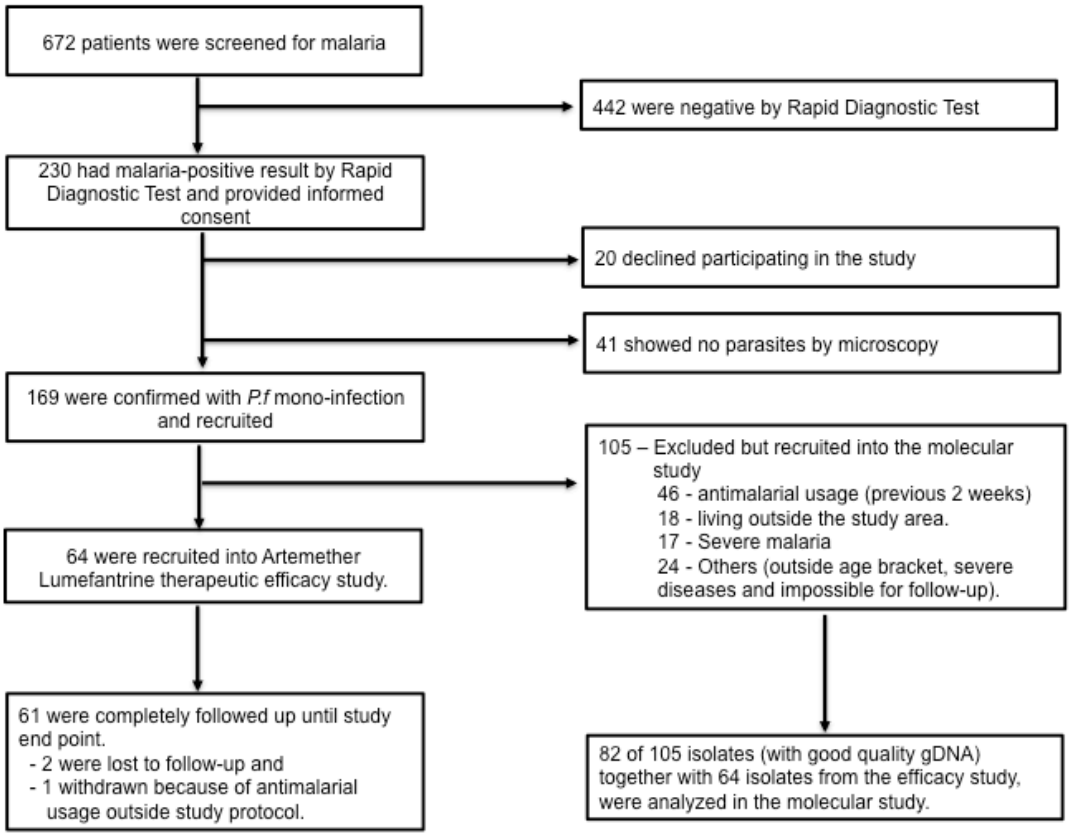


Figure 8. Study Design of Artemether-lumefantrine therapeutic efficacy study.

Table 1 Characteristics of 61 individuals in artemether-lumefantrine efficacy study at enrollment

Characteristic	**< 5 years (n = 49)**	**≥ 5 years (n = 12)**
Gender ratio, Male/female	32/17	5/7
Age (years)	2.7 ± 13.0	5.9 ± 18.8
Temperature (ºC)	38.4 ± 1.3	38.5 ± 1.0
Geometric mean parasitaemia (No. of parasites/μL)	10,429.8 ± 10.4	17,746.4 ± 12.4
Haemoglobin (g/dL)	10.0 ± 1.9	10.8 ± 2.5
Gametocytes present (n)	4 (8.2%)	1 (8.3%)

± values are means and SD

2.4.2 Artemether-lumefantrine treatment outcomes

Excellent early response to AL treatment was observed in both age groups (Table 2). Fever was cleared by day 2 in almost all patients. Only one (1.6%) child (4 years and 9 month-old male) showed microscopically residual parasites on day 3. His parasite density was 1.0% at enrollment, gradually decreased on days 1 (0.46%) and 2 (0.38%), and was still present by day 3 (0.22%, 9960 parasites/μL). The 3 days rescue regimen of dihydroartemisinin-piperaquine was, therefore, administered, and live parasites disappeared. However, pyknotic parasites persisted until day 7. Prevalence of parasite-positive individuals on day 3 as assessed by PCR was 22.9%, whereas it was 1.6% by microscopy. Individuals that were parasite positive by PCR (19,026.9 ± 11.3, n = 11) on day 3 had significantly (p = 0.0111) higher median parasitaemia at enrollment than the PCR negative group (9,987.1 ± 10.5, n = 48). This suggests that parasite biomass before treatment may also be associated with treatment success and PCR parasite-positive outcome on day 3 (Figure 9).

Table 2 Response to artemether-lumefantrine treatment

Characteristic	Age		Total (n = 61)
	< 5 years (n = 49)	≥ 5 years (n = 12)	
Fever (≥ 37.5°C) persistence, n (%)			
Day 1	11 (22.4)	1 (8.3)	12 (19.7)
Day 2	3 (6.1)	0	3 (4.9)
Day 3	0	0	0
Parasite persistence			
Microscopy, n (%)			
Day 1	40 (81.6)	11 (91.7)	51 (83.6)
Day 2	13 (26.5)	5 (4.2)	18 (29.5)
Day 3	1 (2.0)	0	1 (1.6)
PCR, n (%)			
Day 1	45 (91.8)	11 (91.7)	56 (91.8)
Day 2	27 (55.1)	6 (50)	33 (54.1)
Day 3	11 (22.4)	3 (25)	14 (22.9)
Gametocyte persistence, n (%)			
Day 1	4 (8.2)	1 (8.3)	5 (8.2)
Day 2	3 (6.1)	1 (8.3)	4 (6.6)
Day 3	3 (6.1)	1 (8.3)	4 (6.6)
28-day WHO Treatment Outcome, n (%)			
Early Treatment Failure	0	0	0
Late Clinical Failure	2 (4.1)	1 (8.3)	3 (4.9)
Late Parasitological Failure	0	0	0
Adequate Clinical and Parasitological Response	47 (96.0)	11 (91.7)	58 (95.1)
Cure rate, n (%)			
PCR Unadjusted	47 (96.0)	11 (91.7)	58 (95.2)
PCR Adjusted	47 (96.0)	11 (91.7)	58 (95.2)

Abbreviations: **PCR**: polymerase chain reaction; **WHO**: World Health Organization.

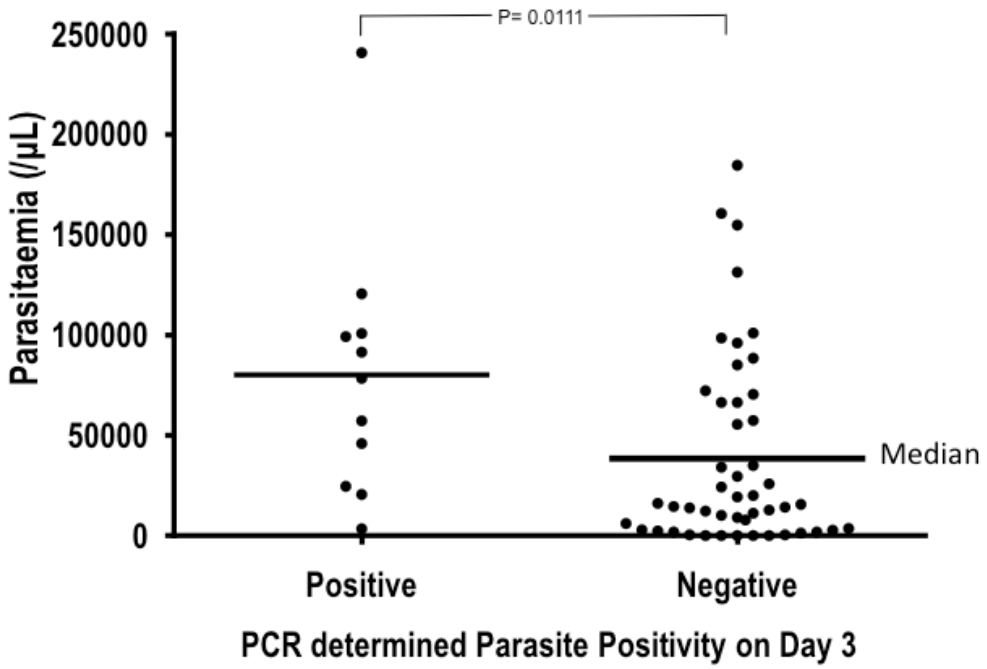


Figure 9 Enrollment parasitaemia as compared between the PCR positive (n=11) and PCR negative group (n = 48) to evaluate its impact on day 3 parasite positivity.

Similar to the excellent early treatment response, efficacy of AL was high with ACPR ratio of 95.2% (Table 2). Only three individuals showed LCF on day 28 and were later confirmed as reinfection by *msp1*, *msp2* and *glurp* genotyping (Supplementary Table 3). These children were all male, aged; 11 months, 3 and 6 years with enrollment temperature of 36.8ºC, 39.2ºC and 39.1ºC and initial parasitaemia of 1,400/µL (0.03%), 99,200/µL (2.2%) and 96,080/µL (2.1%), respectively. Although all were parasite negative by day 2, they had malaria recurrence on day 28 with parasitaemia of 1,840/µL (0.04%), 8,460/µL (0.2%) and 32,120/µL (0.7%), respectively. The children were successfully treated with the rescue regimen. Gametocytes were observed in five individuals (8.2%) at enrolment, and in two cases, persisted until day 7 after AL treatment.

2.4.3 Drug-resistance related alleles at enrollment

In addition to 64 patients for the therapeutic efficacy study, 105 patients who did not meet inclusion criteria for the therapeutic efficacy study were recruited into the molecular epidemiological study for drug resistance. In total, 146 *P. falciparum* confirmed blood samples (82/105 with good quality gDNA + 64 from the therapeutic efficacy study) were used for genotyping of drug-resistance related alleles. Amino acid substitutions in Pfkelch13, which have been associated with artemisinin resistance in the Greater Mekong Sub region (Ariey F., et al 2014) were not detected; almost all (98.6%) carried wild-type allele and only A578S mutation was observed in two samples (1.4%) (Table 3). The six SNPs previously identified as background genetic changes for artemisinin resistance in Southeast Asia, were also genotyped (Miotto O., et al 2015). All isolates harboured wild-type alleles in *fd*, *mdr2*, *arps10*, *pph* and *pibp*. Mutant alleles were only observed in *pfcrt* as mixed alleles (I356 + 356T) (n = 3). Regarding the other polymorphisms in *pfcrt*, K76T, which is known to be the responsible genetic change for chloroquine resistance, was observed in 29.2% of the isolates. In *pfmdr1*, wild-type alleles were nearly fixed at all known polymorphic positions other than 184; position 86 (98.0%), 1034 (98.6%), 1042 (100%) and 1246 (93.7%), respectively. At position 184, the prevalence of wild type alleles was high (80.4%), but as above, was significantly lower than at the other positions ($p < 0.0001$ by Pearson's chi-square test).

Table 3 Prevalence of amino acid substitutions in the putative drug-resistance related genes in *Plasmodium falciparum* isolates collected before artemether-lumefantrine treatment.

Gene	Amino acid Position	Genotypes		
		Wild type (n,%)	**Mutant (n, %)**	**Mixed (n, %)**
Pfkelch13		141 (98.6)	2 (1.4) **A578S**	0
fd	D193Y	127 (100)	0	0
mdr2	T484I	126 (100)	0	0
arps10	V127M	127 (100)	0	0
pph	V1157	122 (100)	0	0
pibp	C1484F	127 (100)	0	0
Pfcrt	K76T	98 (68.1)	42 (29.2)	4 (2.7)
	I356T	122 (97.6)	0	3 (2.4)
Pfmdr1	N86Y	141 (98.0)	3 (2.1)	0
	Y184F	115 (80.4)	14 (9.8)	14 (9.8)
	S1034C	143 (98.6)	2 (1.4)	0
	N1042D	145 (100)	0	0
	D1246Y	134 (93.7)	9 (6.0)	0

2.4.4 *In vivo* selection of drug-resistance related alleles after artemether-lumefantrine treatment

Plasmodium falciparum positivity after AL treatment was assessed by PCR in 61 individuals who were treated and successfully completed 28 days of follow-up. There were 23 PCR positive samples: 14 on day 3, 4 on day 7, and 5 on day 28. In *pfkelch13* and the six background genes for artemisinin resistance (genes encoding for ferredoxin, multiple resistance protein 2, apicoplast ribosomal protein S10, PfCRT protein, protein phosphatase and phosphoinositide-binding protein), all isolates carried wild-type alleles on day 3, 7 and 28 (Figure 10). All these observations were also found when the analysis was focused on a larger sample size involving

103 individuals (64 enrolled in the therapeutic-efficacy study + 39/46 with good quality DNA and who were excluded from the efficacy study due to previous antimalarial use but treated and followed-up) (Figure 11). Amino acid position 76 in *pfcrt*, wild-type alleles were predominant at both day 0 (66.3%) and the follow-up period (50% on day 3, 66.7% on day 7, and 100% on day 28) (Figure 12). In *pfmdr1*, wild-type alleles were nearly fixed at all polymorphic amino acid positions except for 184 on day 0. These alleles were completely fixed on the all follow-up days (day 7 and 28) as well. In contrast, position 184 remained polymorphic throughout the follow-up period without any trend of particular alleles except on day 7 where mutant alleles predominated (Figure 13) in a smaller sample cohort of 61 individuals.

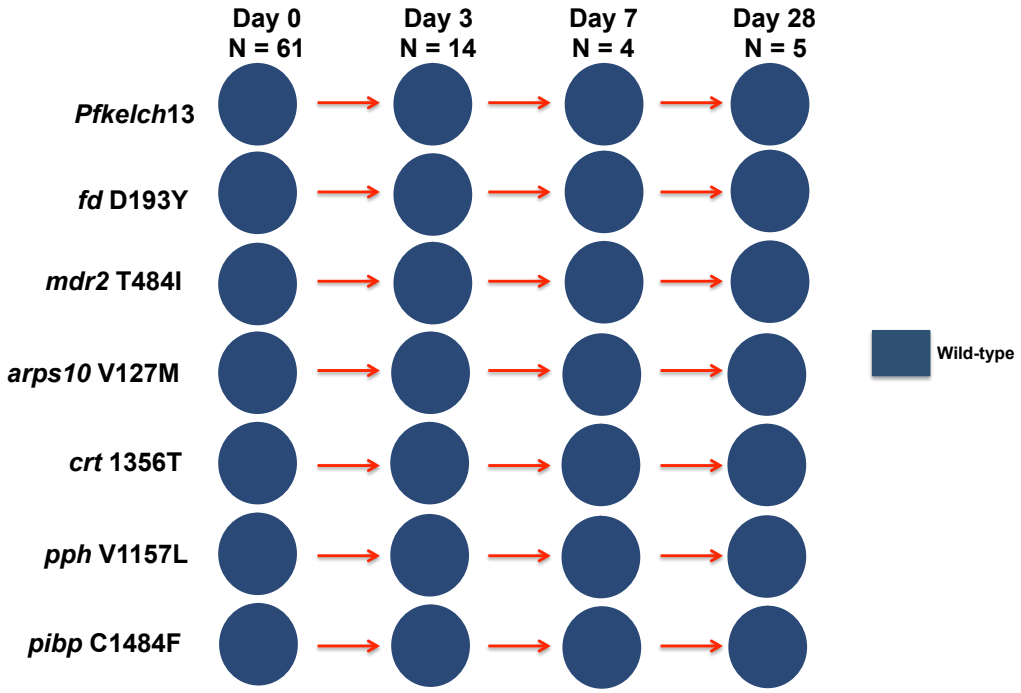


Figure 10 Allele prevalence in *Pfkelch13*, *fd*, *mdr2*, *arps10*, *crt*, *pph* and *pibp* among 61 isolates collected before and after artemether-lumefantrine treatment. Parasite genotypes were characterized at the time of presentation with malaria (day 0) and for infections detected within 28 days after treatment with artemether-lumefantrine (AL). n values represent the number of samples analysed on each day. Wild-type genotypes are also indicated.

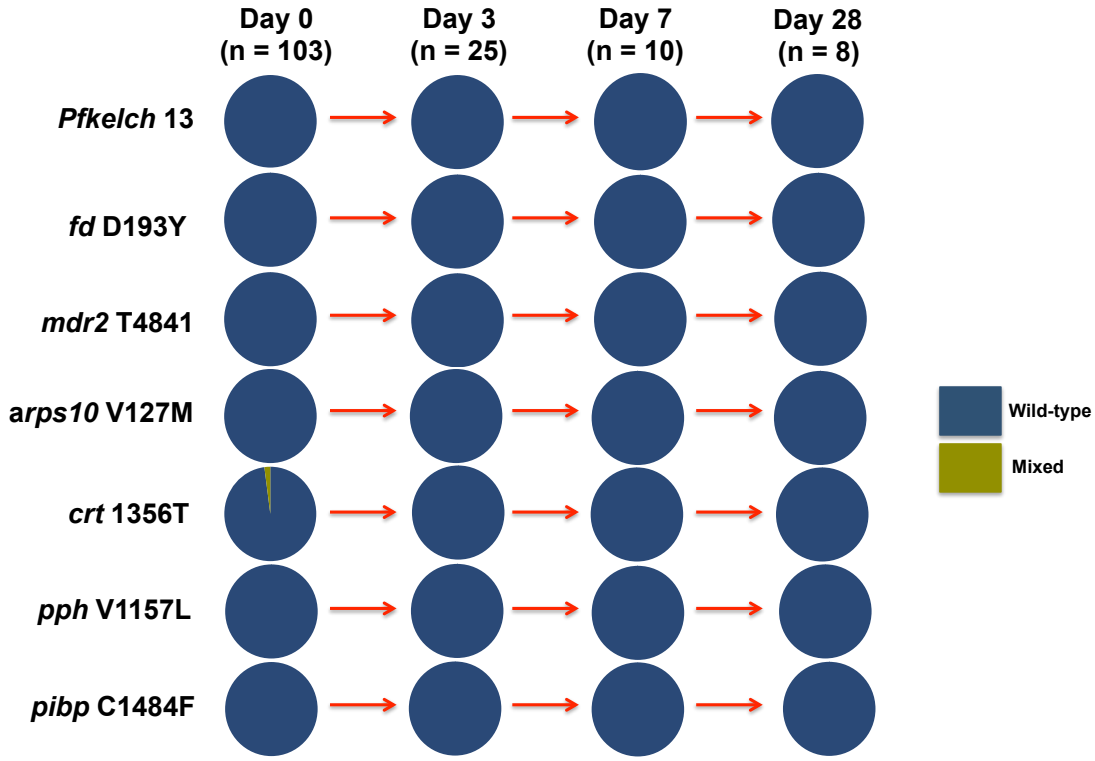


Figure 11 Allele prevalence in *Pfkelch13*, *fd*, *mdr2*, *arps10*, *crt*, *pph* and *pibp* among 103 isolates collected before and after artemether-lumefantrine treatment. Parasite genotypes were characterized at the time of presentation with malaria (day 0) and for infections detected within 28 days after treatment with artemether-lumefantrine (AL). n values represent the number of samples analysed on each day. Wild-type genotypes are also indicated.

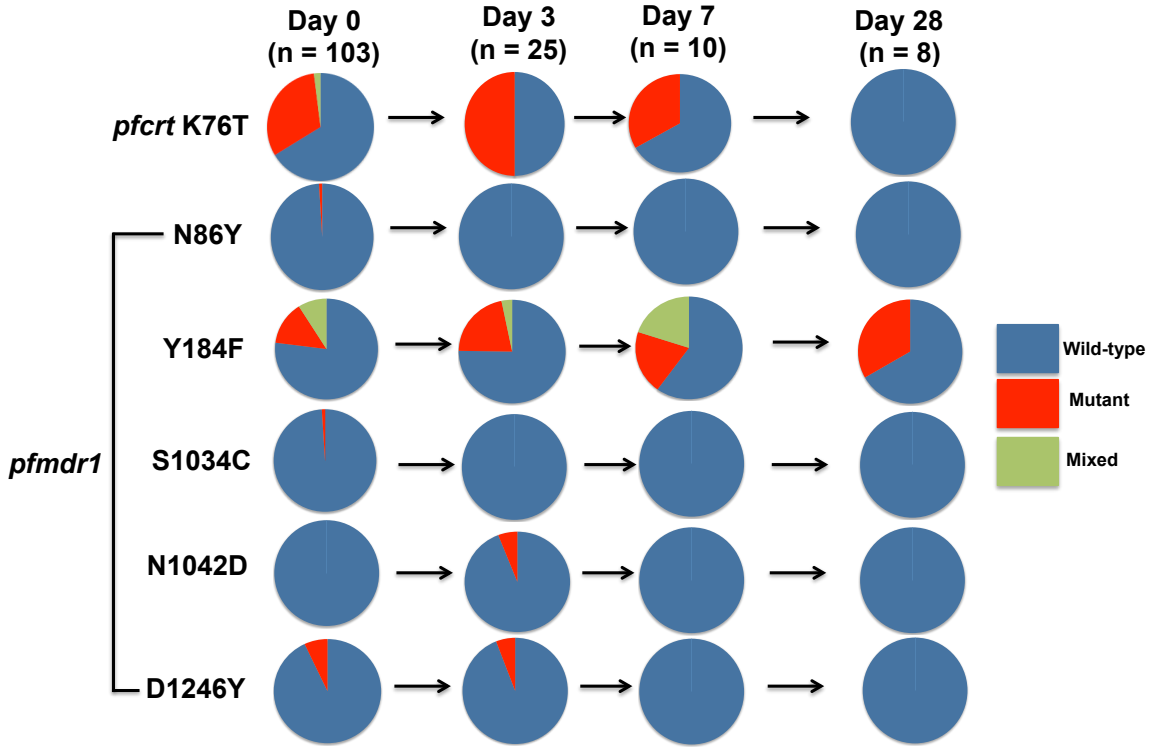


Figure 12 Allele prevalence in *pfcrt* K76T and *pfmdr1*among 103 isolates collected before and after artemether-lumefantrine treatment. Parasite genotypes were characterized at the time of presentation with malaria (day 0) and for infections detected within 28 days after treatment with artemether-lumefantrine (AL). n values represent the number of samples analysed on each day. Wild-type, mixed, and mutant genotypes are indicated.

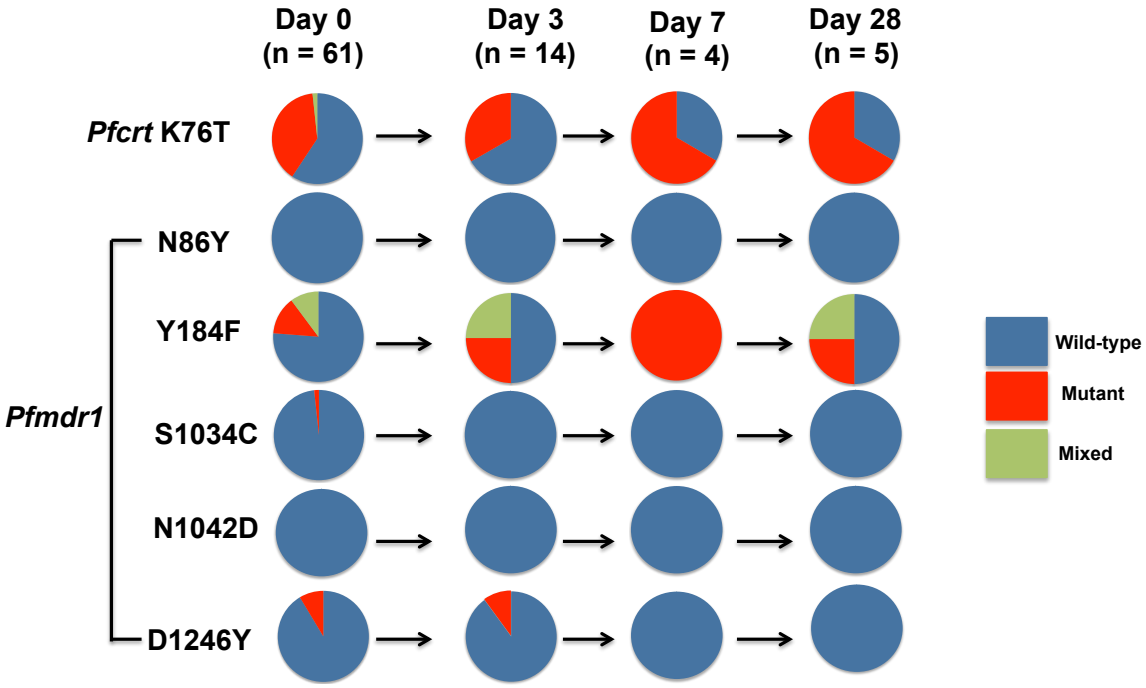


Figure 13 Allele prevalence in *pfcrt* K76T and *pfmdr1*among 61 isolates collected before and after artemether-lumefantrine treatment. Parasite genotypes were characterized at the time of presentation with malaria (day 0) and for infections detected within 28 days after treatment with artemether-lumefantrine (AL). n values represent the number of samples analysed on each day. Wild-type, mixed, and mutant genotypes are indicated.

2.5 Discussion

The present study revealed that both early and late responses to AL were still excellent in this study region of northern Uganda even after 8 years of its actual implementation as first-line treatment. Only one individual (1.6%) showed microscopically detectable parasites by day 3. Overall, similar excellent early response to AL treatment has been reported in other regions in Uganda (Yeka A *et al*., 2016, Yeka A *et al*., 2014, Yeka A *et al*., 2013, Muhindo MK *et al*., 2014, Kapisi J *et al*., 2015, Arinaitwe E *et al*., 2009, Kamya MR *et al*., 2007, Bukirwa H *et al*., 2006, Yeka A *et al*., 2008, Dorsey G *et al*., 2007). According to WHO criteria (WHO. Global plan for artemisinin resistance containment) an endemic region showing ≥ 10% cases with detectable parasites on day 3 after ACT treatment is regarded as an area with suspected artemisinin resistance. The Worldwide Antimalarial Resistance Network (WWARN) proposes a more sensitive benchmark of 5% threshold for sub-Saharan Africa because of higher levels of herd immunity to malaria in the region (WWARN; ACT Africa Baseline Study Group). In all cases, however, the prevalence of day 3 parasite positive individuals in the present study was less than the benchmarks for artemisinin-resistance.

PCR-confirmed parasite positivity after AL treatment was much higher than microscopically confirmed positivity; 91.8% on day 1, 54.1% on day 2, and 23% on day 3. These prevalences were similar to two previous studies that molecularly assessed parasite positivity in Kenya and Tanzania (Beshir KB *et al*., 2013, Carlsson AM *et al*., 2011). Beshir *et al*. reported that PCR-confirmed parasite positivity on day 3 would be a good predictor for malaria recurrence (Beshir KB *et al*., 2013). In this study, however, recurrence frequencies did not differ much between the

PCR-confirmed parasite positive group (7.7%) and the PCR-confirmed parasite negative group (4.2%) on day 3.

Mechanisms of artemisinin resistance have been gradually uncovered albeit the overall picture has not been clarified. Enhanced stress response including activation of unfolded protein response and the PI3K/Pi3P/AKT pathway is thought to be the main mechanism for parasite survival in the presence of artemisinin (Mita T, *et al*., 2016, Paloque L *et al*., 2016). Pfkelch13 has been elucidated to be involved in these processes (Mok S, *et al*., 2011, Dogovski C *et al*., 2015). In the present analysis, however, nearly all parasites harboured wild-type alleles in *pfkelch13* at enrollment. The only mutation observed in Pfkelch13 was A578S, which has been widely distributed in Africa (MalariaGen. 2015, Ménard D *et al*., 2016). Computational modelling reported that A578S could potentially disrupt the normal function of the Pfkelch13 protein (Mohon AN *et al*., 2014). However, only one study has described a close link between A578S and prolonged parasite clearance after artemisinin treatment (Hawkes M *et al*., 2015) and others reported no association (Ménard D *et al*., 2016, Muwanguzi J *et al*., 2016, Ouattara A *et al*., 2015). Very recently, it has been described that introduction of A578S mutation into Dd2 did not change the *in vitro* artemisinin susceptibility determined by ring-stage survival assay (Ménard D *et al*., 2016). This observation partially supports the idea that A578S is not an artemisinin resistance related mutation. However, acquirement of artemisinin resistance would be a consequence of multiple genetic changes. As observed in this study and others (Miotto O *et al*., 2015), genetic background was different between African and Southeast Asian parasites. Since Dd2 clone is derived from Indochina, similar transfection studies using African parasite in addition to further *in vivo* efficacy study and population genetic assessment would be required to

determine the potential role of A578S mutation.

In vivo selection analysis revealed that *pfkelch13* mutation was not observed in the parasite positive samples on day 3, 7 and 28, consistent with the recent observations Muwanguzi J *et al*., 2016). Also, no selection of putative six non-synonymous polymorphisms was observed, suggesting that these genetic changes would not be responsible for parasite persistence in the present study. In contrast, Pfcrt K76 and Pfmdr1 N86/D1246 were observed in all recurrent parasites. Prevalence of Pfmdr1 Y184F (33.3%) in the recurrent patients was higher than baseline (14%), although not statistically significant. These observations support the potential selection of Pfcrt K76 and Pfmdr1 N86/Y184F/D1246 after AL treatment (Sisowath C *et al*., 2009, Happi CT *et al*., 2009). *In vivo* selection of these mutations would increase these allele prevalences in the parasite population. In fact, the analysis herein revealed much higher allele frequencies than previously reported (Ashley EA *et al*., 2014); 68% *vs* 0% in the Pfcrt K76, 98.0% *vs* 9.5% in Pfmdr1 N86 and 93.7% *vs* 16.9% in Pfmdr1 D1246.

2.6 Conclusions

2.6.1 Overall conclusion

The overall conclusion of this thesis is that artemether lumefantrine treatment remains of high efficacy for the treatment of *P. falciparum* malaria in Uganda, and mutations in *pfkelch13* and the six background genes may not play an important role in the *in vivo* selection of polymorphisms associated resistance after artemether-lumefantrine treatment in Uganda. Different mechanisms might rather be associated with the existence of parasites after treatment.

2.6.2 Specific conclusions

1. Resistance to artemisinins does not seem to be a problem currently in Uganda and other African countries as evidenced by the low prevalence of individuals with delayed parasite clearance on day 3
2. *Pfkelch13* and the six background genes are not playing a role in the delay in parasite clearance and malaria recurrence observed in this study since all infections carried wild type alleles.
3. Resistance to lumefantrine may be multigenic, including *pfmdr1* and *pfcrt* and the observation of Pfcrt K76 and Pfmdr1 N86/Y184F/D1246 after AL treatment supports the potential selection of these alleles and supports their use as markers for *in vivo* lumefantrine tolerance, especially in Africa.

3.0 Personal reflections and future perspectives

The emergence of artemisinin resistance seems to be restricted to the Greater Mekong Subregion. Emergence of artemisinin-resistant parasites particularly in Africa, is of great concern. One distinct feature of *Pfkelch13* is that a number of SNPs can confer resistance, and multiple resistant lineages have been evidenced, (Miotto O., et al 2015) which may suggest the risk of potential independent emergence of artemisinin resistance in Africa. The acquirement of artemisinin resistance is also a consequence of accumulation of *Pfkelch13* mutations and multiple background genetic changes such as non-synonymous the six background genes (Miotto O., et al 2015). However, No clear evidence was obtained for the selection of mutant alleles in *pfkelch13* and the six-background genes, all of which have been reported to be associated with artemisinin resistance in Southeast Asia. Close monitoring of AL efficacy is therefore necessary in an effort to understand the influence of anti-malarial treatments and parasite persistence, in different genetic background of parasites and host interactions involved in the mutational process.

3.1 References

Adams, J., Kelso, R., & Cooley, L. (2000). The kelch repeat superfamily of proteins: propellers of cell function. *Trends Cell Biol*, *10*(1), 17-24.

Amm, I., Sommer, T., & Wolf, D. H. (2014). Protein quality control and elimination of protein waste: the role of the ubiquitin–proteasome system. *Biochim Biophys Acta*, *1843*(1), 182-196.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A. C., Khim, N., ... & Lim, P. (2014). A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature*, *505*(7481), 50-55.

Arinaitwe, E., Sandison, T. G., Wanzira, H., Kakuru, A., Homsy, J., Kalamya, J., ... & Tappero, J. (2009). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for falciparum malaria: a longitudinal, randomized trial in young Ugandan children. *Clin Infect Dis*, *49*(11), 1629-1637.

Ashley, E. A., Stepniewska, K., Lindegårdh, N., Annerberg, A., Kham, A., Brockman, A., ... & Nosten, F. (2007). How much fat is necessary to optimize lumefantrine oral bioavailability?. *Trop Med Int Health*, *12*(2), 195-200.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., ... & Sopha, C. (2014). Spread of artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med*, *371*(5), 411-423.

Bardwell, V. J., & Treisman, R. (1994). The POZ domain: a conserved protein-protein interaction motif. *Genes Dev*, *8*(14), 1664-1677.

Beshir, K. B., Sutherland, C. J., Sawa, P., Drakeley, C. J., Okell, L., Mweresa, C. K., ... & Chilongola, J. (2013). Residual Plasmodium falciparum parasitemia in Kenyan children after artemisinin-combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. *J Infect Dis*. *208*(12), 2017-2024.

Björkman, A., & Bhattarai, A. (2005). Public health impact of drug resistant Plasmodium falciparum malaria. *Acta tropica*, *94*(3), 163-169.

Bray, P. G., M. Mungthin, R. G. Ridley & S. A. Ward, (1998) Access to hematin: the basis of chloroquine resistance. *Mol Pharmacol* **54**: 170-179.
Bukirwa H, Yeka A, Kamya MR, Talisuna A, Banek K, Bakyaita N, et al. Artemisinin combination therapies for treatment of uncomplicated malaria in Uganda. PLoS Clin Trials. 2006;1:e7.

Carlsson, A. M., Ngasala, B. E., Dahlström, S., Membi, C., Veiga, I. M., Rombo, L., ... & Mårtensson, A. (2011). Plasmodium falciparum population dynamics during the early phase of anti-malarial drug treatment in Tanzanian children with acute uncomplicated malaria. *Malaria journal*, *10*(1), 380.

CDC Global Health-Division of Parasitic Diseases and Malaria https://www.cdc.gov/malaria/malaria_worldwide/impact.html

Conrad, M. D., LeClair, N., Arinaitwe, E., Wanzira, H., Kakuru, A., Bigira, V., ... & Dorsey, G. (2014). Comparative impacts over 5 years of artemisinin-based combination therapies on *Plasmodium falciparum* polymorphisms that modulate drug sensitivity in Ugandan children. *J Infect Dis*, *210*(3), 344-353.

Cowman, A. F., S. Karcz, D. Galatis & J. G. Culvenor, (1991) A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. *J Cell Biol* 113: 1033-1042.

Cox-Singh, J., & Singh, B. (2008). Knowlesi malaria: newly emergent and of public health importance?. *Trends in parasitology*, *24*(9), 406-410.

Davis, W. J., Lehmann, P. Z., & Li, W. (2015). Nuclear PI3K signaling in cell growth and tumorigenesis. *Front Dev Biol*, *3*, 24.

Dogovski, C., Xie, S. C., Burgio, G., Bridgford, J., Mok, S., McCaw, J. M., ... & Bozdech, Z. (2015). Targeting the cell stress response of Plasmodium falciparum to overcome artemisinin resistance. *PLoS Biol*, *13*(4), e1002132.

Dorsey, G., Staedke, S., Clark, T. D., Njama-Meya, D., Nzarubara, B., Maiteki-Sebuguzi, C., ... & Rosenthal, P. J. (2007). Combination therapy for uncomplicated falciparum malaria in Ugandan children: a randomized trial. *Jama*, *297*(20), 2210-2219.

Duraisingh, M. T., Jones, P., Sambou, I., von Seidlein, L., Pinder, M., & Warhurst, D. C. (2000). The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol*, *108*(1), 13-23.

Eastman, R. T., & Fidock, D. A. (2009). Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat Rev Microbiol*, *7*(12), 864-874.

Enserink, M. (2007). Malaria treatment: ACT two. *Science*, *318*(5850), 560-563.

Ezzet, F., Mull, R., & Karbwang, J. (1998). Population pharmacokinetics and therapeutic response of CGP 56697 (artemether+ benflumetol) in malaria patients. *Br J Clin. Pharmacol*. *46*(6), 553-561.

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., ... & Wootton, J. C. (2000). Mutations in the *P. falciparum* digestive vacuole transmembrane

protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell*, *6*(4), 861-871.

Foote, S. J., Thompson, J. K., Cowman, A. F., & Kemp, D. J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. f alciparum*. *Cell*, *57*(6), 921-930.

Foote, S. J., D. E. Kyle, R. K. Martin, A. M. Oduola, K. Forsyth, D. J. Kemp & A. F. Cowman, (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. *Nature* **345**: 255- 258.

Four Artemisinin-Based Combinations (4ABC) Study Group. (2011). A head-to-head comparison of four artemisinin-based combinations for treating uncomplicated malaria in African children: a randomized trial. *PLoS Med*, *8*(11), e1001119.

Happi, C. T., Gbotosho, G. O., Folarin, O. A., Sowunmi, A., Hudson, T., O'Neil, M., ... & Oduola, A. M. J. (2009). Selection of Plasmodium falciparum multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. *AAC*, *53*(3), 888-895.

Hetz, C., Chevet, E., & Oakes, S. A. (2015). Proteostasis control by the unfolded protein response. *Nat Cell Biol*, *17*(7), 829-838.

Huang, F., Takala-Harrison, S., Jacob, C. G., Liu, H., Sun, X., Yang, H., ... & Ringwald, P. (2015). A single mutation in K13 predominates in Southern China and is associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment. *J Infect Dis*, jiv249.

Hawkes, M., Conroy, A. L., Opoka, R. O., Namasopo, S., Zhong, K., Liles, W. C., ... & Kain, K. C. (2015). Slow clearance of Plasmodium falciparum in severe pediatric malaria, Uganda, 2011–2013. *Emerg Infect Dis*, *21*(7), 1237.

Imagej.nih.gov https://imagej.nih.gov/ij. Accessed 9th December 2016.

Kamya, M. R., Yeka, A., Bukirwa, H., Lugemwa, M., Rwakimari, J. B., Staedke, S. G., ... & Wabwire-Mangen, F. (2007). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment of malaria: a randomized trial. *PLOS Clin Trial*, *2*(5), e20.

Kapisi, J., Bigira, V., Clark, T., Kinara, S., Mwangwa, F., Achan, J., ... & Dorsey, G. (2015). Efficacy and safety of artemether-lumefantrine for the treatment of uncomplicated malaria in the setting of three different chemopreventive regimens. *Malar J*, *14*(1), 53.

Kigozi, R., Baxi, S. M., Gasasira, A., Sserwanga, A., Kakeeto, S., Nasr, S., ... & Dorsey, G. (2012). Indoor residual spraying of insecticide and malaria morbidity in a high transmission intensity area of Uganda. *PloS one*, *7*(8), e42857.

Lakshmanan, V., P. G. Bray, D. Verdier-Pinard, D. J. Johnson, P. Horrocks, R. A. Muhle, G. E. Alakpa, R. H. Hughes, S. A. Ward, D. J. Krogstad, A. B. Sidhu & D. A. Fidock, (2005) A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. *EMBO J* **24**: 2294- 2305.

Langhorne, J., F. M. Ndungu, A. M. Sponaas & K. Marsh, (2008) Immunity to malaria: more questions than answers. Nat Immunol **9**: 725-732.

Leang, R., Taylor, W. R., Bouth, D. M., Song, L., Tarning, J., Char, M. C., ... & Khim, N. (2015). Evidence of Plasmodium falciparum malaria multidrug resistance to artemisinin and piperaquine in Western Cambodia: dihydroartemisinin-piperaquine open-label multicenter clinical assessment. *AAC*. *59*(8), 4719-4726.

Lengeler, C., (2004) Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev: CD000363.

Mabaso, M. L., B. Sharp & C. Lengeler, (2004) Historical review of malarial control in southern African with emphasis on the use of indoor residual house-spraying. Trop Med Int Health **9**: 846-856.

Malaney, P., A. Spielman & J. Sachs, (2004) The malaria gap. Am J Trop Med Hyg **71**: 141-146.

MalariaGen. *P. falciparum* Community Project data (beta release). (2015) Oxford, United Kingdom: MalariaGen, https://elifesciences.org/content/5/e08714. Accessed 18th August 2016.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R. V., ... & Nguon, C. (2015). A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature*, *520*(7549), 683-687.

McNAMARA, D. T., Kasehagen, L. J., Grimberg, B. T., Cole-Tobian, J., Collins, W. E., & Zimmerman, P. A. (2006). Diagnosing infection levels of four human malaria parasite species by a polymerase chain reaction/ligase detection reaction fluorescent microsphere-based assay. *The Am J Trop Med Hyg*. *74*(3), 413-421.

Ménard R. (2005): Knockout malaria vaccine?. Nature. 13;433:113-4.

Ménard, D., Khim, N., Beghain, J., Adegnika, A. A., Shafiul-Alam, M., Amodu, O., ... & Bustos, M. D. (2016). A worldwide map of *Plasmodium falciparum* K13-propeller polymorphisms. *N England J Med*, *374*(25), 2453-2464.

Miotto, O., Amato, R., Ashley, E. A., MacInnis, B., Almagro-Garcia, J., Amaratunga, C., ... & Imwong, M. (2015). Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nat Genet*, *47*(3), 226-234.

Mita, T., Tanabe, K., & Kita, K. (2009). Spread and evolution of Plasmodium falciparum drug resistance. *Parasitol Int*, *58*(3), 201-209.

Mita, T., Tanabe, K., Takahashi, N., Culleton, R., Ndounga, M., Dzodzomenyo, M., ... & Kobayakawa, T. (2009). Indigenous evolution of Plasmodium falciparum pyrimethamine resistance multiple times in Africa. *J Antimicrob Chemother*, *63*(2), 252-255.

Mita, T., Venkatesan, M., Ohashi, J., Culleton, R., Takahashi, N., Tsukahara, T., ... & Ferreira, M. U. (2011). Limited geographical origin and global spread of sulfadoxine-resistant dhps alleles in *Plasmodium falciparum* populations. *J Infect Dis*, *204*(12), 1980-1988.

Mita, T., Tachibana, S. I., Hashimoto, M., & Hirai, M. (2016). *Plasmodium falciparum* kelch 13: a potential molecular marker for tackling artemisinin-resistant malaria parasites. *Expert Rev Anti-Infect Ther*, *14*(1), 125-135.

Mok, S., Imwong, M., Mackinnon, M. J., Sim, J., Ramadoss, R., Yi, P., ... & Socheat, D. (2011). Artemisinin resistance in *Plasmodium falciparum* is associated with an altered temporal pattern of transcription. *BMC Genomics*, *12*(1), 391.

Mok, S., Ashley, E. A., Ferreira, P. E., Zhu, L., Lin, Z., Yeo, T., ... & Nguon, C. (2015). Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. *Science*, *347*(6220), 431-435.

Mohon, A. N., Alam, M. S., Bayih, A. G., Folefoc, A., Shahinas, D., Haque, R., & Pillai, D. R. (2014). Mutations in *Plasmodium falciparum* K13 propeller gene from Bangladesh (2009–2013). *Malar J*, *13*(1), 431.

Muhindo, M. K., Kakuru, A., Jagannathan, P., Talisuna, A., Osilo, E., Orukan, F., ... & Dorsey, G. (2014). Early parasite clearance following artemisinin-based combination therapy among Ugandan children with uncomplicated *Plasmodium falciparum* malaria. *Malar J*, *13*(1), 32.

Müller, O. (2011). *Malaria in Africa: challenges for control and elimination in the 21st century*. Peter Lang.

Muwanguzi, J., Henriques, G., Sawa, P., Bousema, T., Sutherland, C. J., & Beshir, K. B. (2016). Lack of K13 mutations in *Plasmodium falciparum* persisting after artemisinin combination therapy treatment of Kenyan children. *Malar J*, *15*(1), 36.

Noedl, H., Allmendinger, T., Prajakwong, S., Wernsdorfer, G., & Wernsdorfer, W. H. (2001). Desbutyl-Benflumetol, a Novel Antimalarial Compound: In Vitro Activity in Fresh Isolates of Plasmodium falciparumfrom Thailand. *AAC*, *45*(7), 2106-2109.

Noedl H. Artemisinin resistance: how can we find it? Trends Parasitol. 2005;21 (9):404–405.

Noedl, H., Socheat, D., & Satimai, W. (2009). Artemisinin-resistant malaria in Asia. *N Engl Med*, *361*(5), 540-541.

Ntale, M., Ogwal-Okeng, J. W., Mahindi, M., Gustafsson, L. L., & Beck, O. (2008). A field-adapted sampling and HPLC quantification method for lumefantrine and its desbutyl metabolite in whole blood spotted on filter paper. *J Chromatogr B*, *876*(2), 261-265.

Okell, L. C., Drakeley, C. J., Ghani, A. C., Bousema, T., & Sutherland, C. J. (2008). Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. *Malar J*, *7*(1), 125.

Okello, P. E., Van Bortel, W., Byaruhanga, A. M., Correwyn, A., Roelants, P., Talisuna, A., ... & Coosemans, M. (2006). Variation in malaria transmission intensity in seven sites t hroughout Uganda. *Am J Trop Med Hyg*, *75*(2), 219-225.

Ouattara, A., Kone, A., Adams, M., Fofana, B., Maiga, A. W., Hampton, S., ... & Sagara, I. (2015). Polymorphisms in the K13-propeller gene in artemisinin-susceptible *Plasmodium falciparum* parasites from Bougoula-Hameau and Bandiagara, Mali. *Am J Trop Med Hyg*, *92*(6), 1202-1206.

Paloque, L., Ramadani, A. P., Mercereau-Puijalon, O., Augereau, J. M., & Benoit-Vical, F. (2016). Plasmodium falciparum: multifaceted resistance to artemisinins. *Malar J*, *15*(1), 149.

Peel, S. A., (2001) The ABC transporter genes of *Plasmodium falciparum* and drug resistance. *Drug Resist Updat* **4**: 66-74.

Peters, W. 1987. *Chemotherapy and drug resistance in malaria*. 2nd edition. Academic Press. London, United Kingdom. 1091 pp.

PlasmoDB database. http://plasmodb.org/plasmo/app/record/gene/PF3D7_1343700. Accessed 7th January 2015.

Plucinski, M. M., Talundzic, E., Morton, L., Dimbu, P. R., Macaia, A. P., Fortes, F., ... & Udhayakumar, V. (2015). Efficacy of artemether-lumefantrine and dihydroartemisinin-piperaquine for treatment of uncomplicated malaria in children in Zaire and Uige Provinces, angola. *AAC*. *59*(1), 437-443.

Price, R., Van Vugt, M., Nosten, F., Luxemburger, C., Brockman, A., Phaipun, L., ... & White, N. (1998). Artesunate versus artemether for the treatment of recrudescent multidrug-resistant falciparum malaria. *Am J Trop Med Hyg*. *59*(6), 883-888.

Price, R. N., C. Cassar, A. Brockman, M. Duraisingh, M. van Vugt, N. J. White, F. Nosten & S. Krishna, (1999) The pfmdr1 gene is associated with a multidrug- resistant phenotype in

Plasmodium falciparum from the western border of Thailand. *Antimicrob Agents Chemother* **43**: 2943-2949.

Price, R. N., A. C. Uhlemann, M. van Vugt, A. Brockman, R. Hutagalung, S. Nair, D. Nash, P. Singhasivanon, T. J. Anderson, S. Krishna, N. J. White & F. Nosten, (2006) Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clin Infect Dis* **42**: 1570-1577.

Pukrittayakamee, S., Chotivanich, K., Chantra, A., Clemens, R., Looareesuwan, S., & White, N. J. (2004). Activities of artesunate and primaquine against asexual-and sexual-stage parasites in falciparum malaria. *AAC*, *48*(4), 1329-1334.

Rathod, P. K., McErlean, T., & Lee, P. C. (1997). Variations in frequencies of drug resistance in *Plasmodium falciparum*. *PNAS*, *94*(17), 9389-9393.

Reed, M. B., K. J. Saliba, S. R. Caruana, K. Kirk & A. F. Cowman, (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. *Nature* **403**: 906-909.

Renia, L. & S. M. Potter, (2006) Co-infection of malaria with HIV: an immunological perspective. Parasite Immunol **28**: 589-595.

Roper, C., Pearce, R., Nair, S., Sharp, B., Nosten, F., & Anderson, T. (2004). Intercontinental spread of pyrimethamine-resistant malaria. *Science*, *305*(5687), 1124-1124.

Rubio, J. M., Benito, A., Roche, J., Berzosa, P. J., Garcia, M. L., Mico, M., ... & Alvar, J. (1999). Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of *Plasmodium vivax* infection in Equatorial Guinea. *The Am J Trop Med Hyg*. *60* (2), 183-187.

Sakihama, N., Mitamura, T., Kaneko, A., Horii, T., & Tanabe, K. (2001). Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. *Exp Parasitol*, *97*(1), 50-54.

Sidhu, A. B., D. Verdier-Pinard & D. A. Fidock, (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. *Science* **298**: 210-213.

Sisowath, C., Petersen, I., Veiga, M. I., Mårtensson, A., Premji, Z., Björkman, A., ... & Gil, J. P. (2009). In vivo selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with artemether-lumefantrine in Africa. *J Infect Dis*, *199*(5), 750-757.

Sniegowski PD, Gerrish PJ, Johnson T, Shaver A (2000). The evolution of mutation rates: separating causes from consequences. Bioessays. 1;22:1057-66.

Straimer, J., Gnädig, N. F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A. P., ... & Gregory, P. D. (2015). K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science*, *347*(6220), 428-431.

Takahashi, N., Tanabe, K., Tsukahara, T., Dzodzomenyo, M., Dysoley, L., Khamlome, B., ... & Kaneko, A. (2012). Large-scale survey for novel genotypes of Plasmodium falciparum chloroquine-resistance gene pfcrt. *Malar J*, *11*(1), 92.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. (2013) . MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol.;30:2725-9.

Tanabe K, Sakihama N, Kaneko O, Saito A, Kimura M. (1999). A PCR Method for molecular epidemiology of *Plasmodium falciparum Msp-1*. Tokai J Exp Clin Med. 23:375-81.

Trape, J. F., (2001) The public health impact of chloroquine resistance in Africa. AmJ Trop Med Hyg **64**: 12-17.

Uganda Bureau of Statistics. Uganda Malaria Indicator Survey 2009. https://dhsprogram.com/pubs/pdf/MIS6/MIS6.pdf. Accessed 8th January 2016

Uganda Bureau of Statistics (UBOS) and ICF International 2015. Uganda Malaria Indicator Survey 2014-15. https://dhsprogram.com/pubs/pdf/MIS21/MIS21.pdf. Accessed 8th January 2016.

Valderramos, S. G. & D. A. Fidock, (2006) Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci* **27**: 594-601.

Wernsdorfer, W. H., (2004) Coartemether (artemether and lumefantrine): an oral antimalarial drug. *Expert Rev Anti Infect Ther* **2**: 181-196.

White NJ. (1997)**.** Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. *AAC* ; 41:1413-22.

White NJ. (2004). Antimalarial drug resistance. J Clin Invest. 113:1084-92.

White NJ. (2008). Qinghaosu (artemisinin): the price of success. Science. 18;320:330-4.

White, N. J., Pongtavornpinyo, W., Maude, R. J., Saralamba, S., Aguas, R., Stepniewska, K., ... & Day, N. P. (2009). Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. *Malar J*, *8*(1), 253.

World Health Organization. Global plan for artemisinin resistance containment (GPARC). http://www.who.int/malaria/publications/atoz/artemisinin_resistance_containment_2011.pdf. Accessed 7th January 2016

World Health Organization. Methods for surveillance of antimalarial drug efficacy http://apps.who.int/iris/bitstream/10665/44048/1/9789241597531_eng.pdf. Accessed 10th January 2016.

WHO. World Malaria Report 2011.

WHO. World Malaria Report 2014. Geneva, World Health Organization, 2014. www.who.int/malaria/.../world_malaria_report_2014/wmr-2014-no-profiles.pdf. Accessed 4th January 2016

World Health Organization Global Malaria Programme. Status report on artemisinin resistance, September 2014 (http://www.who.int/malaria/publications/atoz/status_rep_artemisinin_resistance_sep2014.pdf. Accessed 16 November 2015).

WHO: Guidelines for treating malaria Third edition 2015. Third Edition http://apps.who.int/iris/bitstream/10665/162441/1/9789241549127_eng.pdf

World Malaria Report 2016. http://www.who.int/malaria/publications/world-malaria-report-2016/WMR-2016-annexes.pdf

Witkowski, B., Lelièvre, J., Barragán, M. J. L., Laurent, V., Su, X. Z., Berry, A., & Benoit-Vical, F. (2010). Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *AAC*. *54*(5), 1872-1877.

Worldwide Antimalarial Resistance Network (WWARN) http://www.wwarn.org/about-us/malaria-drug-resistance

Worldwide Antimalarial Resistance Network-Tools and resources/procedures; http://www.wwarn.org/tools-resources/procedures. Accessed 8th December 2016.

WWARN Artemisinin based Combination Therapy (ACT) Africa Baseline Study Group. (2015) Clinical determinants of early parasitological response to ACTs in African patients with uncomplicated falciparum malaria: a literature review and meta-analysis of individual patient data. BMC Med;13:212.

Yeka, A., Dorsey, G., Kamya, M. R., Talisuna, A., Lugemwa, M., Rwakimari, J. B., ... & Bukirwa, H. (2008). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treating uncomplicated malaria: a randomized trial to guide policy in Uganda. *PLoS One*, *3*(6), e2390.

Yeka, A., Kigozi, R., Conrad, M. D., Lugemwa, M., Okui, P., Katureebe, C., ... & Staedke, S. G. (2016). Artesunate/amodiaquine versus artemether/lumefantrine for the treatment of uncomplicated malaria in Uganda: a randomized trial. *J Infect Dis*, *213*(7), 1134-1142.

Yeka, A., Lameyre, V., Afizi, K., Fredrick, M., Lukwago, R., Kamya, M. R., & Talisuna, A. O. (2014). Efficacy and safety of fixed-dose artesunate-amodiaquine vs. artemether-

lumefantrine for repeated treatment of uncomplicated malaria in Ugandan children. *PLoS One*, *9*(12), e113311.

Yeka, A., Tibenderana, J., Achan, J., D'Alessandro, U., & Talisuna, A. O. (2013). Efficacy of quinine, artemether-lumefantrine and dihydroartemisinin-piperaquine as rescue treatment for uncomplicated malaria in Ugandan children. *PloS one*, *8*(1), e53772.

3.2 Paper citation

Balikagala, B., Mita, T., Ikeda, M., Sakurai, M., Yatsushiro, S., Takahashi, N., … Tsuboi, T. (2017). Absence of in vivo selection for K13 mutations after artemether–lumefantrine treatment in Uganda. *Malaria Journal*, *16*, 23. http://doi.org/10.1186/s12936-016-1663-1

3.3 Supplementary Data

Supplementary Table 1. Eligibility Criteria for recruitment into the therapeutic efficacy study and Molecular study

	Therapeutic Efficacy Study
Inclusion criteria	Children aged 6 months to 10 years
	Fever (axillary temperature ≥ 37.5ºC) or history of fever within the previous 24 hours.
	Body weight ≥ 5kg
	Uncomplicated malaria with asexual *P.falciparum* mono infection with parasitaemia ≤ 200,000/μL.
	Hemoglobin concentration ≥ 5g/dL
	No history of hypersensitivity reactions or contradictions to the study drug.
	No evidence of concomitant febrile illness
	Provision of informed consent by a parent or guardian and assent for children ≥ 7 years and agreement to complete 28 days of follow-up.
	No regular medication, which could interfere with antimalarial drug, *e.g.* prophylaxis with cotrimoxazole for the prevention of *Pneumocystis carinii* pneumonia in children born to HIV positive women.
	Absence of severe malnutrition (defined as a child whose growth standard is below –3 z-score, with symmetrical oedema involving at least the feet or mid-upper arm circumference < 110 mm).
Exclusion Criteria	Concomitant severe disease (cardiac, renal, hepatic diseases) or co-infection with other malaria species, which would place the subject at undue risk or interfere with the results of the study.
	Danger signs or evidence of severe malaria defined as: - Unarousable coma (if after convulsion, > 30 min) - Recent convulsions (1 to 2 within 24 hours) - Altered consciousness (confusion, delirium, psychosis, coma) - Lethargy - Severe anaemia (Hb < 5.0 g/dL) - Respiratory distress (breathing difficulties/labored breathing at rest) - Vomiting - Prostration
	Antimalarial drug usage within 2 weeks prior to enrollment into the study.
	Presence of other conditions that in the opinion of the Investigator would jeopardize the safety or rights of a child in the study or would render the child unable to comply with the follow-up.
	Molecular Study
Inclusion criteria	1. Aged ≥ 6 months
	2. Uncomplicated malaria with asexual *P.falciparum* mono infection of any density.
	3. Provision of informed consent by a parent/guardian and assent for children ≥ 7 years.
Exclusion Criteria	None

Supplementary Table 2. List of primers and probes for multiplex and qPCR for SNP typing in *P. falciparum*

Primer Name	Full name of target genes	PlasmoDB ID	Type	Sequence (5′ - 3′)	# mer	Tm (°C)
PfARPS10-F4	Apicoplast ribosomal protein S10 precursor	PF3D7_1460900.1	Multiplex PCR	ATTGTAGCAGGCCCAATTCCCCAAAAGACA	30	77.1
PfARPS10-R7	Apicoplast ribosomal protein S10 precursor	PF3D7_1460900.1	Multiplex PCR	CAGAATTTTTTATAGGAACAGATGAGTTCA	30	64.9
PfFD-F1	Ferredoxin	PF3D7_1318100	Multiplex PCR	TTGAATTTCCTAAGTAATAATCAGCTAGCT	30	63.2
PfFD-R1	Ferredoxin	PF3D7_1318100	Multiplex PCR	TATTAGGAAATTTTATCATTCCCCATTTCA	30	66.9
PfMDR2-F1	Multidrug resistance protein 2+ (heavy metal transport family)	PF3D7_1447900	Multiplex PCR	TGATGAAAATAGTGGCTTATTAGGAACAGA	30	66.9
PfMDR2-R1	Multidrug resistance protein 2+ (heavy metal transport family)	PF3D7_1447900	Multiplex PCR	ACCTGTATGACCTACAAGAGCACATGTTGT	30	69.7
PfPIBP-F2	Phosphoinositide-binding protein	PF3D7_0720700	Multiplex PCR	TACTTGAGAAGAATGTCATGAAAGA	25	60.7
PfPIBP-R1	Phosphoinositide-binding protein	PF3D7_0720700	Multiplex PCR	TGTTAGACATATCGTACATATCATACGCGT	30	66.5
PfCRT-F2	Chloroquine resistance transporter	PF3D7_0709000	Multiplex PCR	GAAAACCTTCGCATTGTTTTCCTTCT	26	68.6
PfCRT-R2	Chloroquine resistance transporter	PF3D7_0709000	Multiplex PCR	ATGATACGTTGTACCATCATAAACA	25	60.8
PfPPH-F3	Protein phosphatase	PF3D7_1012700	Multiplex PCR	GAAAAGTCAAGAGTCCTCTCAAGTGATTGA	30	69.1
PfPPH-R2	Protein phosphatase	PF3D7_1012700	Multiplex PCR	TCGATATTGAGTCAATATCGTACGCACAAC	30	70.3
PfARPS10-Fq	Apicoplast ribosomal protein S10 precursor	PF3D7_1460900.1	qPCR	TTCGAATTGCTCTCTACTGTC	21	59.7
PfARPS10-Rq	Apicoplast ribosomal protein S10 precursor	PF3D7_1460900.1	qPCR	AAAGACAATAAGAAAGAGGTAACATA	26	59.4
PfARPS10-probe (WT)	Apicoplast ribosomal protein S10 precursor	PF3D7_1460900.1	Probe qPCR	TTAT+CC+A+C+A+TGGGG	19	63.0
PfARPS10-probe (Mut)	Apicoplast ribosomal protein S10 precursor	PF3D7_1460900.1	Probe qPCR	A+TCCA+T+A+T+GGGG	17	62.9
PfFD-Fq	Ferredoxin	PF3D7_1318100	qPCR	GATTGAAACGCACAAGGAAGAC	22	62.1
PfFD-Rq	Ferredoxin	PF3D7_1318100	qPCR	CCCATTTCAATCATATCCATACCAAA	26	62.0
PfFD-probe (WT)	Ferredoxin	PF3D7_1318100	Probe qPCR	AACTACA+C+G+ACATGTAAT	21	61.8
PfFD-probe (Mut)	Ferredoxin	PF3D7_1318100	Probe qPCR	AACTA+CA+C+T+A+CATGTA	21	62.4
PfMDR2-Fq	Multidrug resistance protein 2+ (heavy metal transport family)	PF3D7_1447900	qPCR	TAGTTGATAGAGGTACCGAGAG	22	59.8
PfMDR2-Rq	Multidrug resistance protein 2+ (heavy metal transport family)	PF3D7_1447900	qPCR	ATAAAGTTAAACCTATAAATAATACACTACC	31	59.7
PfMDR2-probe (WT)	Multidrug resistance protein 2+ (heavy metal transport family)	PF3D7_1447900	Probe qPCR	CCGGC+A+A+C+A+A+TAGAA	21	63.5
PfMDR2-probe (Mut)	Multidrug resistance protein 2+ (heavy metal transport family)	PF3D7_1447900	Probe qPCR	CCGG+CA+A+T+A+A+TAGA	20	61.7
PfPIBP-Fq	Phosphoinositide-binding protein	PF3D7_0720700	qPCR	TTGTTCATGTAAATGTTTGTAATTATAGGA	30	61.6
PfPIBP-Rq	Phosphoinositide-binding protein	PF3D7_0720700	qPCR	TTCATATTCTGTCGAGGATAACAATTC	27	61.7
PfPIBP-probe (WT)	Phosphoinositide-binding protein	PF3D7_0720700	Probe qPCR	CTA+CAAT+GAAAAAA+T+G+TAT+TAA	28	64.9
PfPIBP-probe (Mut)	Phosphoinositide-binding protein	PF3D7_0720700	Probe qPCR	C+CTA+CAA+TGAAAAAA+T+T+TATTAA	29	64.1
PfCRT-Fq	Chloroquine resistance transporter	PF3D7_0709000	qPCR	ACCATGACATATACTATTGTTAGTTGTA	28	61.0
PfCRT-Rq	Chloroquine resistance transporter	PF3D7_0709000	qPCR	ATGATACGTTGTACCATCATAAACA	25	61.0
PfCRT-probe (WT)	Chloroquine resistance transporter	PF3D7_0709000	Probe qPCR	A+G+CA+A+T+A+GCAAT	18	61.1
PfCRT-probe (Mut)	Chloroquine resistance transporter	PF3D7_0709000	Probe qPCR	AGCA+A+C+A+GC+AAT	17	60.8
PfPPH-F1q-1	Protein phosphatase	PF3D7_1012700	qPCR	ACATATATGGATGATTATATGGATGGTAAA	30	61.6
PfPPH-R1q	Protein phosphatase	PF3D7_1012700	qPCR	GTATTATTATTGACTTCGTTCACATGTTT	29	61.8
PfPPH-probe (WT)	Protein phosphatase	PF3D7_1012700	Probe qPCR	TATT+CAAA+T+GT+CAAA+CA+TC	25	66.1
PfPPH-probe (Mut)	Protein phosphatase	PF3D7_1012700	Probe qPCR	TATT+CAAA+T+C+TCAAA+CA+TCA	26	67.0

Supplementary Table 3. Recrudescence versus re-infection test results of all recurrent malaria infections

	Sample ID	PCR Product sizes (bp)										**Results/Comment**
		***msp1* (Block 2 to 6*)**					***msp1* (Block 4a/4b*)**			***msp2***	***glurp***	
		K1	3D7	FCR3	MAD20	Thai838	97S	K1	MAD20	**3D7**	**3D7**	
1	1405-A024_D0		1100				94			240	950	Re-infection
2	1405-A024_D28		1100				94			320	900	
3	1410-C036_D0		1100			1100			97	350	1000	Re-infection
4	1410-C036_D28		-			1100			97	290	900	
5	1410-C088_D0					1100	-		97	260	810	Re-infection
6	1410-C088_D28					1100	94		97	250	1000	

*For *msp1*, block sizes are after Kaneko O et al 1997