THE ACTIVITIES OF VARIOUS ANTIMALARIAL DRUGS ON *PLASMODIUM FALCIPARUM* ISOLATES FROM KILIFI, KENYA AND STUDIES ON MECHANISMS OF RESISTANCE

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PUBLICATIONS

1. **Mwai L**, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K, Borrmann S, Mackinnon M, Nzila A, Chloroquine resistance before and after its withdrawal in Kilifi Kenya. Malar J. 2009 May 18;8:106.
2. **Mwai L**, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A. *In vitro* activities of piperaquine, lumefantrine and dihydroartemsinin on Kenyan *P.falciparum* isolates and polymorphisms in *pfcrt* and *pfmdr1*. Antimicrob Agents Chemother. 2009 Dec;53(12):5069-73
3. Nzila A**, Mwai L.** *In vitro* selection of Plasmodium falciparum drug-resistant parasite lines**.** J Antimicrob Chemother. Mar; 2010 Mar; 65(3):390-8.
4. Sasi P, Abdulrahaman A*,* **Mwai L**, Muriithi S, Straimer J, Schieck E, Rippert A*, Bashra*heil M, Salim A, Peshu J, Awuondo K, Lowe B, Pirmohamed M, Winstanley P, Ward S, Nzila A, Borrmann S, *In vivo* and *in vitro* efficacy of amodiaquine against *Plasmodium falciparum* in an area of continued use of 4-aminoquinolines in East Africa. J Infect Dis. 2009 Jun 1**;** 199(11):1575-82.
5. **Leah Mwai**, Abdi Diriye, Victor Masseno, Steven Muriithi, Theresa Feltwell, Jennifer Musyoki, Jacob Lemieux, Avi Feller, Gunnar R. Mair, Kevin Marsh, Chris Newbold, Alexis Nzila, Céline K. Carret , Genome wide adaptations of *Plasmodium falciparum* in response to Lumefantrine selective drug pressure; (submitted).

ABBREVIATIONS

CQ	Chloroquine
PQ	Piperaquine
LM	Lumefantrine
AQ	Amodiaquine
DEAQ	Desethylamodiaquine
DHA	Dihydroartemisinin
SP	Sulphadoxine-Pyrimethamine
QN	Quinine
MFQ	Mefloquine
HLF	Halofantrine
PYN	Pyronaridine
PMQ	Primaquine
ART	Artemisinin
DNA	Deoxyribonucleic acid
RNA	ribonucleic acid
RFLP	Restriction Fragment length polymorphism
WHO	World Health Organization
ACT	Artemisinin Combination Therapy
AS	Artesunate
AQ-AS	Amodiaquine –Artesunate
AQ-SP	Amodiaquine-Sulphadoxine-Pyrimethamine
LM-ATM	Artemether-Lumefantrine
DHA-PQ	Dihydroartemisinin-Piperaquine

CPG-DAP	Chlorproguanil-Dapsone
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
PfMDR1	*Plasmodium falciparum* multidrug resistance gene
PfATPASE6	*Plasmodium falciparum* ATPASE6
PfNHE	*P.falciparum* Na+/H+ exchanger
PfMRP	*P. falciparum* multidrug associated protein
IC_{50}	50% inhibitory concentration
IC_{99}	99% inhibitory concentration
PABA	Para amino benzoic acid
SCC	Scientific Coordinating Committee
CO_2	Carbon dioxide
O_2	Oxygen
N_2	Nitrogen
HCl	Hydrochloric acid
ATV	Atovaquone
PYR	Pyrimethamine
DE	Differentially expressed
qPCR	quantitative real time polymerase chain reaction
FC	Fold Change
GO	Gene ontology
BP	Biological process
MDR	Multidrug resistant
ARMD	Acclerated resistance to multi drug
DHFR	Dihydrofolate reductase

ABSTRACT

Drug resistance is a significant challenge in the fight against malaria. Importantly, reduced efficacy has been reported against artemether (ATM)/Lumefantrine (LM) (LM-ATM), amodiaquine (AQ)/artesunate (AS) (AQ-AS), two important combination treatment regimens in Africa, and against piperaquine (PQ), a drug which has been evaluated as a potential alternative in Africa, in combination with dihydroarteminisin (DHA). Chloroquine (CQ) resistance in *P.falciparum* is associated with two main transporters PfCRT and PfMDR1. I investigated the mechanisms of resistance to PQ, LM and AQ, with the overall goal of identifying molecular markers that can be used to track resistance. I used CQ as a reference.

The key antimalarial drugs were highly active against clinical isolates from Kilifi, Kenya with median inhibitory concentrations (IC_{50}s) of <5nM for DHA and <55 nM for CQ, AQ, PQ, LM and DEAQ (desethylamodiaquine, the active metabolite of AQ). *pfcrt-76* and *pfmdr1-86* mutations were associated with AQ, DEAQ and LM but not DHA or PQ activity. Interestingly, > 20% of analysed isolates had decreased susceptibility to LM (IC_{50} >100nM); these isolates were the most susceptible to CQ and carried wild type genotypes at *pfcrt-76* and *pfmdr1-86*. I observed that CQ resistance had been declining in Kilifi since 1993 (prior to CQ withdrawal) to 2006 (7 years after its withdrawal), similar to observations in Malawi. My results support the hypothesis that susceptibility to antimalarial drugs returns when drug pressure is removed, and suggest that the use of LM-ATM may hasten the return of CQ susceptibility.

Continued monitoring of drug susceptibility is crucial. *pfcrt-76* and *pfmdr1-86* may be useful molecular markers of LM-ATM efficacy in Kilifi and other African sites. Using a microarray approach, I identified additional genes (including various transporters) that may contribute to LM resistance. I recommend further studies to clarify the exact roles of the identified genes.

CHAPTER ONE

1 Introduction and literature review

1.1 Malaria

Malaria in humans is caused by four main species of Plasmodium i.e. *Plasmodium falciparum (P.falciparum), Plasmodium Vivax (P.vivax), Plasmodium ovale (P.ovale) and Plasmodium malariae (P.malariae*), and it is now recognized that the monkey malaria parasite *Plasmodium knowlesi (P.knowlesi)* also infects humans [1, 2]. More than 2 billion people are at risk of malaria globally, and it is estimated that each year, there are over 300 million malaria cases, which result in about 1 million deaths [3-5].

P.falciparum is responsible for most malaria related deaths and morbidity [5]. The effects of *P.falciparum* malaria are mostly experienced in sub-Saharan Africa, where young children bear the biggest disease burden [3, 6]. In addition to the human cost, malaria also exerts a heavy economic burden in endemic countries; it is estimated that every year, African nations lose US $12 billion to malaria alone [3, 7]. The emergence and spread of drug resistant malaria has been a major setback in the global fight against malaria [8-11].

1.2 Life cycle of *Plasmodium falciparum*

P.falciparum malaria is transmitted to humans by the bite of a female anopheline mosquito [12, 13], which deposits malaria sporozoites from its salivary glands into the dermis of the host [14-16]. The life cycle of the malaria parasite is shown in Figure 1-1.

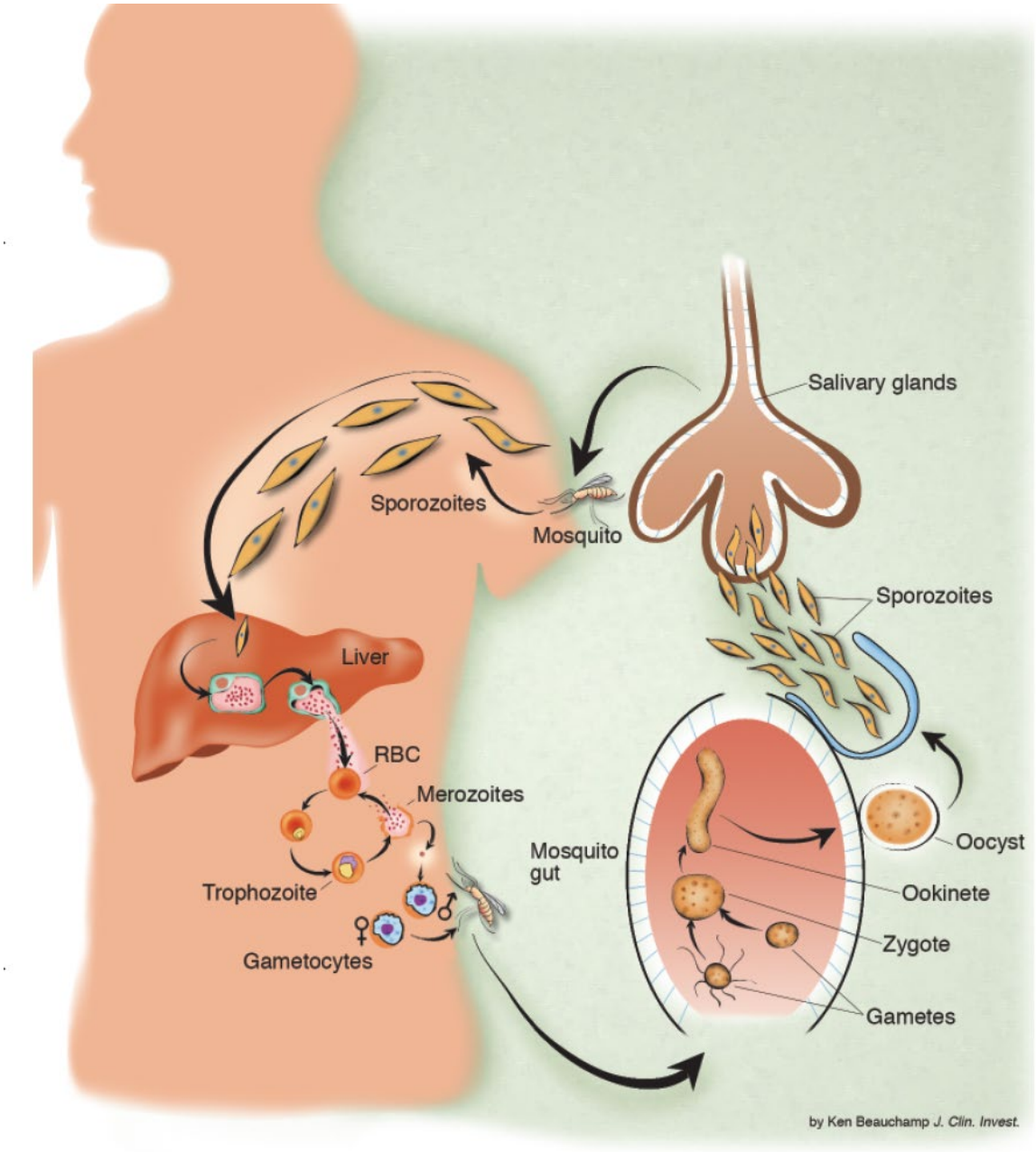


Figure 1-1: The life cycle of the malaria parasite in the human host and anopheline mosquito. Adapted from Ref [17]. **[Reprinted with permission from Elsevier: White, N. J. (2004). 'Antimalarial drug resistance', J. Clin Invest. 113(8), 1084-1092. doi: 10.1172/JCI21682.]**

Inoculated Plasmodium sporozoites have various fates; a proportion remains in the dermis after exhaustion of their motility, whilst others rapidly move through the blood stream into the liver to invade hepatocytes. It has been shown that a proportion of sporozoites invade the lymphatic vessels. It is notable that although the majority of lymphatic sporozoites are degraded there, recent studies have shown that some sporozoites may partially differentiate into exo-erythrocytic stages in the lymphatic vessels [18].

The liver stage is an asymptomatic phase whereby the parasite undergoes silent logarithmic multiplication. In *P.ovale* and *P.vivax* infections, the latent liver stage (referred to as hypnozoite) can be prolonged. A prolonged latent liver stage does not occur in *P.falciparum* infections. Rather, the liver stage lasts only about 6 days in *P.falciparum* infections, after which each sporozoite yields tens of thousands of merozoites that invade and develop within human red blood cells, initiating the blood stage of the infection.

The blood stage of the infection comprises 2 distinct forms: an asexual erythrocytic form whereby the parasites undergo repeated 48 hour cycles of multiplication, and the sexual forms (male and female), referred to as gametocytes. To undergo further development, the sexual forms must be ingested by mosquitoes. During the asexual erythrocytic stage, the parasite undergoes complex transcriptional and morphological changes as it matures through the ring, trophozoite and schizont stages [19, 20]. For its survival, it requires haemoglobin as a food source, which it uses in complex metabolic pathways. At the end of 48 hours, each schizont bursts, releasing 8-32 new merozoites, leading to an exponential increase in parasite levels, to numbers as high as up to 10^{13} parasites per host. The asexual erythrocytic parasites are the pathogenic forms causing a febrile illness, which initially presents as non-specific symptoms, but which may develop into a severe disease, affecting different organ systems.

The genome of the malaria parasite is haploid, except briefly after fertilization. Sexual reproduction in the Plasmodium parasite occurs in the anopheles mosquito. Sexual stage parasites (gametocytes) are non-pathogenic and are transmitted from the host to the anopheles vector during a blood meal. Ingestion of gametocytes by a mosquito activates the formation of gametes (gametogenesis) in the mosquito mid gut lumen, which fuse within minutes to form a diploid zygote. Soon after zygote formation, meiosis is executed and genetic recombination occurs. The zygote then transforms into a motile ookinete, which migrates to the basal site of a midgut cell where it arrests and transforms into an oocyst. The oocyst undergoes several mitotic divisions to form sporoblasts. Sporozoite budding from sporobalasts takes 10-14 days

after the blood meal, rendering this the longest developmental phase of the Plasmodium life cycle. The genetically distinct sporozoites actively egress the oocyst by proteolytic activity, entering the hemolymph, the circulatory system of the mosquito. When passing through the salivary glands, the sporozoites invade the acinar cell layer from the basal side, accumulating in the salivary duct of the mosquito. Transmission continues when the mosquito deposits sporozoites into the dermis of its subsequent blood meal donor [21]. The high rate of sexual recombination that occurs in the mosquito is an important factor in the generation of multiple drug resistant phenotypes [22].

The unicellular malaria parasite employs its tool-kit of more than 5000 genes to undergo the critical metamorphoses in its life cycle needed for the numerous environments and physiological barriers it encounters. At each of the developmental stages, this life cycle can be interrupted or altered. Thus, studies aimed at understanding the biology of the parasite can aid in the development of tools aimed at treating, preventing or monitoring infection, disease or transmission. The ability to culture malaria parasites *in vitro[23],* coupled with biotechnological advances in molecular biology techniques and more recently, advances in generating the full genome sequence of the parasite [24], have presented new opportunities for developing such innovative tools.

1.3 Malaria disease

Infection with *P.falciparum* malaria can either result in asymptomatic infection, uncomplicated (non-severe) malaria or severe malaria. In asymptomatic infection, the individual is able to tolerate the presence of malaria parasitaemia without having any symptoms. Following several repeated malaria infections, malaria specific partial immunity usually develops. In high transmission areas, this immunity is built up in childhood and most of the malaria morbidity and mortality occurs in young children [25, 26]. Thus, in high transmission settings, symptomatic malaria is less common in adults and older children and

malaria infections in this group can often be contolled by the body's immune mechanisms without the need for antimalarial treatment. This is in contrast with malaria infections in settings with low or sporadic transmission, where due to little acquired immunity, infection results in symptomatic disease in all age groups.

Symptomatic malaria is related to the asexual blood stage of the parasite. Uncomplicated (non-severe malaria) accounts for possibly 99% of all malaria disease events and consists of a febrile illness with non-specific symptoms such as malaise, headache and gastrointestinal symptoms. As maturing infected erythrocytes circulate in peripheral blood, they adhere to blood vessels in the brain, gut and other organs, a process referred to as cytoadherence. Cytoadherence leads to sequestration of infected blood cells in the microvasculature, a pathophysiological process characteristic of *P falciparum* [27]. Sequestration is thought to play a key role in some of the life threatening complications characteristic of severe malaria such as acidosis and coma [28]. Prompt and effective treatment of uncomplicated malaria cases is crucial in preventing disease progression to severe malaria or death, and in minimizing malaria related morbidity.

1.4 Interventions used for malaria control in endemic countries

Although research has greatly advanced our understanding of malaria biology and epidemiology, a safe and effective malaria vaccine for use in humans remains elusive. However, significant improvements in the control and management of malaria have been observed in various African endemic countries in the recent past [29].

These improvements have been attributed to increased funding for malaria control, which has resulted in the scaling up of existing malaria control measures, notably: i) the improved diagnosis and treatment of cases with artemisinin-based therapies (ACTs) or other highly effective drug combinations (see section 1.5) and ii) increased coverage of preventive

interventions such as insecticide treated nets (ITNs), indoor residual spraying (IRS), and intermittent preventive therapy (iPTP) in pregnant women [29-32]. Importantly, since ITNs have been the most widely distributed of these interventions [33], it is plausible that they have contributed the most to the observed positive changes. The general improvements in health and socio-economic status and technological advances that have occured in some of the endemic sites may also have contributed, at least in part, to the observed improvement in the malaria situation.

Importantly, there is increasing concern that the gains achieved could be lost if current drugs and insecticides lose their effectiveness, or if there is a resurgence of malaria in places where malaria cases had previously declined [34]. It is also clear that control can only be maintained in the context of health systems robust enough to conduct surveillance and to co-ordinate the delivery of available interventions in a timely and sustainable manner. If developed, the deployment of a safe and effective vaccine in the future would greatly aid in achieving effective malaria control, and perhaps eradication. Therefore, long-term commitment to continued control efforts and to the discovery of new tools such as drugs, insecticides and vaccines is crucial.

1.5 Antimalarial drugs

Antimalarial drugs have previously been classified and evaluated based on the stages of the malaria life cycle that they target, their molecular targets, chemical structure, half-life or cost [35, 36].

Due to variation in stage specific biology, antimalarial drugs may act on different stages of the malaria parasite. For example, tissue schizonticides kill hepatic schizonts and prevent invasion of erythrocytes, thereby preventing the establishment of clinical malaria. Therefore, they are sometimes termed causally prophylactic. Hypnozoiticides kill persistent intra-hepatic

stages (occuring in *P.vivax* and *P.ovale* infections) thus prevent relapses from these dormant stages of infection. Of the antimalarials in clinical use, only primaquine, an 8-aminoquinoline is available for the elimination of hypnozoites [35]. Gametocytocides eliminate the intraerythrocytioc sexual forms (gametocytes), and thus prevent human to human transmission [36]. Only artemisinins [37] and the 8-aminoquinoline primaquine [38] have reported gametocytocidal activity. Most antimalarials in clinical use are blood schizonticides, which work by eliminating the asexual erythrocytic parasite forms [36]. As there are no dormant hepatic stages in *P.falciparum* malaria, blood schizonticides are sufficient to manage infections. Figure 1-2 shows the key antimalarials that are used as blood schizonticides clinically, and their proposed sites of action.

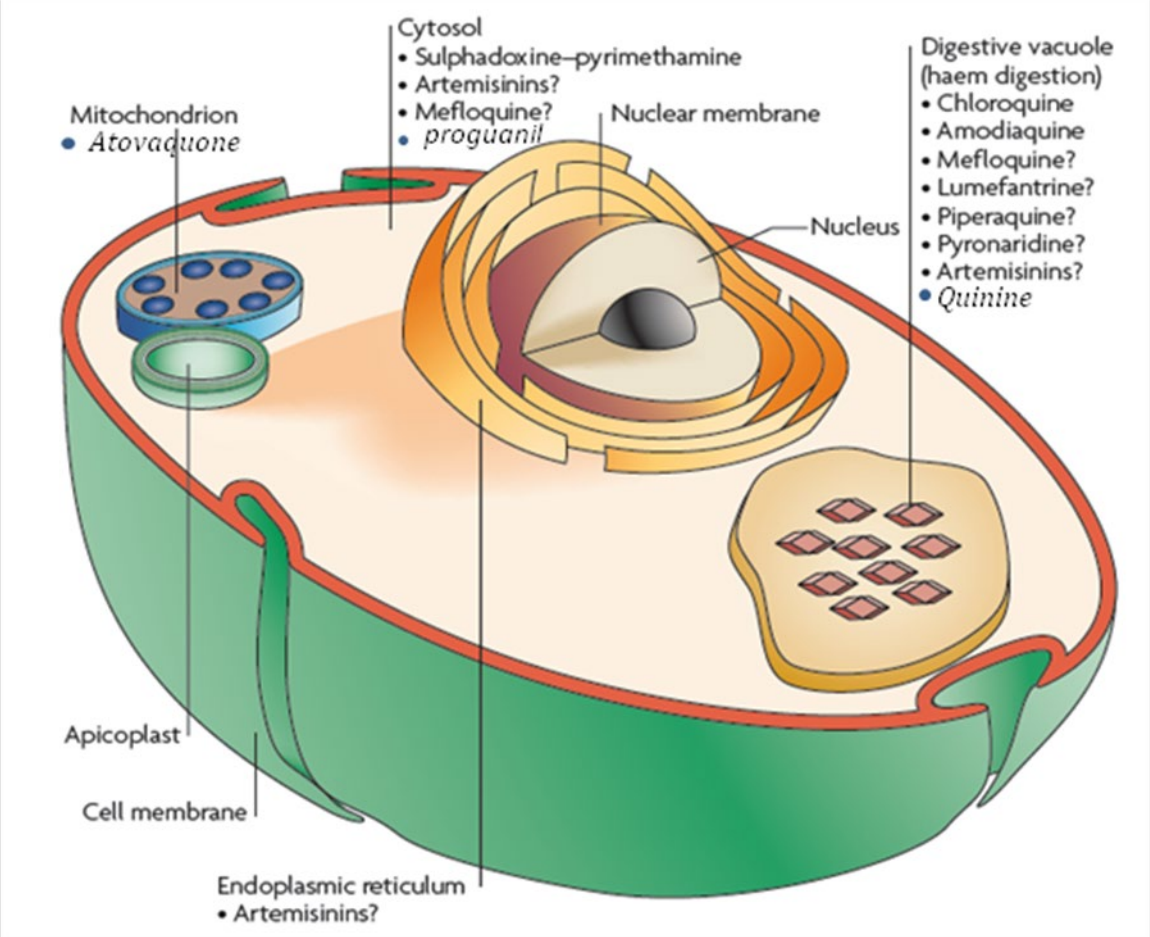


Figure 1-2: The sites of action of antimalarial drugs.The diagram depicts an intra-erythrocytic malaria parasite, and the proposed sites of action of key antimalarial drugs. Adapted with modification from Ref [39]. The drugs shown in italics are additions to the original figure.[Adapted by permission from Macmillan Publishers Ltd: Eastman, R. T. & Fidock, D. A. (2009). 'Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria', Nature Reviews Microbiology 7, 864-874. doi:10.1038/nrmicro2239].

As part of this DPhil programme of work, I investigated the activity and mechanisms of resistance of several antimalarial drugs that belong to the chemical class of quinolines. I have provided an overview of quinoline antimalarials and their proposed mode of action below (see section 1.5.1).

1.5.1 Mode of action of quinoline antimalarial drugs

Quinoline drugs used in the clinical management of *P.falciparum* malaria can be categorized into two main subgroups; a) 4-aminoquinolines (including chloroquine (CQ), amodiaquine (AQ), Piperaquine (PQ) and pyronaridine (PYN) and b) quinoline methanols (also referred to as aryl amino alcohols and include quinine (QN), mefloquine (MFQ), halofantrine (HLF) and

lumefantrine (LM). The quinoline antimalarial drugs studied in this DPhil project were CQ (Figure 1-3A), PQ (Figure 1-3B), LM (Figure 1-3C), and AQ (Figure 1-3D).

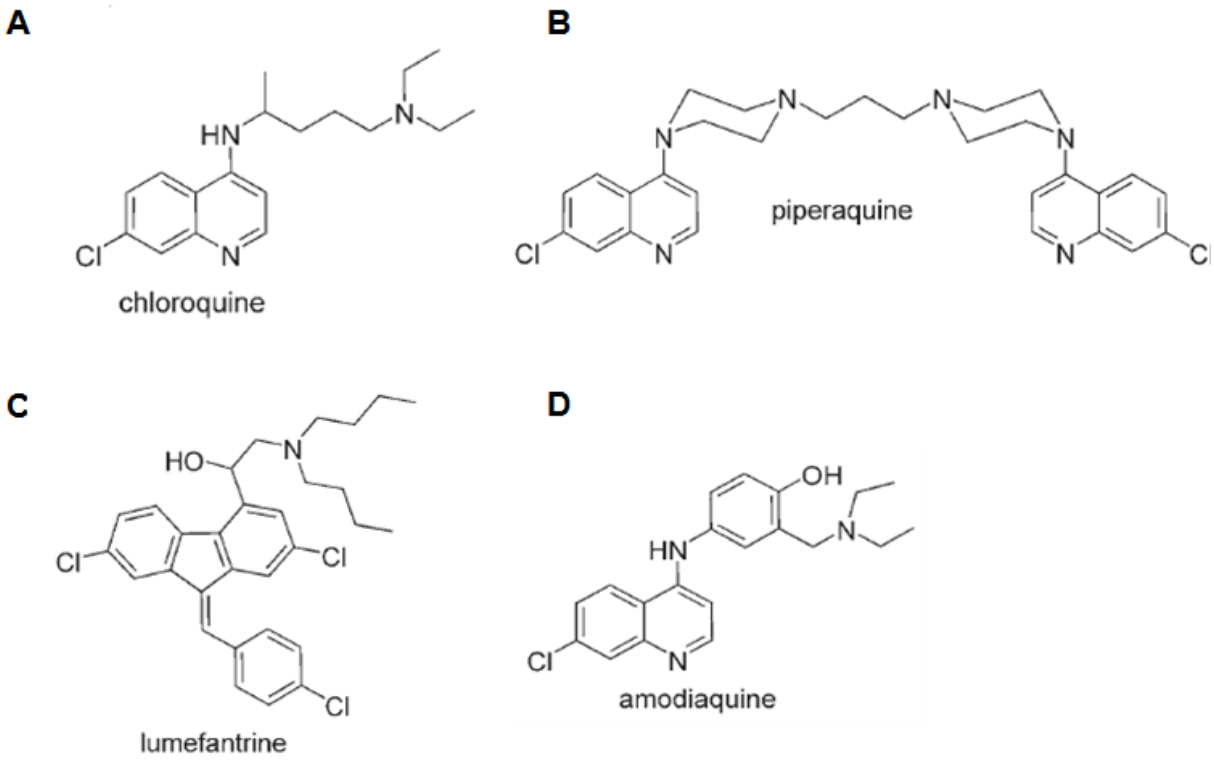


Figure 1-3: chemical structures of the quinoline antimalarial drugs

The quinoline drugs studied in this project. were A) chloroquine; B) piperaquine; C) Lumefantrine; D) Amodiaquine

The exact mechanisms by which quinolines exert antimalarial activity are still unclear. They are known to accumulate in the acidic digestive vacuole, a component of the endolysosomal system of the intra-erythrocytic malaria parasite [40].

Evidence suggests that the malaria parasite requires unsaturated fatty acids derived from membrane recycling for haemoglobin detoxification [40, 41], and that membrane recycling in the endolysosomal system is maintained through the process of vesicular docking [40, 42]. Further, it has been proposed that similar to other eukaryotic cells, the endolysosomal system of malaria parasites is regulated via a calcium signalling pathway [40, 43, 44].

Notably, although the quinoline methanols and the 4-aminoquinolines both act on the endolysosomal sytem, it has been proposed that they may kill the intraerythrocytic malaria parasite in different ways [40]. This is based on the observation that although treatment with the quinoline methanols MFQ and QN leads to morphologic changes similar to those observed after treatment with the 4-aminoquionoline CQ, treatment with the quinoline methanols does not lead to accumulation of haemoglobin and undimerized ferriprotoporphyrin IX (toxic heme) as is observed after treatment with CQ [45-50]. On the contrary, quinoline-methanols have been shown to prevent and partially reverse these CQ induced abnormalities in susceptible malaria parasites [49-55].

It is hypothesized that undimerized ferric heme (ferriprotophyrin IX , FP) is the target of CQ, and that binding of CQ to FP promotes accumulation of FP by inhibiting its dimerization to the non-toxic malaria pigment haemozoin (also known as ß-hematin) [56]. It is proposed that the CQ-FP complex is responsible for the morphologic changes and intrinsic biological toxicities observed in malaria parasites treated with the 4-aminoquinoline CQ. These include the impairment of membrane function [57-62], peroxidation of lipids [63, 64], destruction of glutathione [65], inhibition of proteolytic enzymes [66], release of calcium from the acidic store [67], redistribution of neutral aminopeptidase [53] and masking of the lipid that promotes FP dimerization[54].

The absence of FP accumulation after treatment with quinoline methanols led to the hypothesis that these compounds have a target other than FP in malaria parasites. This is supported by the observation that the quinoline methanols MFQ and QN bind with high affinity to membrane and purified phospholipids, suggesting that these molecules may be their biological targets [68-72].

The existence of an alternative phospholipid target is consistent with the observation that in *P.berghei*, the degree of MFQ accumulation was similar in erythrocytes infected with

unpigmented CQ resistant parasites (hence low amounts of FP) and erythrocytes infected with pigmented CQ susceptible parasites (hence high amounts of FP). Therefore, it is likely that the quinoline methanols prevent or partially reverse CQ induced abnormalities by impairing membrane recycling within the endolysosomal system of the intracellular parasite [47, 48], and that it is this impairment that ultimately leads to impaired heme degradation and death of the parasite. Further, it has been suggested that the observed impairment of membrane recycling possibly occurs via the inhibition of ca^{2+} ion release which results in the inhibition of vesicular docking [40]. The observation that CQ does not bind with high affinity to phospholipids [68] suggests that these molecules may not be CQ targets. However, undimerized FP binds with high affinity to phospholipid, suggesting that CQ may interact indirectly with phospholipids, with FP serving as a bridge.

Thus, although, the 4-aminoquinolines and quinoline methanols accumulate in the acidic food vacuole where they impair heme detoxification, these drugs may have different (or additional) targets in the malaria parasite.

1.5.2 Artemisinin combination therapy (ACT)

For decades, CQ was the mainstay of uncomplicated malaria therapy. The eventual appearance and spread of CQ resistance led to the introduction of SP which soon met with rapid development of resistance, rendering it increasingly ineffective in many endemic areas [73]. To date, *P.falciparum* clinical treatment failure has been reported for all the common classes of antimalarial drugs apart from artemisinin compounds, although there are recent reports of reduced artemisinin sensitivity in Cambodia [74, 75].

To reduce the pace of selection of resistance, WHO recommends that all antimalarial therapies be deployed as combinations that include an artemisinin derivative as one of the partner drugs, a strategy referred to as Artemisinin Combination Therapy (ACT). The introduction of ACT in malaria endemic countries together with other malaria control

interventions (see section 1.4) is reported to have contributed to the recent declines in malaria mortality and morbidity observed in some settings [76]. I have outlined the main components of ACT below.

1.5.2.1 Artemisinins

Extracts of *Artemisia annua* (a Chinese herb also known as sweet worm wood or qinghaosu) have been used in China for as long as 2000 years in the treatment of fever [36]. The isolation of the active ingredient, the sesquiterpene lactone artemisinin (ART) in 1972 [77] was a major breakthrough in malaria treatment. To improve on the poor bioavalilability of ART, the ART derivatives artemether (ATM), artesunate (AS) and dihydroartemisinin (DHA) were developed. Figure 1-4 shows the chemical structures of artemisinin (Figure 1-4A), dihydroartemisinin (Figure 1-4B) and artemether (Figure 1-4C).

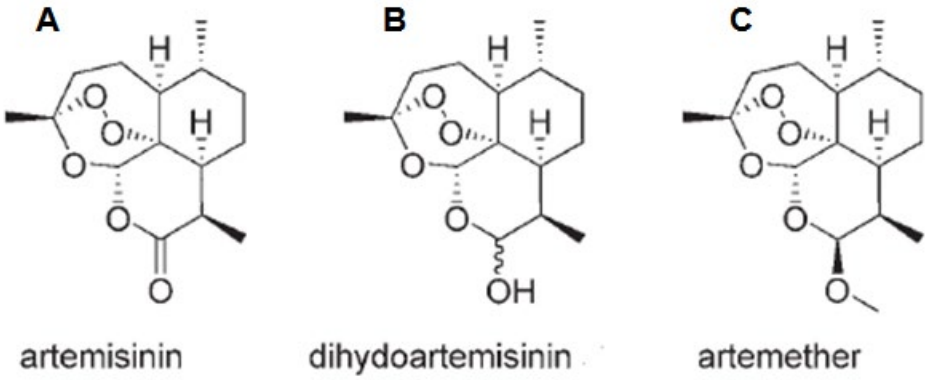


Figure 1-4: chemical structures of artemisinins.
The structures of artemisinin (A) and its derivatives dihydroarteminin (B) and artemether (C) are shown.

ART is highly potent against CQ and SP resistant *P.falciparum* parasites *in vitro* and *in vivo*, producing faster parasite clearance and fever resolution than any other licensed antimalarial [78].

The exact mode and site of action of ART compounds is still unclear. It has been proposed that their activity results from reductive scission of their peroxide bridge by reduced heme iron (produced inside the digestive vacuole as the parasite digests haemoglobin); this process is thought to lead to the formation of toxic heme adducts [79-83]. An alternative hypothesis suggests that that ART peroxide bond cleavage might be activated by non-heme iron sources (such as intracellular iron-sulphur-redox centres found in multiple Plasmodium enzymes). It has been suggested that the activated ART might in turn generate free radicals that alkylate and oxidize various proteins and possibly lipids in parasitized erythrocytes [84-86]. It is this alkylation that is thought to cause parasite death. Recent *in vivo* and *in vivo* studies have suggested that ART may specifically target *PfATP6*, a *P.falciparum* SERCA type Ca^{2+} dependent ATPase localized in the endoplamic reticulum[87, 88]

Despite their high potency, ART antimalarials are limited by their short half-lives, which translate into long treatment durations and high treatment failure rates when used as mono therapy. Combining ART derivatives with long half-life partner drugs effectively shortens treatment duration to standard 3-day regimens, delays the development of resistance and may reduce the transmissibility of malaria through reduced gametocyte carriage [89]. PQ, LM, AQ, SP, MFQ, and PYN are longer half-life partner drugs that are currently being used in ACT (see section 1.5.2 for an overview of ACT). Below, I give an overview of LM, PQ and AQ, the artemisinin partner drugs which I investigated in my Dphil project.

1.5.2.2 Lumefantrine

LM (also known as benflumetol) (see Figure 1-3C) is a quinoline methanol (aryl amino alcohol) antimalarial drug. As LM is structurally related to quinine, mefloquine and halofantrine, it is likely that they have similar modes of action i.e. interfering with heme detoxfication (see section1.5.1 for a detailed overview of their modes of action).

LM and ATM were reported to have synergistic effects *in vitro* [90], leading to the development of the fixed dose combination of LM-ATM (known as Coartem®; Norvatis/Chinese Academy of Medical military Sciences). Studies have demonstrated that the 3 day 6 dose regimen of LM-ATM is safe and highly effective in the treatment of uncomplicated *P.falciparum* malaria [91-93]. In Kenya, LM-ATM replaced SP as first-line treatment in government facilities in 2006 [94].

LM-ATM has several disadvantages including a complicated dosage regimen, poor and highly variable oral bioavailability of the LM component, necessitating administration with a fatty meal to improve bioavailability, and a relatively high cost of treatment despite substantial subsidies. Therefore, other regimens such as DHA-PQ (see below section 1.5.2.3) and AS-AQ (see below section1.5.2.4) have been considered as alternatives to LM-ATM in Africa.

LM and ATM have different pharmacokinetic properties. Like most artemisinin derivatives, ATM is a very short acting drug, with a half-life of less than 2 hours [95, 96] while LM is a long acting drug, with a half-life > 4 days [96-99]. Despite the ACT strategy to decrease the rate of development of resistance, there are concerns about the development of clinical resistance to LM-ATM. It has been proposed that LM, being the partner drug with a longer elimination half-life, would provide the main selective pressure for resistance to LM-ATM combination [100] (see section 1.6.2.3 for a more detailed explanation).

1.5.2.3 Piperaquine

PQ (Figure 1-3B) is an aminoquinoline, structurally related to CQ. Although the studies on the exact mode of action of PQ have been limited, it is likely to work in a similar manner to 4-aminoquinolines such as CQ (see section 1.5.1 for a detailed overview of quinoline mode of action).

The fixed dose combination of DHA-PQ, (Artekin™) is now being evaluated as an alternative to LM-ATM in several African countries including Kenya. It offers several distinct advantages over LM-ATM including a once daily regimen for 3 days, which is likely to improve adherence and hence, effectiveness. In addition, DHA-PQ is relatively cheap (costing approximately US$ 1 per adult treatment) compared to LM-ATM (US$ 8).

PQ was developed and heavily used as monotherapy in China during the 1970s and 1980s in response to increasing CQ failure rates. Notably, its use was accompanied by a rapid selection of resistance; *in vitro* evidence showed that PQ resistance increased from 18 to 48% during the late 1980s and from 48 to 98% during the 1990s [101-103]. This *in vitro* resistance was followed inevitably by the emergence of *in vivo* resistance.

The rapid selection of resistance when PQ was used as monotherapy suggests that selection of resistant parasites could occur relatively quickly, even when this drug is used in combination with DHA. Compared to the short acting DHA, PQ is a long acting drug, with a terminal elimination half-life of up to 33 days [104, 105]. It has been proposed that the selective pressure for DHA-PQ resistance would be primarily exerted by sub-therapeutic concentrations of PQ at time of re-infection (unprotected by DHA)[106] (see section 1.6.2.3 for a more detailed explanation).

1.5.2.4 Amodiaquine

AQ (Figure 1-3D) is a key antimalarial that was developed during world war II as an alternative to quinine [107]. Although there were initial reports of agranulocytosis and hepatitis when AQ was used as prophylaxis, a detailed analysis revealed that AQ was more effective than CQ and relatively safe [108]. Indeed, AQ has been demonstrated to be significantly more effective than CQ in the treatment of uncomplicated malaria in Africa, even in areas of high CQ resistance [109].

Being an aminoquinoline closely related to CQ, AQ is thought to act in a similar way to CQ (see section 1.5.1 for an overview of quinoline mode of action). *In vivo*, AQ has a short half-life of about 3 hours. It is rapidly metabolized into its active metabolite desethyl amodiaquine (DEAQ). DEAQ has a longer elimination half-life than AQ (1-3 weeks) and hence is responsible for most of the antimalarial activity [110].

Several AQ containing combinations have been recommended for use in Africa. For example, WHO currently recommends the use of AQ-AS combination in areas where AQ resistance is less than 20%, and AQ-SP combination was recommended as an interim option where ACT could not be made available, provided the efficacy of both drugs is high [89]. AQ has also been considered for intermittent preventive treatment in infants [111].

Inadequate response or treatment failure of AQ has been reported in endemic sites in Asia [112, 113], South America [114], Papua New Guinea [115] and more recently in East Africa [116-120]. Interestingly, differential AQ and CQ responses have been documented for isolates from different Geographic settings. Whilst AQ resistance is correlated to some extent with CQ resistance in Africa (where CQ resistance is predominant), CQ resistant parasites in this setting appear to have lower levels of resistance to AQ [121-123]. On the other hand, only moderate levels of CQ resistance but high levels of DEAQ resistance are observed in South America [124]. Further, AQ and CQ resistance levels have continued to be stable in South America for decades, despite withdrawal of the drugs [125]. This contrasts with observations in several endemic sites in Africa and Asia where a re-emergence of CQ-sensitive *P.falciparum* has been reported after only a few years of drug withdrawal (see section1.10). Furthermore, following chemosensitization with verapamil, CQ resistance is more readily reversed in African than South American resistant parasites [126]. Clearly, different phenotypes of CQ and AQ resistance emerged in Africa and South America, and this may account for the differential responses observed.

Importantly, mutations in the *P.falciparum* chloroquine resistance transporter (*pfcrt*, see section 1.8.1) and multidrug resistance gene 1 (*pfmdr1* see section 1.8.2) have been linked with CQ and AQ resistance [127]. Distinct *pfcrt* haplotypes at codon 72-76 occur in different geographic regions (see Table 1-1for a summary of *pfcrt* haplotypes). Whilst the CVIET haplotype is predominant in Africa and South East Asia, the SVMNT haplotype is found predominatly in South America and the Pacific region. Although AQ has remained relatively effective in East Africa (where the *pfcrt* 72-76 CVIET haplotype is predominant), AQ treatment failure has been associated with a significant selection of parasites carrying the *pfmdr1* 86Y, 184Y and 1246Y mutations [118, 128]. Clearly, the *pfcrt* CVIET allele is insufficient to confer clinical AQ resistance in this setting, and additional *pfmdr1* mutations are required. On the other hand, molecular studies have shown that the *pfcrt* codon 72-76 SVMNT haplotype, which is predominant in South America, is sufficient to confer *in vivo* AQ resistance [129]. This haplotype is therefore considered the most crucial molecular determinant of AQ response.

Importantly, although AQ combinations have been reported to improve treatment outcomes in Africa [130, 131], the observed selection of CQ resistant genotypes after AQ treatment raises concern about future AQ effectiveness. It has been proposed that the selective pressure for resistance to AQ-AS combinations would be exerted by the longer acting DEAQ component (see section 1.6.2.3 for a more detailed explanation). Importantly, the AQ resistant *pfcrt* 72-76 SVMNT allele has already been detected in some endemic areas in sub-Saharan Africa [132-134], and the role of AQ use in the selection of this haplotype has been suggested. Therefore, further research, coupled with continued monitoring of AQ resistance in sub-Saharan Africa is crucial.

1.6 Antimalarial drug resistance and drug tolerance

1.6.1 Definition of resistance

Antimalarial drug resistance has been defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject [135]. Most often, the term resistance refers to therapeutic failure after administration of a standard dose of an antimalarial drug, as defined in the WHO standard *in vivo* test protocol [136]. Importantly, host factors such as immunity, pharmacokinetics, pharmacodynamics and the presence of co-morbidities must be considered when reporting resistance, as they may influence the clinical outcome after treatment with an antimalarial drug.

Sometimes, the term resistance is used to refer to the ability of a parasite phenotype to survive a threshold concentration of drug under standard *in vitro* culture conditions. Notably, the demonstration of *in vitro* resistance may more accurately reflect biological resistance to an antimalarial drug, as it eliminates confounding host factors [137]. However, to be clinically relevant, the definition of true parasite resistance requires a linking of *in vitro* and *in vivo* data, and a clear demonstration of the ability of parasites to survive *in vivo* in the presence of adequate therapeutic concentrations of the drug in serum [138].

1.6.2 Dynamics of resistance

Drug resistance in the malaria parasite arises from rare spontaneous mutations which are then selected by sub therapeutic antimalarial drug concentrations i.e. the drug concentrations efficiently eliminate susceptible parasites but inhibit to a less extent or do not inhibit the multiplication of resistant parasites [139-141]. It is noteworthy that, some field isolates may have 'innate resistance' to certain antimalarials (prior to drug exposure); in such instances, prevailing drug pressure would promote the multiplication of such intrinsically resistant

parasites [142]. Therefore, the current evidence suggests that the genetic events that confer resistance are spontaneous and rare, and in some instances, these may be independent of the drug [140]. Importantly, even when mutation rates are low, antimalarial resistance inevitably arises when mutant alleles are selected under drug pressure.

Drug resistance may be conferred by mutations or copy number changes of genes which encode (or are related to) the drug's target, or its efflux/influx mechanism in the parasite [143]. Interestingly, although other mechanisms of drug resistance such as transferrable resistance genes, the production of drug inhibiting enzymes and the activation of accessory metabolic pathways have been recognized in bacterial pathogens [144], these do not appear to be involved in acquisition of antimalarial drug resistance so far. Antimalarial drug resistance mutations may lead to changes in the rates of drug accumulation or efflux (as in the case of resistance to quinolines and quinoline methanols) or reduced affinity of the drug to its specific drug target (as in the case of resistance to SP and ATV) [145].

1.6.2.1 The evolution of resistance

The development of resistance to an antimalarial drug can be considered in two parts: the first is the initial genetic event which produces the resistant mutant (the *de novo* arising of resistance), and the second is the subsequent selection process in which the survival advantage in the presence of drug promotes preferential transmission and spread of resistance [140]. Various factors may increase the probability of the first step and subsequently of resistance emerging.

For example, when subjected to *in vitro* drug pressure, a multidrug resistant strain acquired resistance to 5-flouro-orotate and atovaquone at 10-1000 fold higher frequencies than sensitive strains, a phenomenon referred to as accelerated resistance to multidrug (ARMD) [146]. Another study demonstrated that resistance to artemisinins is a multifactorial trait and that genetic predisposition may favour the acquisition of high level resistance in some

parasites [147]. Interestingly, the rate at which parasites generate drug resistant clones, referred to as per parasite resistance frequency (PRF) varies according to the gene that confers resistance; PRF is higher for drugs for which resistance involves one gene (as in the case of ATV) compared to drugs for which resistance is multifactorial (as in the case of CQ and ART) [140]. It is noteworthy that mutation frequencies derived from *in vitro* studies are often much higher than those derived from observations *in vivo*. For example the PRF of ATV *in vivo* is estimated to be 1 in 10^{12} , whereas *in vitro* this rate can be as high as 10^{5} [140].

In vivo resistance is more likely to arise from infections with high parasitaemias, which receive inadequate treatment. As host immunity contributes a major antiparasitic effect, it can significantly reduce the emergence of resistant mutants. Also, as *de novo* resistance is more likely to be subsequently selected for in patients with lower blood levels of drug, the use of drugs with variable pharmacokinetics and pharmacodynamics (such as LM and ATV) increases the probability of resistance [140]. The rate of emergence of resistance is decreased by the presence of two or more antimalarial drugs (preferably with different mechanisms of resistance and comparable pharmacokinetic profiles) [140]. Resistance has been observed to arise most frequently and repeatedly in S.E Asia [148-151], and anecdotal evidence suggests that drug resistance to new drugs may occur more easily in that setting than in other parts of the world. The higher frequency of resistance may be attributable to low transmission resulting in low host immunity, which ultimately results in a lot of symptomatic malaria infections exposed to drug treatment [140].

In summary, these observations show that although the *de novo* appearance of resistance in *P.falciparum* is spontaneous and rare, the evolution and spread of resistance is often modulated by several external factors.

1.6.2.2 The role of transmission in the spread of drug resistance

The evolution of drug resistance mutations in itself is not sufficient for the spread of malaria drug resistance. Rather, the survival and subsequent multiplication of drug resistant parasites after drug treatment increases the likelihood of transmission of resistant sexual stages (gametocytes) to the next host, therefore increasing the likelihood of the spread of resistance. Notably, some studies demonstrated that gametocyte carriage and infectivity to mosquitoes was consistently higher in patients infected with drug resistant parasites than in those infected with drug sensitive parasites [152, 153]. Therefore, although elimination of the asexual blood stages is the focus of treatment in individual symptomatic patients, at a population level, reducing the post -treatment carriage of sexual stages (gametocytes) is necessary to limit the transmission of resistant malaria parasites to new hosts.

Interestingly, individuals living in regions where malaria is endemic develop an acquired partial immunity to malaria, which protects them against severe illness without eliminating chronic, mild and asymptomatic infections (see section 1.3). The level of acquired immunity is lower for individuals living in low transmission settings compared to those living in high transmission settings [154]. This acquired partial immunity can affect malaria transmission to mosquitoes and drug resistance in several ways.

For example, because levels of immunity are lower in low transmission settings, many patients are often symptomatic and seek treatment when infected with malaria. On the other hand, owing to the high levels of immunity acquired in high transmission settings, many individuals who are parasitaemic are often asymptomatic, fewer individuals seek treatment when infected with malaria, and consequently, fewer parasites are exposed to drugs [155]. Therefore, based on this basic model, it would be expected that selective pressure would be lower in high transmission settings because infections from asymptomatic carriers have a higher transmission advantage than those from patients carrying resistant infections.

However, using a mathematical model, it was recently demonstrated that the spread of resistance was not dependent on the intensity of transmission but rather on the ratio of the periods of infection in treated and untreated infections, and on the rates of transmission of resistant parasite phenotypes from infectious humans [154]. Clearly, the spread of resistance is a highly dynamic process.

Furthermore, because humans infected with *P.falciparum* often carry several distinct infections (different parasite clones), the intrahost dynamics involved after treatment may also affect the transmission of drug resistant parasites [156-158]. Importantly, studies based on mathematical models have demonstrated that treatment does not always eliminate infection in a population [154]. For instance, as antimalarial treatment does not usually eliminate mature gametocytes, an individual who has successfully been treated with an effective antimalarial may become asymptomatic but remain infectious until the gametocytes die off naturally, or until an antimalarial that eliminates gametocytes is administered. Therefore, it has been argued that treatment with gametocytocidal drugs such as artemisinins can prevent the spread of malaria drug resistance [152]. However, recent evidence suggests that gametocytocidal activity promotes (rather than reduces) transmission of malaria drug resistance through a population, the underlying reason being that gametocytocidal activity reduces transmission of drug sensitive parasites to a greater extent than drug resistant ones, thereby increasing spread of the latter [159]. In addition, it has been shown that resistance spreads faster in regions with better access to drugs, and with increasing drug use [154, 160]. Therefore, in endemic settings it is important to consider not only the ability of antimalarials to cure clinical malaria, but also their effect on the transmission of drug resistant phenotypeshe at a population level.

It has been suggested that there exist critical treatment rates above which resistance spreads and eventually becomes fixed, and below which resistance cannot spread [154].Therefore, several strategies have been proposed to prevent the transmission of drug resistant phenotypes. To reduce infectious periods and infectivity of individuals, timely and effective

antimalarial treatment, increased compliance to antimalarial regimens and early identification of drug resistance in patients are recommended. Additionally, measures that increase the mortality rate of adult mosquitoes such as house spraying have been shown to prevent the spread of drug resistant malaria by decreasing the number of resistant parasites that are transmitted [154, 161]. In a population that has developed resistance to a particular drug, restraining the use of the drug for some time could result in a re-emergence of sensitive parasites (see section 1.10). Therefore, depending on the context, several public health strategies may be applied to curb transmission of drug resistance in malaria endemic settings.

1.6.2.3 The role of tolerance in evolution of drug resistance

Previously, the evolution of antimalarial drug resistance was considered to be a single step process, whereby parasites that are fully sensitive to drug become fully resistant, thus leading to a dichotomous classification of clinical malaria infections i.e. any clinical infection classified as ‘resistant’ or ‘sensitive’ to an antimalarial at therapeutic doses. However, the use of such a strict and generalized dichotomy in classification of resistance has been questioned [106]. This is because whilst such a strict distinction between sensitivity and resistance may be appropriate for antimalarial drugs to which resistance arises in a single step (e.g. ATV to which resistance arises through a single mutation in the *cytb* gene [162], this dichotomy does not take into account the important intermediate stage of drug tolerance.

Drug tolerance is now considered a biologically important stage in the step-wise evolution of malaria drug resistance. Importantly, studies suggest that the drug elimination profile is an important parameter that determines the evolution of tolerance and ultimately resistance [163, 164]. These studies suggest that selective pressure for resistance to antimalarial combinations is exerted by the longer acting antimalarials, which persists in the body below effective concentrations long after treatment, promoting the selection of tolerance and ultimately resistance. Further, studies suggest that that even when true clinical resistance is not apparent,

drug tolerance might be associated with specific biological processes in the parasite [165-167].

If the concept of the existence of selective pressure because of long elimination half-life applies to all antimalarials, it is expected that selective pressure to the ACTs LM-ATM, DHA-PQ and AS-AQ would be exerted by LM, PQ and DEAQ which are the partner drugs with longer half lives (compared to the short acting artemisinin components).

As part of my work towards this thesis, I investigated whether selection for resistance to LM-ATM, DHA-PQ and AS-AQ was associated with LM, PQ and DEAQ respectively.

1.7 The role of membrane transport proteins in antimalarial drug resistance

The parasite-infected erythrocyte consists of multiple compartments with numerous discrete membrane systems. As the parasite grows in the host erythrocyte, it is separated from the extracellular environment by the erythrocytic (host cell) membrane and a 'parasitophorous vacuole membrane' which is closely juxtaposed with the parasite plasma membrane. Within the parasite, there are numerous organelles, which include a large acidic digestive vacuole and other smaller acidocalcisomes (all bounded by single membranes), a single mitochondrion (bounded by a double membrane), and an apicoplast (bounded by four membranes).

Membrane transport proteins (which consist of pumps, porters or channels) are integral proteins which play a key role in the survival of the parasite by mediating the movement of various important solutes (such as nutrients, waste materials and drugs) and by generating and maintaining the electrochemical gradients across the various membranes [168].

In human cancer cells and in many pathogens (including malaria parasites), drug resistance has been linked to mutations or changes in the expression patterns of various transport

proteins [169-174]. In malaria parasites, the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt,* see more details in section 1.8.1) and the p-glycoprotein homologue gene *pfmdr1,*(see more details in section1.8.2) are important mediators of aminoquinoline drug resistance [175], whilst the *P.falciparum* ATPase6 (*pfatpase6)* gene is a proposed mediator of artemisinin drug resistance *[175]*. Evidence suggests that the *P.falciparum* Na+/H+ exchanger *(*PfNHE*)* may alter quinine susceptibility [176] and the *Plasmodium falciparum* multidrug associated protein *(*PfMRP) may influence the susceptibility of malaria parasites to several antimalarial drugs [177]. In a genome wide association study, 11 putative transporter genes (including *pfcrt, pfmdr1* and *pfmrp1)* were reported to be associated with decreased susceptibility to CQ and quinine [178]. Other candidate transporters were shown to be differentially expressed in drug resistant or drug treated parasites [179-181]. Although the exact mechanisms by which transporters modulate antimalarial drug resistance are still largely unclear, studies on CQ have linked resistance to a reduction in drug accumulation resulting from impaired drug uptake [56], enhanced drug efflux [182], and/or modulation of ΔpH across the food vacuole membrane [183, 184]. Studies of *pfcrt* expression in oocytes proposed that *pfcrt* was also a direct or indirect activator or modulator of other transporters [185]. Therefore, transporter genes may modulate antimalarial activity in different ways and it is possible that this role requires a functional interplay between them.

A recent comprehensive search revealed that genes encoding transporters and channels account for at least 2.5% of the Plasmodium genome, indicating that *P.falciparum* encodes for more transporters than had been originally thought [186, 187]. An improved understanding of the mechanisms that underpin antimalarial drug resistance will require more studies on the role of such transporters. I have highlighted the key transporters associated with quinoline resistance below.

1.8 Transporters associated with quinoline resistance

PQ, and AQ belong to the aminoquinoline group of antimalarial drugs such as CQ, which exert their antimalarial effects by interfering with heme polymerisation [188, 189], thus are thought to have similar modes of action. It has been proposed that LM (a quinoline-methanol) also exerts its antimalarial effects by interacting with heme polymerisation, similar to CQ [188, 190], (also see section1.5.1 for an overview of quinoline mode of action). Many studies that have examined the mechanism of resistance to CQ and other quinoline based drugs in *P.falciparum*, have implicated *pfcrt* and *pfmdr1* as the two transporter genes that mediate resistance. Because of their structural similarity, it is logical to hypothesize that the same tranporter genes may contribute to AQ and PQ, and perhaps to some extent, LM resistance. The two transporter genes are outlined below (see sections1.8.1 and 1.8.2)

1.8.1 pfcrt

PfCRT(see Figure 1-5) is a member of the drug/metabolite super family of transporters [191]. *pfcrt* resides on chromosome 7 and encodes a 45-kDa protein with ten predicted transmembrane domains, located on the membrane of the parasite digestive vacuole (an acidic lysosome –like compartment in which hemoglobin is degraded and detoxified, and in which CQ is known to accumulate in sensitive parasites and bind in its diprotonated form to hematin) [192, 193]. Polymorphisms in *pfcrt* are a key determinant of CQ-resistance both *in vivo* and *in vitro* [194, 195].

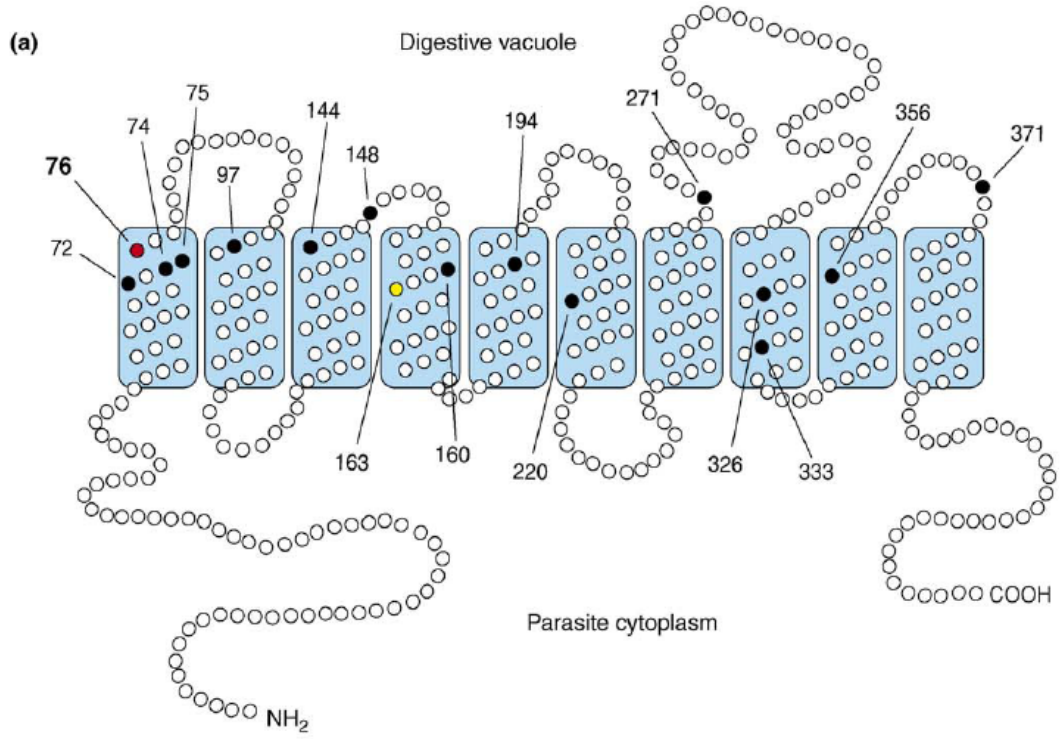


Figure 1-5: The predicted structure of PfCRT.

PfCRT has 10 transmembrane domains shown (blue shaded regions). Known point mutations are indicated as shaded circles. Adapted from Ref [175] and [196].[Reprinted with permission from John Wiley and Sons: Bray, P. G. et al. (2005). 'Defining the role of PfCRT in Plasmodium falciparum chloroquine resistance', Molecular Microbiology 56(2), 1365-2958. Available at http://dx.doi.org/10.1111/j.1365-2958.2005.04556.x] and from Elsevier: Valderramos, S. G. & Flock, D.A. (2006). 'Transporters involved in resistance to antimalarial drugs', Trends in Pharmacological Sciences 27(11), 594-601. Available at http://dx.doi.org/10.1016/j.tips.2006.09.005].

The polymorphism documented to date in PfCRT is considerable (Table 1-1), with a total of 15 SNPs located between codons 72 and 371. PfCRT haplotypes differ by geographic origin of the parasite (see Table 1-1), with the CIET allele (wild type 72 and mutants at 74, 75 and 76 codons) being the ubiquitous mutant allele in Asia and Africa [148]. Other important PfCRT haplotypes include the CIDT allele (found in South East Asia), CIKT (found in the Pacific region) and the SMNT (found in South America and the Pacific) (seeTable 1-1).

Table 1-1: PfCRT haplotypes in parasites from different geographic regions

Residues that differ from the wild type indicated in grey shading. Adapted from Ref [175]. Reprinted with permission from Elsevier: http://dx.doi.org/10.1016/j.tips.2006.09.005

		PfCRT position and amino acid														
Region	**Reference line**	**72**	**74**	**75**	**76**	**97**	**144**	**148**	**160**	**194**	**220**	**271**	**326**	**333**	**356**	**371**
All	**CQ sensitive (wild type)** (HB3, Honduras)	C	M	N	K	H	A	L	L	I	A	Q	N	T	I	R
	CQ Resistant															
Asia and Africa	DD2 (Indochina)	C	**I**	**E**	**T**	H	A	L	L	I	**S**	**E**	**S**	T	**T**	**I**
South East Asia	734 (Cambodia)	C	**I**	**D**	**T**	H	**F**	**I**	L	**T**	**S**	**E**	N	**S**	I	R
Pacific Region	2300 (Indonesian Papua)	C	**I**	**K**	**T**	H	A	L	L	I	**S**	**E**	**S**	T	I	**I**
	PH2 (Phillipines)	**S**	M	N	**T**	H	**T**	L	**Y**	I	A	Q	**D**	T	I	R
South America and Pacific	7G8 (Brazil)	**S**	M	N	**T**	H	A	L	L	I	**S**	Q	**D**	T	L	R

PfCRT mutations are found mainly (but not exclusively) in the transmembrane domains (see Figure 1-5). Mutations in domain 1, 4 and 9 are proposed to play a particularly critical role in accumulation of quinoline drugs in the digestive vacuole [197]. Of all the mutations, the PfCRT K76T mutation is the most critical determinant of CQ resistance *in vitro* and *in vivo,* making it a useful marker of CQ resistance [194, 195, 198, 199].

Several mechanisms are proposed to account for the exact mechanism by which mutated *pfcrt* mediates reduced concentration of CQ in the food vacuole of CQ resistant parasites. It was initially postulated that diprotonated CQ passively leaks out of the food vacuole along an electrochemical gradient, the mutated PfCRT acting as a channel [200]. It has also been proposed that mutated PfCRT modulates drug accumulation indirectly by altering the pH of the digestive vacuole either alone [201] or through interaction with other transporters (such as

the V-type H+ ATPase (PVP2), PfMDR1 or the sodium hydrogen exchanger (PfNHE) [183, 185, 202-204]. PfCRT has also been proposed to have a role in the transport of amino acids and small peptides within the digestive vacuole, although it is not clear how this might influence CQ action [205, 206]. It has also been postulated that *pfcrt* may be an energy dependent CQ transporter which actively pumps CQ out of the food vacuole [207-209]. Therefore, the exact mechanisms by which PfCRT modulates drug susceptibility are yet to be elucidated.

Mechanisms other than PfCRT mutations may also account for the reduced CQ concentrations observed in the parasite food vacuole. For example, the recent identification of a number of putative binding sites for nuclear receptors in the proximal promoter region of *pfcrt* provides some evidence that *pfcrt* may alter CQ susceptibility via a nuclear mediated mechanism [210]. Notably, similar to observations in mammalian cancer cells, CQ resistance in *P.falciparum* can be reversed by verapamil, although the level of CQ sensitivity obtained is not equivalent to that seen in CQ sensitive wild type parasites [211-213]. The observation that a component of CQ resistance remains verapamil insensitive supports the hypothesis that CQ resistance in malaria parasites is multigenic [214]. Importantly, expression of *pfmdr1* in mammalian cells modulated CQ accumulation, and this effect was verapamil insensitive [215]. This led to the suggestion that *pfmdr1* may be responsible for the verapamil insensitive component of CQ resistance in *P.falciparum*.

Interestingly, mutations in *pfcrt* also significantly alter parasite susceptibility to many other antimalarials including QN, DEAQ , HLF and ART [193, 198, 199, 216]. Indeed, mutations in *pfcrt* associated with resistance to CQ (a 4-aminoquinoline) were associated with increased susceptibility to QN and MFQ suggesting different responses to this class of drugs by malaria parasites [199]. It is proposed that the significant impact of *pfcrt* on the activity of antimalarials other than CQ could explain the extraordinary sequence diversity of *pfcrt* alleles in geographically distinct isolates [148, 175].

1.8.2 pfmdr1

The *Plasmodium falciparum* multidrug resistance transporter (PfMDR1,see Figure 1-6) is a *P.falciparum* ortholog of mammalian p glycoproteins that mediate verapamil reversible multidrug resistance in mammalian cancer cells [182]. *pfmdr1* (also referred to as *pgh1)* is located on chromosome 5 and encodes a 162-kDa ABC type transporter, that localizes to the membrane of the digestive vacuole [175]. PfMDR1 consists of two homologous halves, each with six predicted transmembrane domains and a conserved nucleotide binding domain [175, 217].

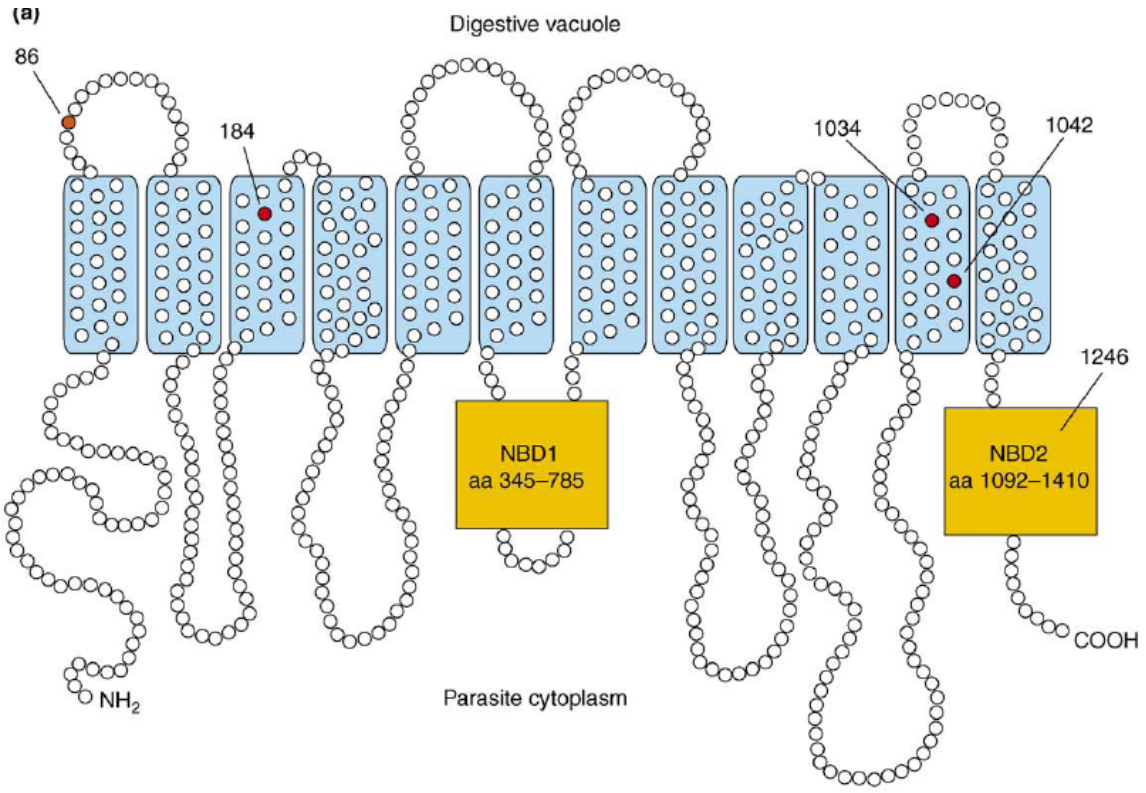


Figure 1-6: Predicted structure of PfMDR1 PfMDR1 consists of 2 homologous halves each with 6 TM domains.The nucleotide bindings domains and known polymorphisms are shown. Adapted from Ref [175]. **[Reprinted with permission** from Elsevier: Valderramos, S. G. & Flock, D.A. (2006). 'Transporters involved in resistance to antimalarial drugs', Trends in Pharmacological Sciences 27(11), 594-601. Available at http://dx.doi.org/10.1016/j.tips.2006.09.005].

Table 1-2: PfMDR1 polymorphisms from different geographic regions.Residues that differ from the wild type are indicated in grey shading. Adapted with modification from Ref [175]. **Reprinted with permission from Elsevier: http://dx.doi.org/10.1016/j.tips.2006.09.005**

		PfMDR1 position and amino acid					
Region	**Reference line**	**86**	**184**	**1034**	**1042**	**1246**	**Copy number**
All	Wildtype:3D7(Netherlands)	N	Y	S	N	D	1
Honduras	HB3 (CQ sensitive)	N	F	S	D	D	1
Asia and Africa	FCB(South East Asia)	N	Y	S	N	D	≥2
	K1(Thailand)	Y	Y	S	N	D	1
South America	7G8 (Brazil)	N	F	C	D	Y	1

PfMDR1 polymorhisms occur at five amino acid positions; 86, 184, 1034, 1042 and 1246 [218] (see Figure 1-6 and Table 1-2). *pfmdr1* is thought to play an ancillary role in CQ resistance, although the evidence has sometimes been conflicting. Indeed, although some studies reported an association between the *pfmdr1* N86Y mutation and CQ resistance [219-223], many studies have shown that *pfmdr1* mutation status on its own is not sufficient to confer CQ resistance in a *pfcrt* wildtype background (147, 150, 167, 195, 199-201). However, some studies have demonstrayed that the *pfmdr1 N86Y* mutation may increase *in vitro* CQ resistance in isolates already carrying the mutant *pfcrt 76T* allele [221, 223, 224] [175, 178, 195, 221, 225-227], demonstrating that *pfmdr1* indeed plays a role in modulating the level of CQ resistance. The contribution of *pfmdr1* to CQ resistance has also been demonstrated in mammalian cells where expression of *pfmdr1* led to increased accumulation of CQ (187). Therefore, although *pfmdr1* mutations may not confer CQ resistance *per se*, they may modulate the level of CQ resistance, once *Pfcrt* mutations are selected. It has also been suggested that *pfmdr1* polymorphisms may play a compensatory role in CQ resistant parasites [228], or that they may modulate the degree to which CQ and other drugs or solutes accumulate in the food vacuole, by interacting with PfCRT and possibly other transporters [226, 229, 230]. *pfmdr1* point mutations alter the degree of parasite resistance to other major quinoline and quinoline-methanol like drugs including mefloquine, halofantrine, quinine and artemisinin. Notably, most studies have reported an inverse relationship between CQ resistance and resistance to these drugs (MFQ, LM), which are also thought to act on the parasite food vacuole [226, 229, 231].

In Africa, parasites carrying the CQ sensitive wild type *pfmdr1* 86N allele (either alone or together with the wild type *pfmdr1* 184F, *pfmdr1* 1246D or *pfcrt* 76K alleles) are likely to tolerate residual levels of LM after treatment with LM-ATM; this feature supports the hypothesis that a strong selective pressure for LM resistance exists *in vivo* [232-234]. Another study demonstrated that AQ and LM-ATM select distinct alleles of *pfmdr1*, and exert opposite within host selective effects on the *pfmdr1* gene [235] i.e. treatment with LM-ATM

led to the selection of *pfmdr1* wildtype alleles, whilst treatment with AQ led to the selection of *pfmdr1* mutant alleles. Importantly, most field studies reported the significant selection of CQ sensitive alleles after treatment with LM-ATM, suggesting that they may be important molecular markers of LM-ATM efficacy in the African setting. These observations support the hypothesis that CQ and AQ (4-aminoquinolines) and LM (an aryl amino alcohol) exert opposite effects on the *pfmdr1* gene in malaria parasites.

Several studies have linked *pfmdr1* over-expression with drug resistance in *P.falciparum,* similarly to the multidrug resistance mechanism observed in mammalian cancer cells .For example *pfmdr1* transcript levels increased after treatment with CQ, MFQ and QN (all quinoline based drugs), but not after treatment with pyrimethamine (an antifolate) [236]. This suggested that induction of *pfmdr1* was specifically associated with the quinoline class of antimalarials. However, in other reports, p*fmdr1* expression levels did not vary between CQ-sensitive and CQ-resistant parasites for certain strains suggesting that the role of *pfmdr1* over expression was not universal but strain specific [237]. *pfmdr1* amplification or increased expression has also been inversely correlated with CQ resistance, with parasites selected for CQ resistance showing reduced amplification of *pfmdr1* and decreased resistance to MFQ [238], whilst parasites selected for MFQ resistance showed amplification of *pfmdr1*, decreased resistance to CQ and cross resistance to other antimalarials including HLF and QN [239-241]. The observation of deamplification of *pfmdr1* after *in vitro* selection of CQ resistance in a CQ resistant line led to the suggestion that over expression of *pfmdr1* was not compatible with high levels of CQ resistance [238]. In laboratory and field studies (in South East Asia), amplification of *pfmdr1* to copy numbers of up to 5 and increased expression of *pfmdr1* (rather than *pfmdr1* point mutations) have been positively correlated with MFQ [242-246] and LM resistance [166, 245-247]. Although amplified *pfmdr1* copy number is prevalent mostly in Asian isolates, it has also been reported at low levels in field isolates from Gabon [248] and Kenya [249]. Possibly, due of its low prevalence, *pfmdr1* amplification has not been associated with LM failure in Africa.

Clearly, *pfmdr1* amplification and altered *pfmdr1* expression are important mechanisms of development of resistance to the amino alcohols MFQ and LM. However, although *pfmdr1* amplification is a useful predictor of LM resistance in South East Asia, LM resistance has only been linked with p*fmdr1* point mutations (and not *pfmdr1* amplification) in Africa.Therefore it is possible that the mechanisms of LM resistance may be different for Asian and African isolates. It remains to be seen if the prevalence of *pfmdr1* amplification in Africa will increase with the continued use of LM-ATM.

Mechanisms other than *pfmdr1* polymorphisms may also play a role in mediating altered drug responses in parasites. For example, in a recent study, increased expression of *pfmdr1* (but not *pfcrt*) was associated with decreased susceptibility to CQ in both CQ resistant and CQ sensitive parasite lines which had been pre-treated with the anticonvulsant phenorbabitone [210]. A number of putative binding sites for nuclear receptors were identified in the proximal promoter region of both *pfmdr1* and *pfcrt*, suggesting that nuclear-receptor mediated responses may be a mechanism of *pfmdr1* gene regulation in *P.falciparum* and that this mechanism may account at least in part, for drug resistance in malaria parasites.

Therefore, although the exact mechanisms by which *pfmdr1* modulates drug activity are not clear, alternative mechanisms such as gene regulation may explain some of the the conflicting evidence on the effects of *pfmdr1* polymorphisms on antimalarial activity.

As part of my work towards this thesis, I examined the impact of pfmdr1 and pfcrt polymorphisms on the activity of PQ, LM, AQ, DEAQ and DHA. I used CQ as a control. The main aim was to identify molecular markers that can be used to monitor resistance to these drugs.

1.9 Investigating other genes that mediate antimalarial drug resistance

1.9.1 The role of other transporters

Although *pfmdr1* and *pfcrt* are the main transporter genes linked with antimalarial drug resistance, different parasite clones carrying the same *pfcrt* and *pfmdr1* alleles often show a wide range of *in vitro* responses to QN and CQ. For example, IC_{50} values of CQ resistant isolates range from 2.5 to 200 fold higher than those observed for CQ sensitive isolates, leading to the hypothesis that antimalarial drug resistance is multifactorial and genes other than *pfcrt* and *pfmdr1* might be involved [176, 178, 250].

In support of this hypothesis, a systematic quantitative trait loci analysis (QTL) of an established cross of *P.falciparum [176]* provided some evidence for the involvement of PfNHE*,* a NA^+/H^+ exchanger, in quinine (QN) resistance. High levels of QN resistance were associated with elevated PfNHE activity in the strains used for QTL anlaysis above [251]. whilst, decreased expression of the *pfnhe* gene in a transfection experiment was associated with a statistically significant increase in QN susceptibility [252]. *pfnhe* polymorphism has been associated with decreased drug susceptibility in field isolates, [253, 254].

Another genome wide study proposed 11 novel putative transporters as potential modulators of CQ and quinine sensitivity [178]. Interestingly, the disruption of one of the genes identified above, a gene encoding a putative multidrug resistance associated protein (PFMRP, a member of the ABC transporters) was recently reported to be associated with increased sensitivity to multiple antimalarial drugs including CQ, PQ, QN, ART, and PMQ [177]. In recent work, additional candidate transporter genes were reported to be differentially expressed in drug resistant or drug treated parasites [179-181], and a strong linkage disequilibrium has been observed between polymorphisms in transporter genes of parasites

from malaria endemic areas [178, 218, 255]. Taken together, this evidence supports the hypothesis that there is a functional interplay between multiple transporters in mediating antimalarial drug resistance.

The recent advances in generating the whole genome of *P.falciparum* have provided fresh opportunities for the investigation of other transporters potentially involved in *P.falciparum* drug resistance. For example, by using DNA microarrays, thousands of malaria genes can be analyzed simultaneously in a single experiment. Such studies can aid in the rapid identification of genetic markers of resistance to LM, PQ and AQ, thereby facilitating the tracking of resistance to these drugs in malaria endemic areas.

1.9.2 The generation of resistant phenotypes in vitro

In vitro selection of resistance is a useful tool in antimalarial drug resistance studies. However, the technique has not been fully exploited due to the difficulty of obtaining stable resistant phenotypes [256]. To date, there are only few examples of the selection of stable drug resistance phenotypes in *P. falciparum in vitro*, mainly using CQ and MFQ [239, 257, 258].

Together, the majority of *P.falciparum in vitro* studies with CQ, MFQ and ART to date suggest that the selection of *P.falciparum* resistance *in vitro* occurs in 2 stages: the first phase gives rise to an unstable phenotype followed by the selection of a stable one [165, 179-181, 236, 239, 257-260]. The initial phase, arising relatively quickly may be associated with gene amplification or deletion and/or the over-expression or down-regulation of various proteins [179, 236], and in some studies, the genes involved initially after *in vitro* drug exposure (first phase) have been shown to be the same as those already identified to be associated with stable resistance (second phase). For example, treatment with CQ and ART was associated with altered expression of *pfmdr1*, one of the main genes implicated in stable resistance to these drugs [179, 236, 259]

Notably, despite their low amplitudes, the changes observed in the *P.falciparum* transcriptome in response to *in vitro* drug pressure in the initial phase have been shown to be highly reproducible and dose dependent, suggesting that they represent physiologically relevant processes and may involve functionally related genes [261]. Therefore, some of the mechanisms associated with unstable resistance during *in vitro* selection may indeed be similar to those that mediate stable resistance in the field. These observations suggest that despite the difficulty in obtaining stable phenotypes, *in vitro* selection of resistance is a potentially useful tool in the study of antimalarial drug resistance. Further, evidence suggests that the efficiency of this process might be increased by selecting resistance against multi drug resistant isolates, increasing the parasitaemia exposed to drug pressure and increasing the duration of drug exposure (see section 1.6.2.1).

As part of my work towards this thesis, I selected LM resistance in vitro against V1S (a multidrug resistant isolate) for 16 months. Using the selected line, I searched for genes that were differentially expressed in response to LM using a P.falciparum DNA microarray. I hypothesized that transporter genes would be amongst those differentially expressed.

1.10 The effects of withdrawing antimalarial drug pressure

Studying the effects of withdrawing a drug can help in clarifying the mechanisms of resistance and in the formulation of rational drug policies. Two hypotheses currently exist to predict the effects of removing drug pressure from a population. The initial observations that mutations that confer drug resistance to an organism come with a fitness cost [262], led to the first hypothesis suggesting that organisms would revert back to the fitter wild-type genotypes after removal of drug pressure. In *P.falciparum*, this feature has been shown experimentally for antimalarial drugs such as CQ, pyrimethamine and atovaquone [263-266].

Other studies support the alternative hypothesis that rather than revert back to their wild-type forms, mutant organisms often develop additional compensatory mutations that restore the fitness of the mutants, allowing them to survive when drug pressure is withdrawn [267-269]. In malaria parasites, significant associations have been observed between the occurrence of the *pfcrt-K76T* and *pfmdr1-N86Y* mutations in parasites from various endemic regions [195, 221, 223, 225, 255]. It has been proposed that parasites carrying the *pfcrt-K76T* mutation incur a fitness deficit [270-272], and that the *pfmdr1-N86Y* mutation might compensate for this deficit [195, 223]. It is important to understand which of these alternative hypotheses would more accurately predict the effects of removing failing antimalarial drugs from malaria endemic areas.

The hypothesis that drug sensitive malaria parasites may have a survival advantage after withdrawal of drug pressure is supported by observations in Malawi, where due to high rates of CQ treatment failure, antimalarial policy was changed from CQ to SP in 1993. This made Malawi the first country in sub-Saharan Africa to withdraw CQ from clinical use. This withdrawal was accompanied by an effective nation-wide campaign promoting the use of SP as the first-line treatment for malaria. Interestingly, a recovery of CQ sensitivity in Malawi was observed after 5 to 7 years of CQ withdrawal [271, 273], evidenced by a dramatic decline in the prevalence of the *pfcrt-K76T* mutation (the molecular marker of CQ resistance). Notably, CQ resistant genotypes had disappeared in 2001, 7 years after CQ withdrawal [270]. This was associated with a steady but slower decline in the prevalence of the *Pfmdr1-N86Y* mutation [270]. These *in vitro* findings were confirmed in a clinical trial in 2006 which reported high clinical efficacy of CQ in Malawian children, 12 years after it was withdrawn from clinical use [272].

The observations in Malawi have been attributed to the re-emergence and the expansion of the fitter wild-type malaria parasites that have a survival advantage in the absence of CQ drug pressure [274]. However, the case of Malawi remains unusual. The possibility of return of CQ

sensitivity following CQ withdrawal has been reported in few other sites only, including Gabon [275], Vietnam [276] French Guiana [277] and China [278], and in all these sites, the observed decline in CQ resistance was less dramatic than that observed in Malawi. Many other endemic sites in Africa [279, 280], Asia and South-America [276, 281-283] have continued to maintain high levels of CQ resistance.

In Kenya, the official change of policy from CQ to SP was implemented in 1998 [284] , although it is noteworthy that according to some reports [285], CQ has nevertheless continued to be widely available through the informal sector [285]. Therefore, the effects of CQ withdrawal have varied greatly across different malaria endemic sites.

The differences observed in the effects of CQ withdrawal in different settings suggest that other factors such as genetic background of the parasites, differences in transmission intensity, the prevailing regulation and drug use policies may be crucial in determining the effects of withdrawing failing antimalarial drugs. Consequently, understanding and monitoring the effects of removing antimalarial drug pressure is important in informing antimalarial treatment policy. Importantly, the hypothesis that resistance to an antimalarial drug reverts when drug pressure is removed suggests that it may be possible to re-introduce antimalarial drugs that had previously been withdrawn, providing new opportunities to manage the challenge of drug resistant malaria.

As part of my work towards this thesis, I investigated the effects of withdrawing CQ in Kilifi by examining the prevalence of the pfcrt-76T mutant parasites over time, since CQ withdrawal in 1998. I compared the trends observed in Kilifi to those of Malawi.

1.11 Justification of the study and specific objectives

Monitoring and preventing resistance to ACT combinations remains an ongoing challenge. LM-ATM, DHA-PQ, AQ-AS are important or potentially important ACTs in our setting. Strategies to monitor their efficacy and extend their therapeutic lifespan require a detailed understanding of the mechanisms of resistance to PQ, LM and AQ. Therefore, the specific aims of this project were as follows:-

a) To establish the baseline activity of CQ, PQ, LM, DEAQ and DHA in *P.falciparum* isolates from Kilifi.

b) To investigate the role of quinoline molecular markers in mediating resistance to LM, PQ, DEAQ and DHA in Kilifi *P.falciparum* isolates.

c) To assess whether LM, PQ and DEAQ exert selective pressure for *in vivo* resistance to LM-ATM, DHA-PQ, and AQ respectively.

d) To investigate the trends in CQ resistance in Kilifi since its withdrawal and to compare these trends with those observed in Malawi.

e) To select LM resistance *in vitro* and to identify novel genes that may mediate the reduced-susceptibility of *P.falciparum* to LM.

The overall goal was to identify molecular markers that can be used to monitor LM, PQ and AQ resistance. Based on their common quinoline strucure, I hypothesized that CQ resistance would have a significant impact on resistance to LM, PQ and AQ. I therefore used CQ as a reference in investigating the mechanisms of resistance to these drugs.

1.12 Chapters of the thesis

In chapter one, I have provided an overview of malaria and important literature in antimalarial drug resistance. In chapter two, I describe the materials and methods used in this project. In chapter three, I present baseline data on antimalarial drug activity in Kilifi and the impact of CQ molecular markers on resistance to key antimalarials. In chapter four I explore whether *in vivo*, LM, PQ and DEAQ (all artemisinin partner drugs with long-elimination half–lives) exert a strong selective pressure for resistance to LM-ATM, DHA-PQ and AQ respectively. In chapter five, I present data on trends of CQ resistance before and after its withdrawal in Kilifi, Kenya and compare these results with those of Malawi. In chapter six, I present data on experiments carried out to identify additional genes that may alter the *P.falciparum* response to LM and similar antimalarials. In chapter seven, I present my conclusions and recommendations

CHAPTER TWO

2 Materials and Methods

2.1 Introduction

In this chapter, I describe important features of the study site and the procedures used in the course of this project. I have provided a list of the reagents and consumables used in appendix 1, a list of all the primers used in appendix 2, and the consent forms and ethical review documents used in appendix 7.

2.2 Study site

The studies presented in this thesis were conducted at the Kenya Medical Research Institute (KEMRI) /Wellcome Trust Research Programme, which is based at the Kilifi district hospital, on the coast of Kenya. *P.falciparum* isolates were collected at a clinical trial facility located at Pingilikani, 20km south of Kilifi town (Figure 2-1). The Pingilikani clinical trial facility is part of the Kenya Medical Research Institute (KEMRI)/Wellcome Trust Research Programme, and is adjacent to a primary health care clinic that is part of the local Ministry of Health.

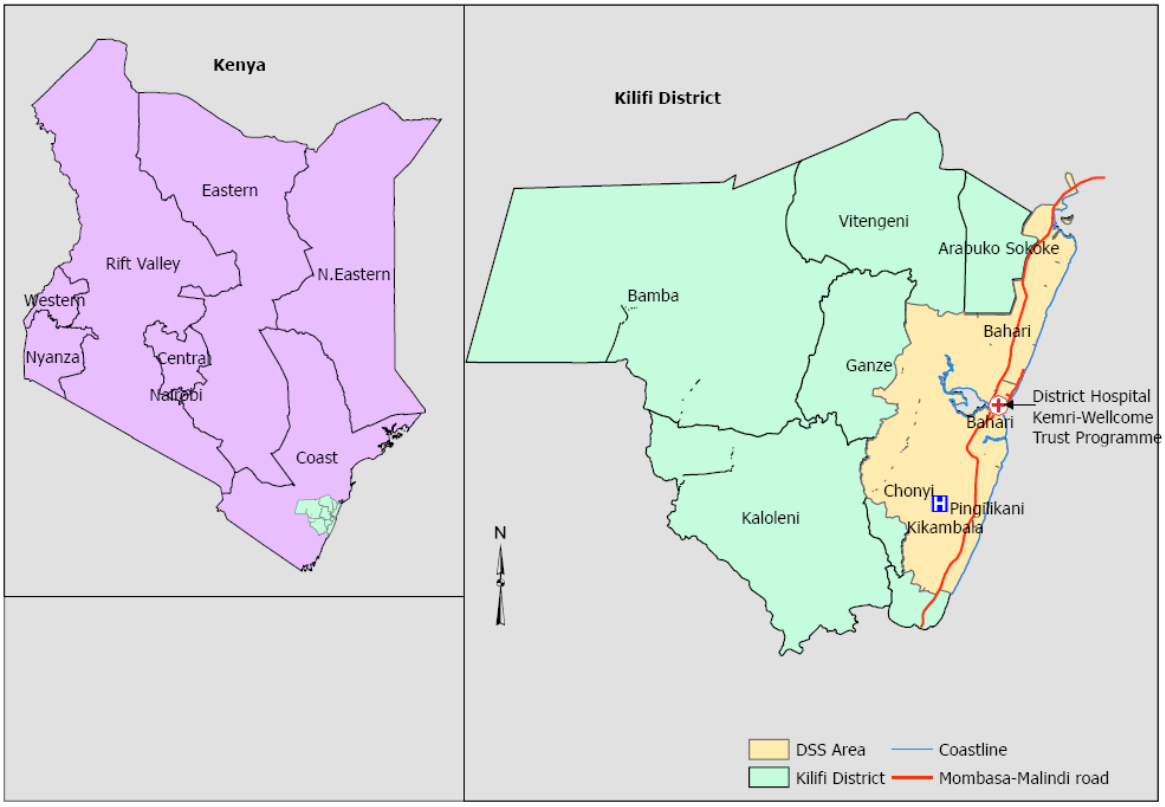


Figure 2-1: A map of Kenya showing the location of Kilifi. The map on the right hand side is a close-up map of Kilifi district showing the location of the KEMRI / Wellcome Trust Research Programme at the Kilifi District Hospital and the Pingilikani facility to the south.

Kilifi District has an estimated population of 600,000 people. *P. falciparum* malaria is endemic in this region, with seasonal transmission peaking after the long rains (April to June) and short rains (October to November). Transmission of malaria has declined over the past 10 years in the entire coastal region of Kenya. In Kilifi this decline has been evidenced by declining prevalence of parasitaemia in the community, which has led to decreased malaria hospital admissions (see Figure 2-2 and Figure 2-3). This has been accompanied by increasing mean age of malaria admissions, shifting presentation of severe disease, and an overall decline in malaria mortality [286].

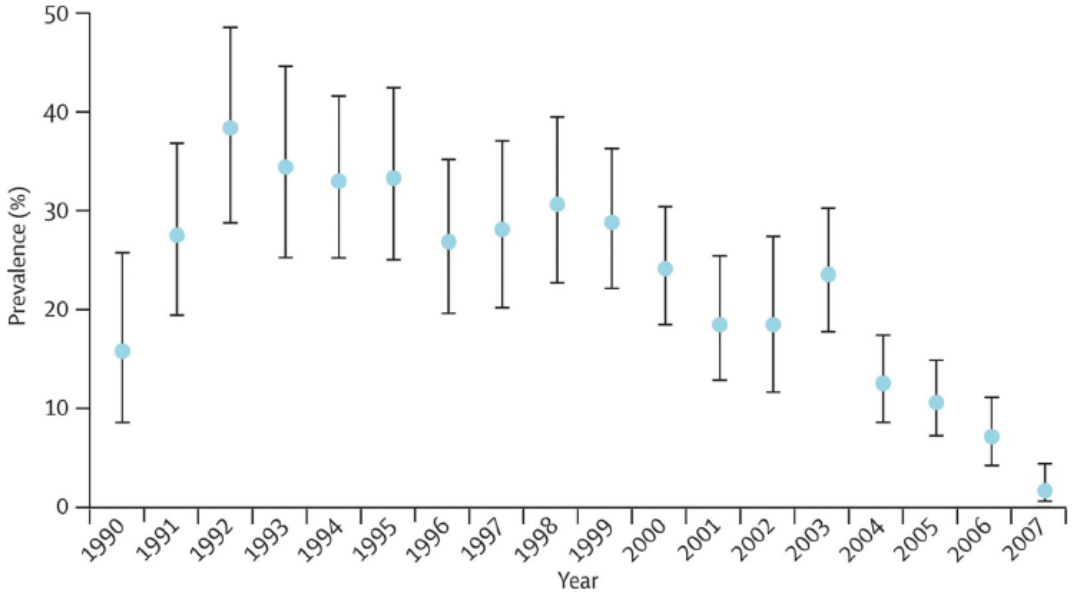


Figure 2-2: Declining annual parasite prevalence in patients admitted with trauma in Kilifi. Adapted from Ref [286]. [Reprinted from the Lancet with permission from Elsevier. doi: 10.1016/S0140-6736(08)61655-4]

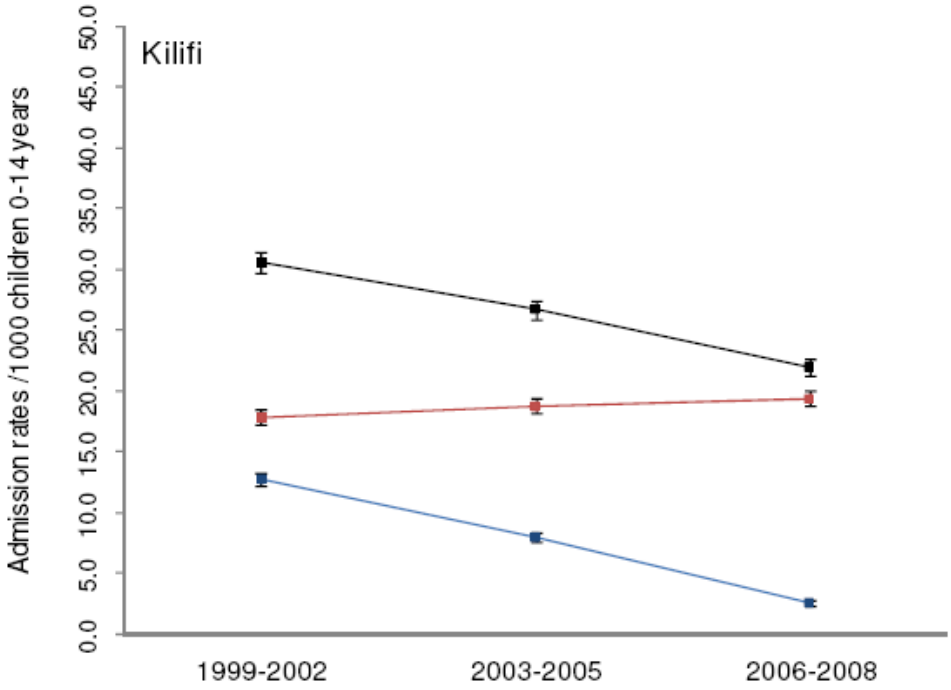


Figure 2-3: Declining paediatric malaria admissions over time in Kilifi. Malaria (blue line), non-malaria (red line) and all cause admissions (black line). Adapted from Ref [287].

During the period the clinical studies were conducted (2005-2009), the risk of malaria infection in Kilifi was associated with medium to low parasite prevalence rates of <10% [286, 288] and between 2006-2008, the average annual malaria admission rate for children aged 0-14 years at risk per annum was estimated to be 2.56 per 1000 [287].

2.2.1 Antimalarial drug use in Kilifi

CQ which was introduced as an antimlarial in the 1930s [36] was the mainstay of treatment for uncomplicated malaria in Kenya for decades. CQ resistance was first detected in non-immune tourists to Kenya and Tanzania in 1978 [289-291]. Thereafter, the first case of resistance was reported in a Kenyan infant in 1985 [292] and the levels of resistance escalated throughout the 1980s [284]. In 1998 the Kenya Ministry of Health implemented a change of policy to SP due to the widespread CQ resistance [284]. At the time of this policy change, >50% of childhood fevers were treated with drugs purchased from local shops in Kilifi, and there was an active shopkeepers training programme to promote antimalarial access in the community through the informal retail sector [293].

SP clinical efficacy declined significantly by the second half of 2003, with 7 of 9 surveillance studies (more than 75%) indicating SP failure rates in excess of 25% [294]. Consequently, a policy decision to adopt LM-ATM as the replacement first line therapy was announced in 2004. Due to delays in policy implementation, LM-ATM became available in government health facilities in 2006, and has since been made available to paediatric patients free of charge throughout the public health sector [94].

AQ had been available as treatment for malaria second line to SP and was being used as first line treatment for paediatric case management in Kilifi during the interim period between change of policy from SP to AL [295].

2.3 Collaborating sites

I performed some of the experiments described in this thesis in collaborating laboratories. I performed microarray hybridization experiments (see section 2.8.5) at the Wellcome Trust Sanger Institute, Cambridge, UK. I did microarray analysis (see section 2.8.6) at the Insitute of Molecular Medicine in Lisbon, Portugal with the support of Dr Celine Carret. I got additional statistical support for microarray analysis from Mr Avi Feller of the Department of Statistics, University of Oxford, UK. I performed qPCR experiments (see section 2.8.9) at the Weatherall Institute of Molecular Medicine, University of Oxford at Prof Chris Newbold's laboratory. I performed the sequencing experiments (see section2.6.2) and the analysis of *Pfmdr1* copy numbers by qPCR (see section 2.6.5) at Dr Steffen Borrmann's laboratory, Institute of Hygiene, University of Heidelberg, Germany.

2.4 Ethical considerations

The studies described in this thesis received ethical approval from the Kenya Medical Research Institute National Ethical Review board (SCC 946, 1031 and 1285).The guardians of all the children who participated in the field studies gave full informed consent prior to sample collection. I provide copies of the consent and the ethical approval forms in appendix 7.

2.5 Procedures used for *in vitro* malaria culture

I employed the procedures described in this section to cultivate malaria parasite isolates *in vitro* and to analyse the activity of the antimalarial drugs CQ, PQ, LM, AQ, DEAQ and DHA. I present the results of these experiments in chapter 3.

2.5.1 Preparation of culture media

To prepare the media used for *in vitro* malaria culture, each vial of powdered RPMI/1640 was dissolved in one litre of distilled water and supplemented with 5.94g/L HEPES, 10ug/L folic acid, 0.5ug/L PABA and 15ug/L of gentamicin. All materials and reagents used in preparation of media were either supplied as sterile by the manufacturer, or sterilized by filtration through a 0.22uM nitrocellulose filter. When adjusted to pH 7.2 with 2g/L sodium bicarobonate, this media could be used for cell washing purposes and is referred to here as 'yellow RPMI'. Yellow RPMI was stored at 4 ^{o}C for a maximum of 4 weeks. For parasite culture, yellow RPMI was supplemented with 15% v/V pooled serum from malaria non-exposed donors, and is referred to here as complete culture media. Complete culture media was stored at 4^{o}C for a maximum of one week.

2.5.2 Preparation of transport media

Transport media was used for collection and storage of malaria parasites isolates in the field prior to parasite adaptation (section 2.5.6) or cryopreservation (section 2.5.4). To prepare transport media, each vial of powdered RPMI/1640 was dissolved in 1 litre of distilled water, and supplemented with 5.94g/L HEPES, 3.2g/L trisodium citrate, 2.65g/L sodium bicarbonate, 5g/L bovine albumin and 15ug/L gentamicin. Once sterilised by filtration, this transport media was kept at 4^{o}C for up to 4 weeks and used as described below for sample collection in the field (section2.5.3).

2.5.3 Sample collection

I (with the support of a field and laboratory team) collected the *P. falciparum* field isolates used in this study during the course of either of the following clinical studies that took place at the Pingilikani clinical trials facility (see section 2.2 for details of the study site).

a) A randomized trial to assess DHA-PQ versus LM-ATM conducted between 2005 and 2008 (KEMRI SCC number 946, Controlled-trials.com registration number ISRCTN16263443). In this study, parasite isolates were collected in patients recruited into the study at baseline and in any recurrent episode of parasitaemia up to 84 days after treatment. The details of this study are as outlined in ref [296].

b) A clinical audit of AQ conducted in 2006 (KEMRI SSC number 1031). In this study, parasite isolates were collected in patients recruited into the study at baseline and in any patient who experienced a recurrent episode of parasitaemia, up to 28 days after treatment. The details of this study are as outlined in ref [295].

c) Some clinical isolates were available for assay in the malaria culture laboratory at the KEMRI/Wellcome trust Programme in Kilifi, having been collected as part of integrated studies of the development of natural immunity to malaria (Dr Pete Bull's study, OXTREC NO:030-06).

To collect field isolates, one milliliter of patient venous blood was taken aseptically from a child presenting with uncomplicated malaria (either before or after treatment for samples taken as part of drug trials) after informed consent was given by the parent or guardian. The blood was immediately transferred into a vacutainer tube containing 4 millilitres of 'transport media' (containing RPMI 1640 and albumin as described in section 2.5.2). The sample was kept at 4^{o}C, where it could be kept for up to 3 days before parasite adaptation (section 2.5.6.) or cryopreservation (section 2.5.4).

2.5.4 Cryopreservation of parasite isolates

P.falciparum isolates were cryopreserved in a glycerolyte cryopreservation solution. To prepare the cryopreservation solution, 70ml of glycerol was added to 180ml of a 0.9% sodium chloride and 4.2% sorbitol solution. The solution was filtered through a 0.45uM nitrocellulose

filter and stored at 4 degrees. To cryopreserve the parasites, an infected pellet was obtained from culture or from a vacutainer by centrifugation. 2/3 X the pellet volume of the cryopreservation solution was added slowly over 5 minutes with continuous shaking. 1ml aliquots of the suspension were transferred into 1.2ml cryovials, labelled and stored overnight in a -80 degrees freezer before being transferred to a liquid nitrogen tank for long term cryopreservation.

2.5.5 Thawing of isolates from liquid nitrogen

Once removed from liquid nitrogen, parasites were thawed rapidly by enclosing the cryovial in a fist. The suspension was transferred to a 15ml centrifuge tube and centrifuged at 2000 rpm for 5 minutes. The cryopreservation solution was removed and parasites re-suspended in an equal volume of ice-cold 3.5% sodium chloride solution. The suspension was centrifuged again at 2000 rpm and the sodium chloride solution removed. The parasites were then washed in 5ml of yellow RPMI. 100ul of pooled human AB serum from non-exposed donors was added to the resulting pellet and left to stand for 5 minutes at room temperature before starting *in vitro* culture

2.5.6 Parasite adaptation

Field isolates were either cultured directly or recovered from cryopreservation while all reference laboratory isolates were recovered from cryopreservation and maintained by diluting with fresh O positive red blood cells from a voluntary donor. *P.falciparum* isolates were cultured in 7ml complete culture media contained in $25cm^2$ culture flasks (4-6% haematocrit) at 37^o C according to standard methods [23]. Media was changed after every 48 hours or more frequently when the parasitaemia was >5% and the cultures gassed with 3% CO_2, 5% O_2, 92% N_2 mixture after each media change.When parasitaemia increased, cultures were diluted as required. Getting a field isolate completely adapted was often a slow process; and usually tookbetween 3-6 weeks. Bacterial contamination was often a challenge.

Substitution of culture media with 15ug/ml of gentamicin was done to decrease the risk of bacterial contamination. *P.falciparum* cultures were routinely tested for Mycoplasma infection and treated when necessary (see section 2.5.7).

2.5.7 Testing and treatment of in vitro Mycoplasma infections

P.falciparum isolates in culture were tested routinely for Mycoplamsa infections using a modification of a previously published assay [297], using *A. laidlawaii and M. pirum* as positive controls. Briefly, 100 microlitres of parasite culture was transferred to a 1.5mililitre Eppendorf tube and centrifuged at 5200g for 5 minutes. After removing the supernatant, the remaining pellet was taken through three cycles of freezing and thawing. The pellet was then washed with 500 µl of PBS, centrifuged at 5200g after each wash. Thereafter the pellet was re-suspended in 100 µl of distilled water and placed in a block preheated to 95ºC for 10 minutes. The pellet was then kept at 4 ºC to be used as a template for the PCR assay.

For the PCR reactions, two master mixes consisting of 10X NH_4 buffer (2.5 ul), 50mM $MgCl_2$ (0.75ul), 40mM dNTPs (0.625 ul), primers (0.56 ul), DNA template (1 ul) were prepared using the different sets of primers for first and second reaction To 24ul of master mix containing the first set of primers (contained in a 200ul PCR tube), 1 ul of DNA template was added and amplified on a mastercycler Personal PCR Machine, (Eppendorf, Germany) under the following conditions: Hot start 94ºC for 30 s and 30 cycles consisting of hybridization at 50 ºC for 30 s and extension at 72 ºC for 1 minute. The product of this first amplification was used as a template for the nested PCR under conditions similar to those used in the first amplification reaction.

Analysis of PCR products was done as described in section 2.6.4. Samples with a band range of 200-365bp were scored positive for mycoplasma while those with bands in the range of 219-426 were scored positive for acholeplasma.

2.5.8 Determination of in vitro parasitaemia

Parasitaemia was expressed as the percentage of infected cells when assessed by light microscopy. A 200 µl aliquot of culture was transferred with into a 0.5ul Eppendorf tube and centrifuged to obtain a cell pellet. The pellet was then transferred onto a clean glass slide and a thin film prepared by spreading the cells with another slide inclined at 45^0. The smear was then air dried and fixed with methanol and staining done for 10 minutes with a 10% giemsa staining solution. Excess stain was washed off under tap water and the slides dried using a blow drier. The number of microscope fields consisting of a monolayer of evenly spaced red blood cells required to count 10000 red blood cells were estimated. Counting was then done at 100X magnification under oil emulsion and number of infected cells expressed as a percent.

The growth rate was calculated using the following formula:

Growth rate (GR) = (current % parasitaemia / initial % parasitaemia X Dilution) $^{2/days}$

Additionally to determine the dilution factor required to achieve a desired parasitaemia within a specific time in days, the flowing formulae was used:

Dilution= (current % parasitaemia / desired parasitaemia) X GR) $^{days/2}$

2.5.9 In vitro chemo sensitivity testing

Susceptibility of isolates to CQ, PQ, LM, AQ , DEAQ DHA was measured by a modification of the standard radio isotopic method [298]. CQ was dissolved in distilled water, LM, PQ and DEAQ in 90% methanol plus 10% Hydrochloric acid (HCl) and AQ and DHA in 70% HCl. Working drug solutions were prepared in complete media. To prepare drug test plates, drug solutions of known concentrations were serially diluted in duplicate to a final working volume of 25ul in each drug well of a 96-well flat bottomed microculture plate. The top 8 wells of each plate were drug free controls: 4 wells for parasitized red blood cells (positive controls)

and 4 wells for non-parasitized red blood cells (negative controls). For each assay, the test isolate was diluted with fresh O positive red blood cells and complete media to a parasitaemia of 0.5% and hematocrit of 1.5%. 200ul of this diluted suspension was added to each well of the 96 well plate.

Prepared test plates were incubated at 37^0C in a humidified airtight box flushed with 3% CO_2, 5% O_2, 92% N_2 mixture. After 48hours, 25ul of ^{3}H-hypoxanthine (tritium labelled, 0.5uCi) diluted in complete media was added to each well and incubated for a further 18 hours. Well contents from each plate were then harvested onto glass fibre filter mats using a 96 well cell harvester attached to a vacuum (TomTec Inc and Perkin-Elmer). The filters were washed thoroughly with distilled water to ensure that all red blood cells were adequately lysed and excess tritiated hypoxanthine was washed away. The filter mats were then dried in an oven for 2 hours and placed in individual scintillation bags, followed by the addition of 2 ml of scintillation cocktail. The amount of radiation was determined using a Wallac 1450 microBeta counter. Results were expressed as the drug concentration required for 50% inhibition of hypoxanthine incorporation into parasite nucleic acid (IC_{50}), obtained by non-linear regression of the dose response curve as previously described [299]. Two reference laboratory isolates V1/S (multidrug resistant) and 3D7 (CQ sensitive) were used as controls in the chemosensitivity assays.

2.6 Analysis of molecular markers associated with PQ, LM and AQ resistance

The procedures described in this section were used in the genotyping of *P.falciparum* isolates to investigate the relationship between polymorphisms in *pfcrt* and *pfmdr1* genes and CQ, PQ, LM, AQ, DEAQ, and DHA activity, and to analyse the temporal trends of drug resistance in Kilifi. The results of these experiments are presented in chapter 3, 4 and 5.

2.6.1 Genotyping at pfcrt-76 and pfmdr1-86

To prepare samples for molecular analysis, an aliquot of each adapted isolate (~50 µl) was spotted onto filter paper, air-dried and stored in plastic bags with silica gel at ambient temperature. DNA material was fixed with methanol and extracted by a boiling method according to a published protocol [300] and fragments of *pfcrt-76* and *pfmdr1-86* were amplified by nested PCR according to protocols described at (http://medschool.umaryland.edu/CVD/2002_pcr_asra.asp).

Briefly, master mixes consisting of 1X PCR buffer, 2.5mM MgCl, 0.2mM dNTPs 0.17uM of forward and reverse primers and 1.5 IU Taq polymerase were prepared using the different sets of primers for first and second reaction Five microlitres of filter paper DNA extracted as described (see 2.6.1) was added to 20ul of mastermix and amplified using the following PCR protocol: 95ºC for 5 minutes, 92 ºC for 30 seconds, 45 cycles (at 45 ºC for 30 seconds and 65 ºC for 45 seconds) followed by an extension at 72 ºC for 15 minutes. One microlitre of this primary product was used as a template in a nested PCR reaction using the same PCR protocol used for the primary reaction; 5-8ul of the PCR product from the nested PCR was then incubated overnight at 37 ºC to digest restriction enzyme, with each digest done in a separate tube. Products of this digestion were examined using gel electrophoresis as described in section 2.6.4, with positive and negative controls (V1S and 3D7) and "uncut" (no enzyme digest alongside).To analyze *pfmdr1-86*, the restriction enzyme Afl III (New England biolabs, Beverly, MA) was used. For isolates mutant at *pfmdr1-86*, the digest yielded two products of 126 and 165bp. To analyze *pfcrt-76,* the restriction enzyme ApoI was used. For isolates wildtype at *pfcrt-76*, the digest yielded two products of 99 and 46bp.

2.6.2 Sequencing of pfcrt and pfmdr1

I used 3 pairs of primers specific to analyse 5 mutations at codons 86, 184, 1034, 1042 and 1246 of *pfmdr1,* and 2 pairs of primers specific for *pfcrt* to analyze mutations at domain 1

(codons 72, 74, 75, 76), 4 (codon 163) and 9 (codons 352 and 356) of *pfcrt*. DNA was extracted from filter papers as described in section 2.6. Amplification of 500 bp PCR *pfmdr1 and pfcrt* fragments was carried out in a 50ul reaction containing 2.5mM magnesium chloride, 0.2mM dNTPs, 0.66uM of each forward and reverse primer, and 1.5 IU of Taq polymerase. PCR was performed using the following protocol: 94 ºC for 3 minutes, 94ºC for 30 seconds, 35 cycles at 58 ºC for 30 seconds and 60ºC for 1 minute, followed by a final extension at 60ºC for 10minutes. Fragments were sent to GATC Biotech (Germany) for clean up and bidirectional sequencing using the Sanger method. The sequencing primers were the same as those used in the PCR reaction.

2.6.3 Genotyping at dhfr-51, 59 and 108

Point mutations in codons 51, 59 and 108 of *P. Falciparum dhfr* were analysed by a nested PCR amplification and restriction enzyme digestion protocol described previously [301-303]. Briefly, three master mixes consisting of 1X PCR buffer, 2.5mM MgCl, 0.22mM dNTPs 0.17uM forward and reverse primer and 1.5 IU Taq polymerase were prepared using the different sets of primers for first and second reaction. 5 ul of filter paper DNA extracted as described (see 2.6.1) was added to 20ul of mastermix and amplified using the following PCR protocol: 92ºC for 30 seconds followed by 45 PCR cycles (at 45 ºC for 30 seconds, 65 ºC for 45 seconds) and a final extension at 72 ºC for 15minutes. 5 ul of this PCR product was used as a template in a nested PCR using the same protocol as in the primary reaction. Thereafter 5-8ul of PCR product was digested with 1 IU of enzyme overnight at 37ºC in a total 20ul reaction in restriction buffer (New England biolabs). Products were examined using gel electrophoresis as described (see section 2.6.4), with positive and negative controls (the reference isolates V1S and 3D7) and an “uncut” (no enzyme digest alongside). To analyse mutation at codon 51, the PCR product from the secondary reaction was digested using the restriction enzyme EcoRI (New England Biolabs, Beverly, MA); for wildtype parasites, this yielded two products of 35 and 78bp. To analyse mutation at codon 59, the PCR product from

the secondary reaction was digested with the restriction enzyme BsrGI (New England Biolabs, Beverly, MA), resulting in two products of 43 and 65bp in wild type parasites. To analyse mutation at codon 108, the primary PCR product was used as a template in a separate nested PCR following the following PCR protocol: 92ºC for 30 seconds and 25 cycles at 42 ºC for 30 seconds, and 65 ºC for 45 seconds followed by a final extension at 72 ºC for 15minutes. The product of this secondary reaction was digested using the restriction enzyme Alu I (New England Biolabs, Beverly, MA), resulting in two products of 46 and 210 bp in wild type parasites.

2.6.4 Analysis of PCR products

Products of PCR amplification were analysed using gel electrophoresis. In general, 7ul of PCR products were run on a 1.5% agarose gel for 30-35 minutes after which the gel was stained in Ethidium bromide (EtBR) solution for 15minutes.To visualize PCR bands, I used a transilluminator, Gel Doc 2000 BIORAD, Milan Italy. The appropriate ladders (depending on the size of the PCR product) and controls were run alongside the products.

2.6.5 Analysis of pfmdr1 copy number

I used a relative quantification Taqman PCR assay to estimate *pfmdr1* copy number in *P.falciparum* isolates, [242]. In summary, the relative gene copy number of *pfmdr1* in each isolate was compared to that of a housekeeping gene (B-tubulin) using specific fluorescently labelled probes (FAM and JOE respectively), in a real time PCR assay. Amplification was done in a multiplex PCR assay in MicroAmp 96 well plates (Applied Biosystems) in 25ul reactions containing Taqman Buffer (8% glycerol and 0.625IU DNA polymerase, 5.5mM $Mgcl_2$, 300uM dNTPs, 600 nM passive reference dye ROX (5- carboxy-X-Rhodamine), pH 8.3), 300nM of each forward and reverse primer, 100nM of each probe and 5uL of template DNA. Fifty cycles were performed (95ºC for 15sec and 58ºC for 1minute). Every Taqman run contained three calibrator DNA reference isolates with known copy numbers i.e. DD2 (2-3

copies), D10 (1 copy) and 3D7 (1 copy). The reference strains were provided by Dr Steffen Borrmann's laboratory, University of Heidelberg, Germany. All assays were run in triplicate on an Applied Biosystems 7500 real time PCR system.

2.7 Analysis of temporal trends in drug resistance

I employed the methods described in this section to analyse changes in the prevalence of *pfcrt, pfmdr1* and *dhfr* mutations over time in Kilifi and to compare these data with those obtained in Malawi after CQ withdrawal. Dr. Margaret Mackinnon (KEMRI / Wellcome Trust Research Programme, Kilifi, Kenya) provided statistical support for this analysis. I present the results of this analysis in chapter 5.

2.7.1 Samples

This was a retrospective analysis. I used samples that had been collected from patients during several clinical trials of antimalarial drugs conducted in Kilifi from 1993 to 2006. The 1993–2003 samples originated from several clinical trials of SP, CPG-DAP, and SP-AS [93, 304-306] and had been collected before drug treatment. The 2006 samples were collected from infected patients both before and after treatment in clinical trials of LM-ATM versus DHA-PQ [296] and AQ[295] in 2006. No samples were collected in 1996, 2004 and 2005. Twenty-five to 30 samples per year were randomly selected and mutation at *pfcrt- K76T* and *pfmdr1-N86Y* (molecular markers of CQ resistance) and at codons 51 and 59 of *dhfr* (molecular markers of SP resistance) were determined as described above (section 2.6.1 and 2.6.3). I compared my data with those from a similar study conducted in Malawi. [270].

2.7.2 Statistical analyses

A detailed account of statistical analyses is provided in a publication that summarized this work [307] (A copy of this paper is provided in the appendix 6). In summary, the frequencies

of mutations at *pfcrt76*, *pfmdr186* and *dhfr* (codons 51, 59 and 108) alleles were calculated as the proportion of samples carrying the mutant form out of the total of all samples carrying either only the mutant form, or only the wild-type form. As most of the samples were collected from patients enrolled in clinical trials, the potential bias to population frequencies caused by drug treatment of the patients from which the parasites were sampled was first analysed. This was done by comparing the frequencies of each mutant before versus after drug treatment using a Fisher's Exact test for each trial separately. After excluding post-treatment data where bias was detected, the trends in the frequencies of mutants through time were analysed by fitting a logistic regression model to the frequency data with generation as a linear covariate, assuming three parasite generations per year. The selection coefficient of the resistant mutant relative to the wild type was calculated from the estimated slope of the logistic regression curve. For comparison, published data from a study in Malawi [270], in which reversal of CQ resistance was observed within 7 years of CQ withdrawal, were analysed in the same manner.

2.8 Identification of additional genes that mediate decreased susceptibility to LM

Using the methods outlined in this section, I generated a parasite line selected for LM resistance *in vitro* (here referred to as $V1S_{LM}$). I used this selected line as a tool to investigate the mechanisms of resistance to LM using microarray and qPCR approaches. To this end, I searched for genes that were differentially expressed in the $V1S_{LM}$ line, which might account for its altered response to LM. Dr Celine Carret (Institute of Molecular Medicine in Lisbon) and Avi Feller (Department of Statistics, University of Oxford) provided statistical support for microarray analysis. I present the results of this section in chapter 6.

2.8.1 Selection of LM resistance in vitro

To select LM resistant parasites *in vitro*, the V1/S isolate (a multi-drug resistant strain sensitive to LM) was continuously cultured in the presence of varying concentrations of LM between August 2006 and December 2007. Initially, parasites were maintained in culture as described above (section 2.5) in 30ml culture media contained in 75cm^2 culture flasks at initial parsitaemais of 5-10% and haematocrit of 4-6%. Parasites were maintained in LM concentrations higher or equal to the IC_{99} (concentration that kills 99% of parasites). Drug pressure was varied after each 48 hour cycle depending on the parasitaemia and the viability of the parasites when observed under a microscope. The drug pressure was reduced to as low as IC_{10} when parasitaemia was <0.5% and then raised steadily to concentrations higher or equal to the IC_{99} and kept at this high concentration. Normal growth was ascertained when 2 to 3 fold increase in parasitaemia was observed. Cultures were scored as growing steadily when they could grow exponentially in a drug concentration for 3 or more consecutive cycles. Thereafter part of the culture (1/3) was subjected to another drug selection cycle. Parasites were cryopreserved at each selection cycle when growth rate had resumed. After achieving a stable growth rate at a particular drug concentration (a process that took several months), the parasites were maintained in routine culture over a period of 10-14 days in the absence of drug. Thereafter, the IC_{50} of the parasite was determined by hypoxanthine incorporation assay (see section 2.5.9) to assess the stability of the resistant phenotype.

2.8.2 Synchronization of malaria parasites in vitro

Synchronization of parasites was required for the analysis of stage specific gene expression of parasites when exposed to LM pressure. To obtain synchronized ring stage parasites, an infected red blood cells pellet was obtained by centrifugation in a 15ml centrifuge tube. The pellet was then re-suspended in 5ml of 5% sterile sorbitol solution and left to stand for 5 minutes. The parasites were then centrifuged again and washed in yellow RPMI before returning them back to *in vitro* culture. For good synchronization, sorbitol treatment was

repeated at the same time for three consecutive cycles. The synchronized parasites were then cultured as and sampled as described below.

2.8.3 Generation of parasite RNA from cultured parasites

Cultures of V1/S (control) and V1/S_{LM} growing steadily in 142 nM LM (a concentration 6X the IC_{50} of the parent line V1S) were maintained in 175cm^2 culture flasks in 50ml culture media at 2% haematocrit according to standard culture protocols described above (section 2.5). Once at least 2-3 fold increase of parasitaemia after every cycle was ascertained, the culture was synchronized with sorbitol (in three 48 hour cycles) and diluted with O positive red blood cells, until the volume was increased to 500ml (contained in175cm^2 culture flasks in 50ml culture media at 2% hematocrit and 8-10% parasiatemia). Thereafter, 1ml infected RBC was sampled at the start of the cycle, (and every 12 hours throughout the 48hours-cycle. The collected samples were centrifuged (1500g, 5 min),and the resulting pellet dissolved in 5ml of Trizol LS reagent (GIBCO Life Technologies) and kept at -80^oC. This sampling experiment was repeated at three different times for both V1S and V1S_{LM} to generate three biological replicates.

2.8.4 RNA and DNA extraction

RNA was extracted using a phenol-chloroform described previously [308]. Genomic DNA was extracted from saponin-lysed parasite pellets using a commercial genomic DNA extraction and purification Kit (Qiagen).

2.8.5 Microarray hybridization experiments

I performed the microarray experiments at the Wellcome Trust Sanger Institute in Cambridge, UK under the supervision of Dr Celine Carret. I employed the PfSANGER high density custom tiling-like arrays and followed the Sanger Institute Affymetrix protocol [309]. PfSANGER Affymetrix arrays are 25-mer custom designed, covering both strands of *P.*

falciparum 3D7 strain, coding and non-coding sequences, for a total of ~2.5 million probes specific for *P. falciparum*. A total of 24 RNA samples generated from 3 biological replicates of synchronized V1S and $V1S_{LM}$ parasites in culture at 4 time points (0h, 12h, 24h and 36h) were treated with Turbo DNAse enzyme (Ambion®) according to manufacturer's instructions to remove any contaminating DNA. RNA samples were then quantified and analyzed for quality using the Agilent RNA 6000 Nano Kit (Agilent Technologies). Once the integrity of the RNA was ascertained using a bioanalyzer, cDNA was synthesized from 8µg of each RNA sample according to the Affymetrix Eukaryotic gene expression protocol. cDNA was then used as a template in an *in vitro* transcription reaction (IVT) to produce amplified amounts of biotin-labelled complementary RNA (cRNA). Twenty µg of cRNA from each time point was fragmented to 25-200bp fragments after which 15ug of fragmented cRNA was hybridized onto a PfSANGER microarray. Hybridization, washing, staining and scanning was as pereformed as recommended by the manufacturer. The hybridization intensity from each scan was computed using the Affymetrix software.

2.8.6 Microarray analysis

The microarray data were preprocessed with Bioconductor software and R (www.r-project.org). The arrays were Robust Multichip average (RMA) background subtracted, quantile normalized, median polished according to methods previously described [310], and summarized according to the June 2007 release of *P. falciparum* genome annotation, representing a total of 5363 genes.

An average of 1.5 fold differences in expression of $V1S_{LM}$ relative to V1S was applied as a cut-off-point for up or down regulation (change in expression) after fitting a linear model to the microarray data (http://bioconductor.org/packages/release/bioc/html/limma.html (limma package) [311]. The cut-off for statistical significance was set at $p<0.05$. Gene ontology (GO) enrichment analysis was performed using the GOstats Bioconductor package [312]. In

addition supplemental ; EDGE analysis [313] was done to identify additional and potentially useful transporter genes..

2.8.7 Identification of candidate transporters for confirmation by qRT-PCR

From the genes identified to be differentially expressed above (section 2.8.6), I searched for genes that are likely to encode for candidate transporters. As a starting point, I considered that a gene might be a transporter if the annotation for that gene predicts at least 7 transmembrane domains (TM). This was based on the observation that the majority of transport proteins annotated to date have >7 TM [186]. I also considered genes that were annotated to have a transport function in the PlasmoDB database (www.plamodb.org.) The PF10_0210 gene, whose expression did not vary greatly throughout the cycle in both the control and the drug treated parasites, was chosen as a control gene for subsequent qPCR confirmation assays. For the control gene and a subset of the genes identified by microarray analysis, primers were designed based on the mRNA sequence of the control and each candidate gene obtained from PlasmoDB (http://www.plasmodb.org/) and using Primer 3 version 0.4.0 design software (**http://frodo.wi.mit.edu/primer3/**). Products were all between 100-150bp in length, and stringent criteria were set in the primer 3 to limit self annealing and /or formation of primer dimers.

For each primer pair, the primer specificity and suitability was first checked by performing an alignment using the Basic Local Alignment Search Tool (BLAST). Performance of the primers was then checked by amplification of 3D7 genomic DNA and running the product on a 3% agarose gel. PCR amplification was done in 200uL PCR tubes in 25uL containing 1X PCR buffer, 2.5mM $MgCl_2$, 0.2mM dNPs mix, 0.17uM of each forward and reverse primer, 1.25units of Taq DNA polymerase and 1uL template. Amplification was done on a thermocycler and consisted of 94ºC hold for 10 minutes and 30 cycles (94ºC for 20seconds, 58 ºC for 20seconds, 68 ºC for 25 seconds). The efficiency of the primers was ascertained by performing a SYBR green based real time PCR assay of serially diluted 3D7 genomic DNA and melting curve analysis as described below (see section 2.8.9).

2.8.8 cDNA synthesis

The total RNA used for cDNA synthesis was obtained from the same V1S (control) and $V1S_{LM}$ RNA used on the Affymetrix arrays. RNA was first checked for any DNA contamination by performing a PCR reaction using primers specific for the *P. falciparum* aldolase (PF10_0210). PCR amplification was carried out according to the same PCR protocols described in 2.8.7. Once DNA contamination was ruled out, cDNA from 5ug of intact RNA was synthesized using $Oligo(dT)_{16}$ primers and Bioscript™ reverse transcriptase, according to the manufacturer's instructions. Reverse transcription for each sample was then confirmed by repeating a PCR reaction using *P.falciparum* aldolase specific primers as described above (section 2.8.7).

2.8.9 SYBR green based real time PCR assays

Confirmation of microarray data was performed by quantitative real time PCR (qPCR) at the Weatherall Institute of Molecular Medicine, University of Oxford. Estimation of gene expression was based on a SYBR green based relative quantification assay, where the expression of candidate genes was compared to that of a reference gene (*Plasmodium falciparum* putative deoxyribose phosphate- aldolase, PF10_0210) whose expression did not vary significantly through the erythrocytic cycle in both V1S and $V1S_{LM}$. Preparation of all master mixes and pipetting was done by a Corbett Robotics CAS-1200™ precision liquid handling system (Corbett Life sciences). Each 10uL amplification reaction was done in 10uL strips containing 1uL template, 167nM/L of forward and reverse primer and 5uL of SYBR® Green PCR Mastermix (supplied as 2X concentration mix containing SYBR® green I dye, Amplitaq® Gold DNA polymerase, dNTPs with dUTP, passive reference dye and optimized buffer components). Quantitative PCR reactions were performed on a Corbett Rotor-gene 6000 thermo cycler and consisted of 95ºC Hold for 10 minutes and 50 cycles (95ºC for 25 seconds, 58 ºC for 25 seconds, 68 ºc for 30 seconds) followed by a melting analysis of PCR products done as follows: ramp from 50-99ºC rising by 1ºC each step, wait 90 seconds of

Premelt conditioning on 1st step, and 5 seconds each step afterwards. Corresponding V1S (control) and $V1S_{LM}$ (drug treated) cDNA samples were quantified in the same real time PCR run. For each sample, PCR reactions were carried out in duplicate. To facilitate relative quantification, a standard curve was generated for each gene by including 4 serial dilutions of 3D7 genomic DNA spanning a wide dynamic range (1, 1:100, 1:10000, 1:1000000). Results are presented as relative expression fold differences between V1/S and $V1/S_{LM}$.

For each sample, the following formula was used to calculate relative expression (fold change, FC) between V1S (control) and $V1S_{LM}$ (drug selected):

$$FC = \frac{\text{Expression of candidate gene } (V1S_{LM})/\text{expression of control gene (PF10\_0210) } (V1S_{LM})}{\text{Expression of candidate gene (V1S)/expression of control gene (PF10\_0210) (V1S)}}$$

CHAPTER THREE

3 The *in vitro* activity of various antimalarial drugs in *P.falciparum* isolates from Kilifi and polymorphisms in *pfcrt* and *pfmdr1*

3.1 Introduction

In this chapter, I provide an introduction highlighting the role *in vitro* methods in studying antimalarial drug resistance and present *in vitro* data collected in Kilifi over the course of my DPhil project. I have outlined the methods used in the generation of these data in section 2.5 and 2.6. The data presented in this chapter were summarised in 3 publications related to this project [295, 307, 314] (see appendix 6 for copies of published papers).

3.1.1 The role of in vitro studies

In vitro drug susceptibility assays are an important tool in the assessment of antimalarial drug resistance [137]. They provide a measure of the intrinsic sensitivity of clinical isolates to antimalarial drugs (including individual components of antimalarial combinations), eliminating confounding host factors such as patient compliance, pharmacokinetics, nutrition and immune status). These assays also allow the identification of parasites with well-characterized sensitive and resistant phenotypes, which can be used to investigate the mechanisms of resistance. Compared to *in vivo* monitoring, molecular markers are a potentially cheap, fast and effective alternative method of tracking antimalarial drug resistance in the field [315].

In this study, I analysed the baseline activities of important antimalarial drugs in Kilifi (CQ, PQ, LM, AQ, DEAQ and DHA), and the impact of *pfmdr1* and *pfcrt* polymorphisms on their activity. The overall goal was to understand the mechanisms of resistance to these drugs and to identify molecular markers that can be used to monitor resistance to LM-ATM, DHA-PQ and AS-AQ.

3.1.2 Rationale

PQ and AQ belong to the 4-aminoquinoline group whilst LM belongs to the quinoline-methanol group of antimalarials [188, 189]. It is thought that 4-aminoquinolines and quinoline-methanols have similar mechanisms of actions, exerting their antimalarial action by interfering with heme polymerisation [188, 190] (see section 1.5.1). Thus, these drugs could share some mechanisms of resistance. Therefore, I used CQ (the most studied of the quinoline drugs) as a reference to study LM, PQ and AQ resistance. Previous studies demonstrate that quinoline resistance is mediated by polymorphisms in two main genes *pfcrt and pfmdr1[175]* (see a detailed outline in section 1.8). I examined the impact of polymorphisms in *pfcrt* and *pfmdr1* polymorphisms on antimalarial drug activity in Kilifi.

3.2 Results

3.2.1 Source of clinical isolates

The isolates used in this study were adapted from several clinical studies carried out in Kilifi from 2005 to 2008. I provide a brief outline of the clinical studies below.

3.2.1.1 LM-ATM vs DHA-PQ clinical trial.

The DHA-PQ vs LM-ATM clinical trial (Controlled trials registry number ISRCTN88705995) was conducted between 2005-2008. The full details of this this trial are described elsewhere [296] and Dr. Steffen Borrmann et al (unpublished). Briefly, four

hundred and seventy five paediatric patients with uncomplicated *P.falciparum* malaria from Kilifi were randomized to 3-day treatment with either DHA-PQ (2.5/20mg/kg once daily) or LM-ATM (1.7/10mg twice daily), and followed up for 84 days. In total, 241 and 243 paediatric patients were recruited into the LM-ATM and DHA-PQ arms respectively. At day 84, the recurrence of asexual parasites was detected in 81 of 217 evaluable patients in the DHA-PQ arm and 98 out of 233 evaluable patients in the LM-ATM arm. The PCR-corrected day 28 cure rate was 90.4% (intention to treat, ITT) and 94.7% (enlarged per protocol, EPP) in the DHA-PQ group, and 90.0% (ITT) and 95.3% (EPP) in the LM-ATM group. Overall, the results of this multicentre trial showed that DHA-PQ was as efficacious as LM-ATM in treating uncomplicated malaria in African children from different endemicity settings, and showed a comparable safety profile [296].

3.2.1.2 Amodiaquine clinical trial

A subset of the samples analysed here were obtained from the clinical trial of AQ in Kilifi [295]. In this trial, 128 paediatric patients with uncomplicated malaria received a supervised oral course of AQ (10mg/kg/day for 3 days) with 28 days of follow up, between March and July 2006. Between days 7 and 28, the recurrence of asexual parasites was detected in 29 patients (12 were determined to be re-infections, 10 to be recrudescences whilst 5 had a mixture of persistent and new alleles). In this trial, the PCR corrected parasitological cure rate on day 28 was 82% showing a relatively high prevalence of *in vivo* resistance to AQ in Kilifi.

3.2.1.3 Other ongoing studies

Some of the isolates analysed here had been collected as part of some integrated parasitology studies in Kilifi (Dr Pete Bull's study, OXTREC NO:030-06)

3.2.2 In vitro adaptation

I chose the method of *in vitro* adaptation as it allowed the cultivation of isolates with low parasitaemia from the field to thresholds required for *in vitro* chemosenitivity testing, as well as the cryopreservation of such isolates for use in further studies. In total, 195 isolates were adapted from the clinical studies (Table 3-1)

Table 3-1: The number *of P.falciparum* isolates adapted from various clinical studies.

Study	Number adapted
DHA-PQ vs LM-ATM trial	96
AQ clinical audit	88
Others	11
Total	**195**

3.2.3 Activity of drugs against reference strains

I used two reference isolates 3D7 (CQ sensitive) and V1S (multidrug resistant) as controls for the chemo sensitivity assays. Table 3-2 shows the IC_{50} (SD) of CQ, LM, DEAQ, AQ, PQ and DHA against the reference isolates.

Table 3-2:IC_{50}nM (SD) of CQ, LM, DEAQ, AQ, PQ and DHA against reference isolates

strains	Description	CQ	LM	DEAQ	AQ	PQ	DHA
3D7	CQ sensitive	7 (2)	96 (12)	3 (0.2)	3 (0.1)	27 (17)	2 (0.2)
V1S	Multidrug resistant	158 (75)	24 (14)	25(0.6)	6 (1.6)	42 (10)	2 (1.2)

AQ and DHA were the most active drugs, with similar activity against V1S and 3D7 (mean IC_{50}<10nM), followed by PQ (mean IC_{50}<50nM). As expected, the CQ resistant V1S was less susceptible to CQ and DEAQ (mean IC_{50}s 158nM and 25nM respectively) than the CQ

sensitive 3D7 (mean IC_{50}s 7nM and 3nM respectively). Interestingly, 3D7 was less susceptible to LM than V1S (mean LM IC_{50} 96nM and 24nM respectively). Lower susceptibility of 3D7 to LM has previously been reported and was associated with polymorphism at codons 184 and 1042 of *pfmdr1* [316].

3.2.4 Activity of drugs against Kilifi P. falciparum isolates

The median IC_{50}s for CQ (n=193), LM.(n=112), DEAQ(n=98), AQ (n=98), PQ (n=109), and DHA.(n=109) were 40nM interquartile range(IQR) 17-72nM), 55nM (IQR 29-100nM), 29nM (IQR 20-52nM), 16nM (IQR 10-23nM), 31nM (IQR 19-46nM), and 2nM (1-3nM) respectively (Figure 3-1).

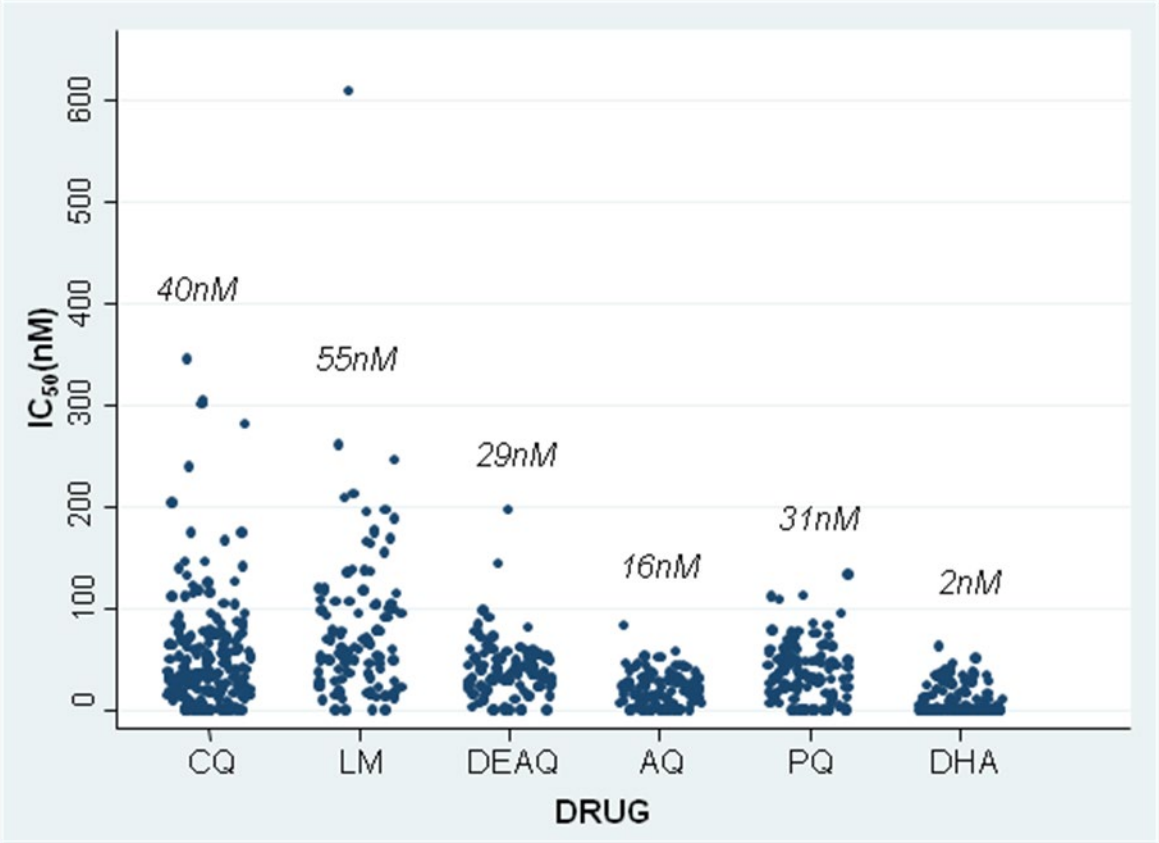


Figure 3-1 : The activity of CQ, LM, DEAQ, AQ, PQ and DHA against Kilifi *P.falciparum* isolates.The figures in italics indicate corresponding median IC_{50}s. The corresponding numbers for each drug were CQ 193, LM 112, DEAQ 98, AQ 98, PQ 109 and DHA 109.

3.2.5 Correlation of in vitro antimalarial activities

The assessment of *in vitro* cross-resistance of antimalarials can give useful insight into the mechanisms of resistance. For example, for drugs with shared mechanisms of resistance, a positive correlation is expected, whilst for those with inverse mechanisms of resistance a negative correlation is expected.

The activities of the aminoquinoline drugs CQ, AQ and DEAQ were significantly correlated ($P<0.001$), as is expected based on their structural similarity (Table 3-3). However, it was interesting to note that there was no significant correlation between the activity of the 4-aminoquinoline PQ and that of the other aminoquinolines ($P>0.5$), despite their structural similarity. This suggests that the mechanisms of resistance to PQ may be different from those of the other 4-aminoquinolines (CQ, AQ and DEAQ).

Table 3-3: The correlation between the activities of different antimalarial drugs.The number of isolates contributing to this analysis for each drug were CQ 193, LM 112, DEAQ 98, AQ 98, PQ 109 and DHA 109. Significant correlations are shown in bold.

Logarithmic IC_{50}	Logarithmic IC_{50}	Pearson Correlation Coefficient (r)	P value
Chloroquine	**Amodiaquine**	**0.35**	**0.0005**
Amodiaquine	**Desethylamodiaquine**	**0.60**	**0.0000**
Chloroquine	**Desethylamodiaquine**	**0.45**	**0.0000**
Piperaquine	Chloroquine	0.15	0.13
Piperaquine	Amodiaquine	-0.41	0.51
Piperaquine	Desesthylamodiaquine	-0.05	0.87
Piperaquine	Lumefantrine	0.13	0.20
Piperaquine	Dihydroartemsinin	0.02	0.85
Chloroquine	**Lumefantrine**	**-0.25**	**0.01**
Lumefantrine	**Dihydroaratemisinin**	**0.33**	**0.0005**
Chloroquine	Dihydroartemsinin	0.19	0.06
Lumefantrine	Amodiaquine	-0.04	0.9
Lumefantrine	Desethylamodiaquine	0.21	0.47
Dihydroartemsinin	Amodiaquine	0.02	0.96
Dihydroartemsinin	Desethylamodiaquine	0.07	0.82

Interestingly, I observed a significant negative correlation between CQ and LM activity, suggesting that these two drugs have inverse mechanisms of resistance. On the other hand, a

significant positive correlation was observed between LM and DHA activity, suggesting some common mechanisms of resistance between these drugs.

3.3 The Impact of *pfcrt* and *pfmdr1* polymorphisms on drug activity

The results presented in this section are based on genetic tests performed on culture-adapted parasites, and thus represent the parasites contributing to the IC_{50} estimates presented in section 3.2.

3.3.1 Pfcrt mutations

3.3.1.1 pfcrt-K76T mutation

The *pfcrt-K76T* mutation (the main molecular marker associated with CQ resistance) was found in 42/65 (65%) of baseline isolates. Figure 3-2 shows the relationship between the *pfcrt-K76T* mutation and the activity of CQ (Figure 3-2A) and LM (Figure 3-2B).

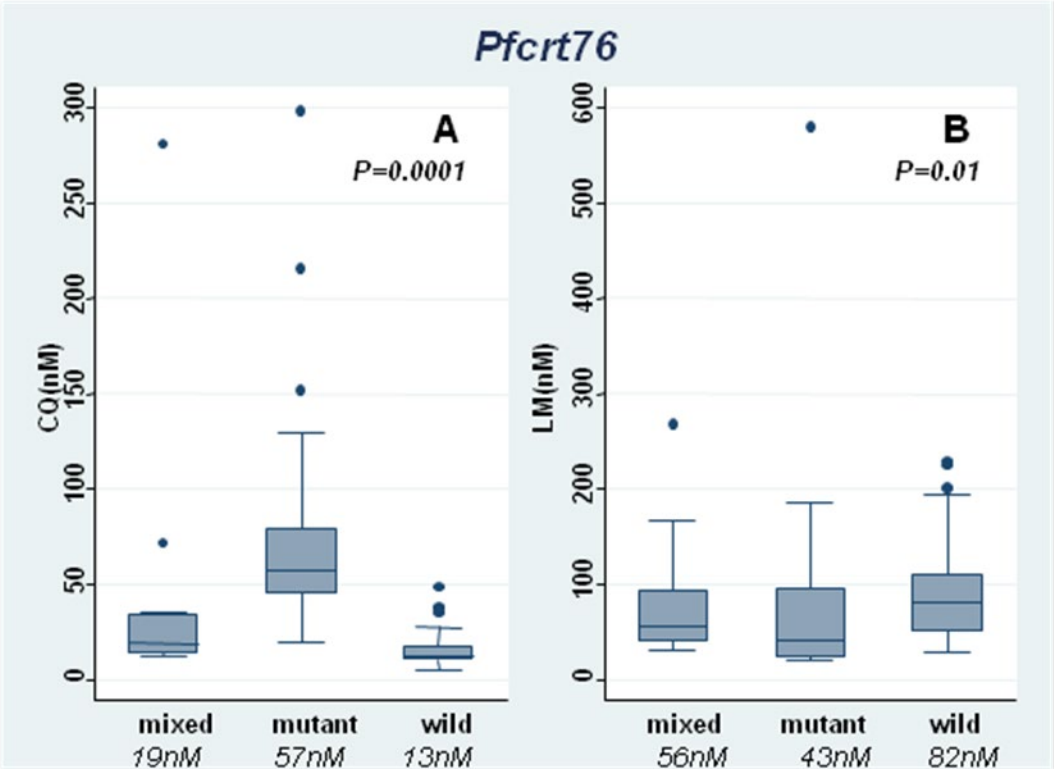


Figure 3-2: Relationship between CQ and LM IC_{50}s (concentration that kills 50% of parasites) and mutation at *pfcrt-76.* The figures in italics indicate corresponding median IC_{50}s. The corresponding numbers were CQ mutant 74, mixed 13, wild 39; LM mutant 45, mixed 11, wild 31. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

Depending on the *pfcrt -76* genotype, Kilifi isolates could be classified into 3 distinct groups. The first group, consisting of mutant parasites, was the least susceptible to CQ (n=74, median IC_{50} of 57nM, IQR 45-80nM), followed by the second group composed of parasites with mixed genotypes which had intermediate CQ IC_{50} values (n=13, median CQ IC_{50} 19nM, IQR 15-34nM). The third group, composed of wild type parasites, was the most susceptible to CQ (n=39, CQ IC_{50} 13nM, IQR 10-18nM). These differences were statistically significant (P=0.0001).

Interestingly, the *pfcrt-K76T* mutation had an opposite effect on LM susceptibility. Wild type isolates were the least susceptible to LM (n=31, LM IC_{50} 82nM, IQR 51-111nM) followed by parasites with mixed genotypes (n=11, median LM IC_{50} 56nM, IQR 42-95nM), whilst mutant parasites were the most susceptible to LM (n=45, median LM IC_{50} 43nM, IQR 26-97nM); these differences were statistically significant (P=0.01).

The *pfcrt-K76T* mutation did not have a significant impact on the activity of PQ (see Figure 3-3A), AQ (see Figure 3-3B), DEAQ (see Figure 3-3C) or DHA (see Figure 3-3D), (p>0.05).

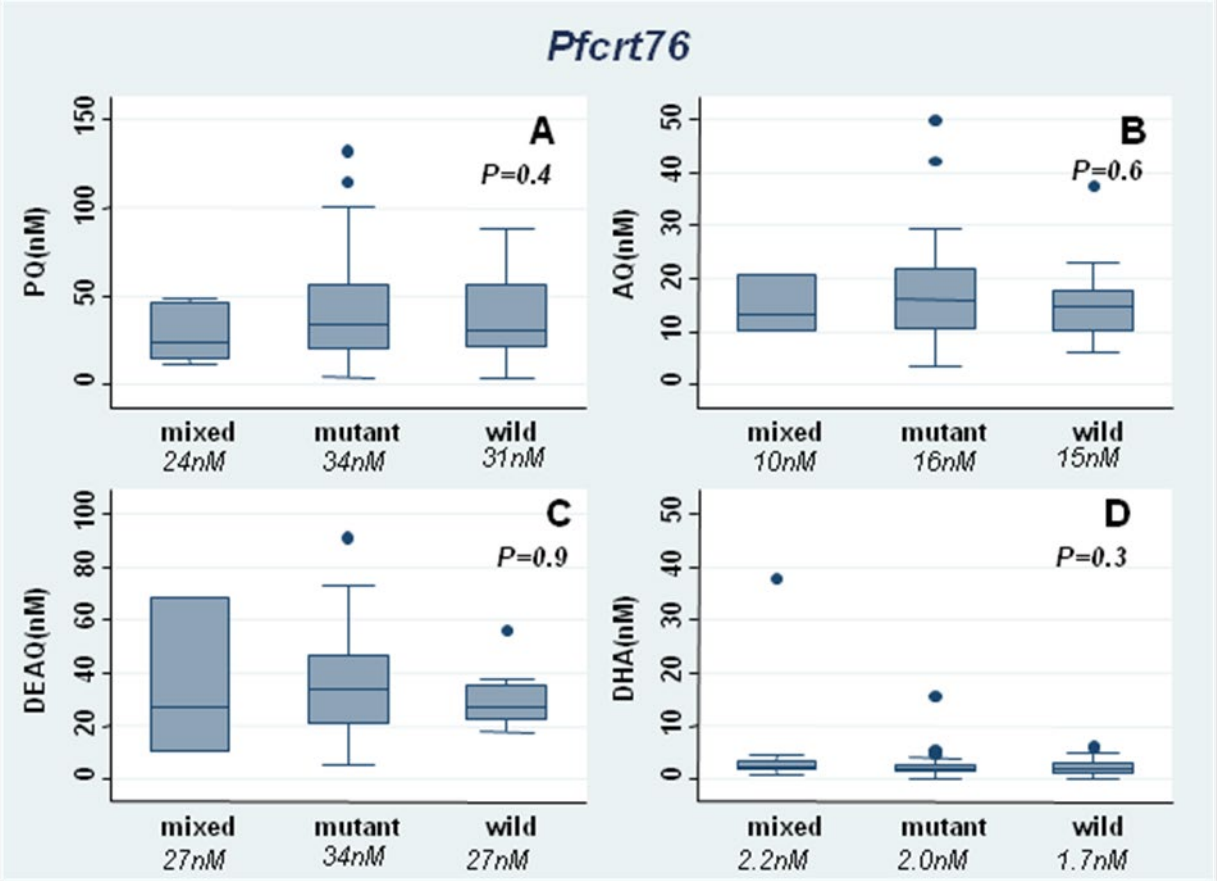


Figure 3-3: Relationship between PQ, AQ, DEAQ and DHA IC_{50}s (concentration that kills 50% of parasites) and mutation at *pfcrt-76*. The figures in italics indicate the corresponding median IC_{50}s. The corresponding numbers were PQ mutant 44, mixed 11, wild 29; AQ mutant 36, mixed 3, wild 12; DEAQ mutant 36, mixed 3, wild 12; DHA mutant 44, mixed 11, wild 30. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

3.3.1.2 Other pfcrt mutations

The *pfcrt* protein consists of 10 transmembrane (TM) domains (see section 1.8.1 for an overview). Point mutations have been detected at up to 15 *pfcrt* residues, and it has been observed that individual CQ resistant parasites may carry 4-8 mutations [175]. Of the known mutations, it has been proposed that those occurring in domains 1, 4 and 9 of *pfcrt* are the most critical determinants of quinoline resistance [197]. Figure 3-4 shows a diagrammatic representation of PfCRT, highlighting mutations that are known to occur in TM 1, 4 and 9.

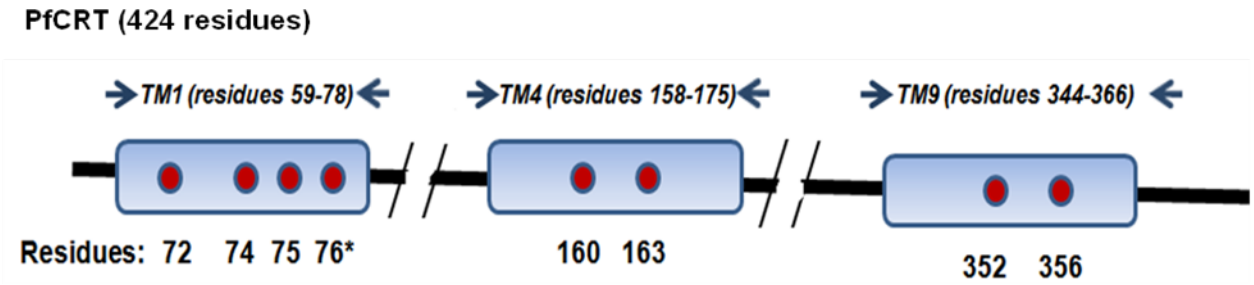


Figure 3-4: A diagram highlighting polymorphisms occurring at TM 1, 4 and 9 of PfCRT. The * mark shows residue 76, the main polymorphism associated with CQ resistance.

I first analysed the impact of the *pfcrt-K76T* mutation on drug activity (see section 3.3.1.1 above). Although the *pfcrt-K76T* mutation is present in all CQR strains regardless of geographic origin, it occurs within different haplotypes at codons 72, 74, 75 and 76 (TM 1) (an overview is provided in chapter one, Table 1-1). Two mutations have been previously identified in TM4 (L160Y and S163R) while two mutations have been identified in TM9 (I356T or I356L and Q352K or Q352R) [175, 197].

To analyse the role of the additional mutations in TM 1, 4 and 9, I sequenced two fragments of the *Pfcrt* gene in a subset of Kilifi isolates: CRTI (bp 322-869, encompassing residues 23-178) and CRTII (bp 1920-2432, encompassing residues 300-366). I analysed the *pfcrt* haplotypes carried by Kilifi isolates at codons 72, 74, 75 and 76.

I sequenced 43 isolates. Of the sequenced isolates, 36 carried the *pfcrt-76T* mutation. All the 36 isolates carrying the mutant *pfcrt-76T* mutation carried the *pfcrt* CIET haplotype (wildtype at codon 72 and mutant at codons 74, 75 and 76). Seven of the 43 isolates were wildtype at *pfcrt-76*; all the 7 wildtype isolates carried the *pfcrt* CMNK allele (wildtype at codons 72, 74, 75, 76). Therefore only two *pfcrt* haplotypes, CIET and CMNK were observed in Kilifi isolates.

I could not obtain reliable reading of sequences at codons 160 and 163, due to poor quality of sequences obtained in this region. All the 43 sequenced isolates carried the wild type *pfcrt-352Q* and *pfcrt-356I* alleles.

3.3.2 pfmdr1 mutations

3.3.2.1 pfmdr1-86

The *pfmdr1-N86Y* mutation was found in 45/57 (79%) of baseline isolates. As expected, there was no association between *pfmdr1-86* mutation and CQ activity (see Figure 3-6A).

AQ susceptibility was modulated by *pfmdr1-86*. Indeed, *pfmdr1-86* mutant were less susceptible to AQ (n=32, median AQ IC_{50} 17nM, IQR 11-22nM) than mixed (n=1, AQ IC_{50} 10nM) and wild type isolates (n=8, median AQ IC_{50} 11nM, IQR 6-13nM) (p=0.04) (see Figure 3-5A)

Interestingly, mutant isolates at *pfmdr1-86* were the most susceptible to LM (n=56, median IC_{50} 48nM , IQR 29-72nM) compared to mixed (n=8, median LM IC_{50} 57nM, IQR 30-67nM) and wild type isolates (n=23, median LM IC_{50} 103nM, IQR 92-155nM);these differences were statistically significant (p=0.0001) (see Figure 3-5B).

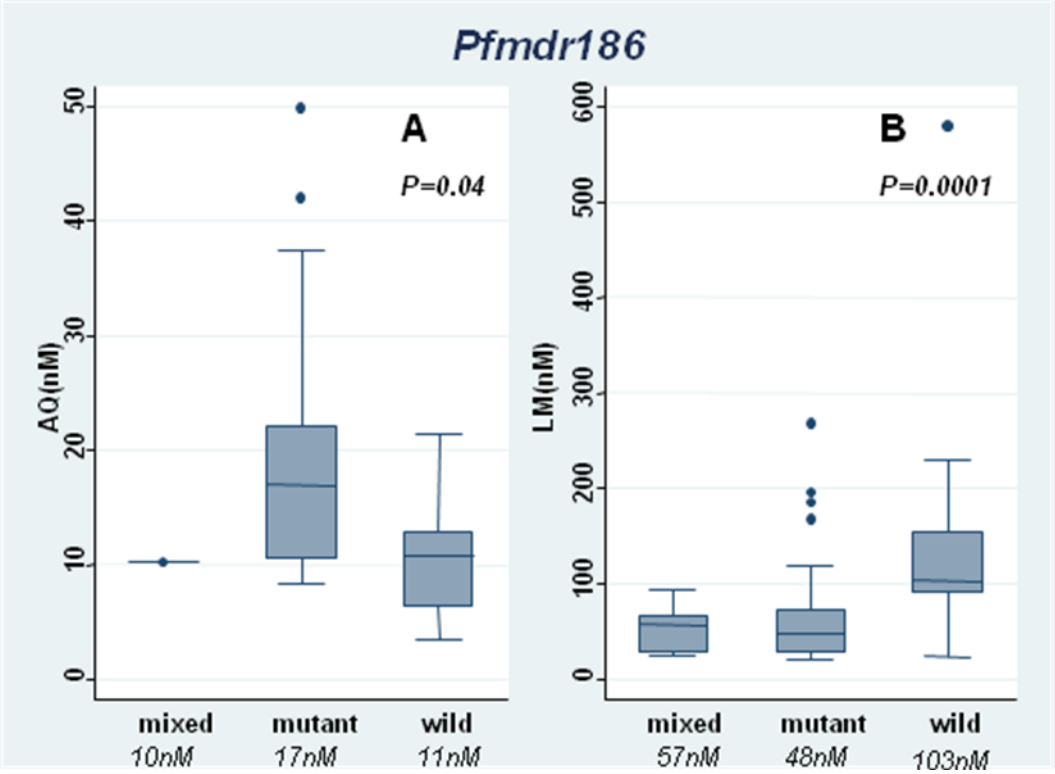


Figure 3-5: Relationship between AQ (A) and LM (B) IC_{50}s (concentration that kills 50% of parasites and mutation at *pfmdr-186*. The figures in italics indicate the corresponding median IC_{50}s. The corresponding numbers were AQ mutant 32, mixed 1, wild 8; LM mutant 56, mixed 8, wild 23. Statistical analysis was done using the Kruskal-Wallis non-parametric test

Pfmdr1-86 mutation did not have a significant impact on the activity of PQ (see Figure 3-6B), DEAQ (see Figure 3-6C), and DHA (see Figure 3-6D).

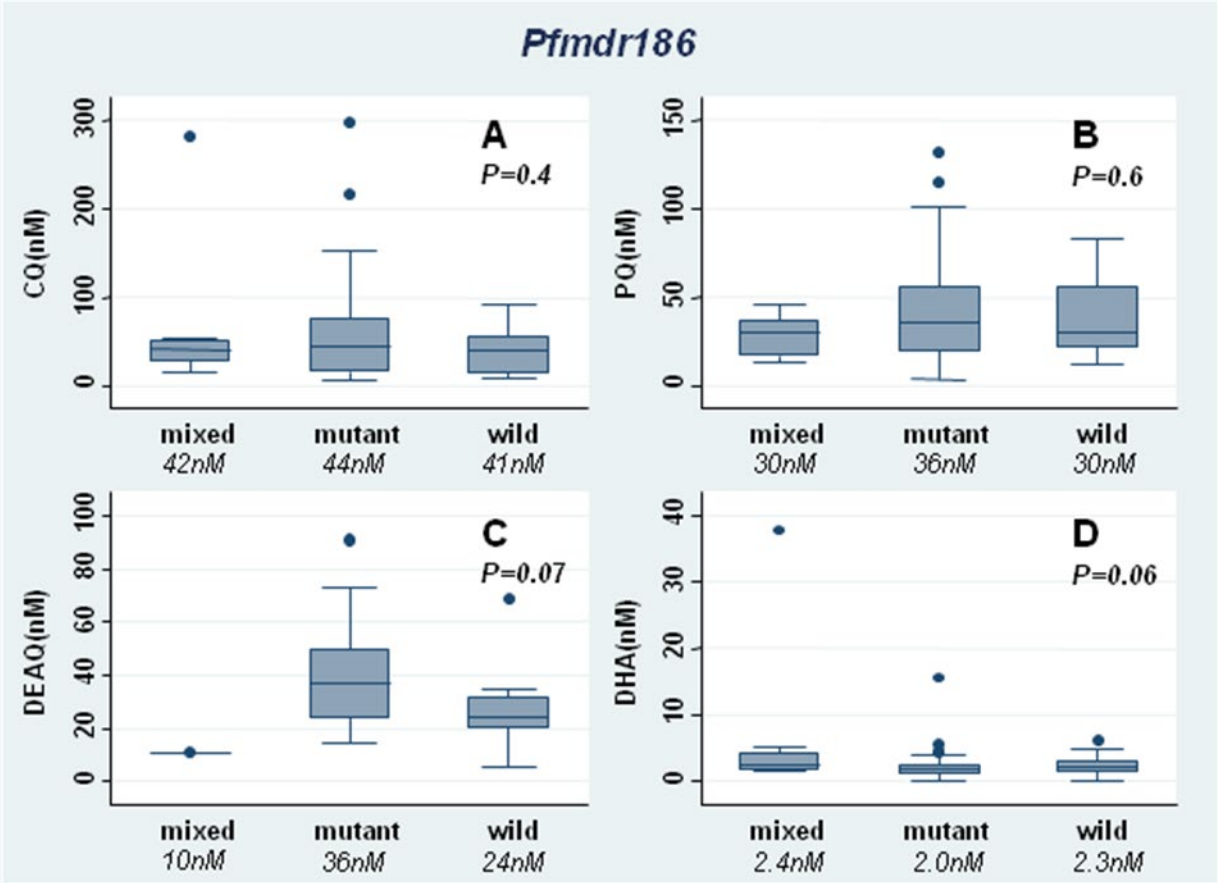


Figure 3-6: Relationship between CQ, PQ, DEAQ and DHA IC_{50} (concentration that kills 50% of parasites and mutation at *pfmdr1-86*.The figures in italics indicate the corresponding median IC_{50}s. The corresponding numbers were CQ mutant 79, mixed 8, wild 29; PQ mutant 54, mixed 8, wild 22; DEAQ mutant 32, mixed 1, wild 8; DHA mutant 55, mixed 8, wild 22. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

3.3.2.2 Other pfmdr1 mutations

In addition to mutation at codon 86, other mutations in *pfmdr1* may be associated with drug susceptibility. Figure 3-7 shows a diagrammatic representation of *pfmdr1* and its known polymorphic residues.

Figure 3-7: a diagram highlighting the known PfMDR1 polymorphic residues. The * sign shows residue 86, the main polymorphism associated with CQ resistance.

pfmdr1 mutations occur at codons 86, 184, 1034, 1042 or 1246 [175]. I first analysed the impact of *pfmdr1-86* on drug activity (see section 3.3.2.1). To analyse the impact of additional *pfmdr1* mutations, I amplified and sequenced 3 fragments of the *pfmdr1 gene*: MDR1A (bp 183-713, encompassing residues 62-244), MDR1B (bp 2971-3482, encompassing residues 994-1160) and MDR1-C (bp 3400-3851, encompassing residues 1134-1283.

In total, I sequenced 80 samples at *pfmdr1*. In addition to polymorphism at codon 86, sequence variation was observed for codons 184 and 1246. The *pfmdr1-Y184F* and *pfmdr1-D1246Y* mutations were present in 8/51 (16%) and 15/52 (29%) of baseline isolates respectively. All sequenced isolates carried the wild type *pfmdr1-1034S* and *pfmdr1-1042N* alleles (see table S4 in appendix 3).

Isolates carrying the *pfmdr1-D1246Y* were less susceptible to CQ (n=31, median CQ IC_{50} 50nM, IQR 21-77nM) compared to wild type isolates (n=49, median CQ IC_{50} 33nM, IQR 15-52nM) (Figure 3-8A). Similarly *pfmdr1-D1246Y* mutant isolates were less susceptible to AQ (n=17, median AQ IC_{50} 20nM, IQR 15-22nM) than wild type parasites (n=31, median AQ IC_{50} 13nM, IQR 10-21nM) (Figure 3-8B). Mutant isolates were also less susceptible to DEAQ (n=17, median DEAQ IC_{50} 42nM, IQR 30-42nM) than wild type parasites (n=31, median DEAQ IC_{50} 24nM, IQR 19-40nM) (Figure 3-8C). These differences were statistically significant ($P<0.05$).

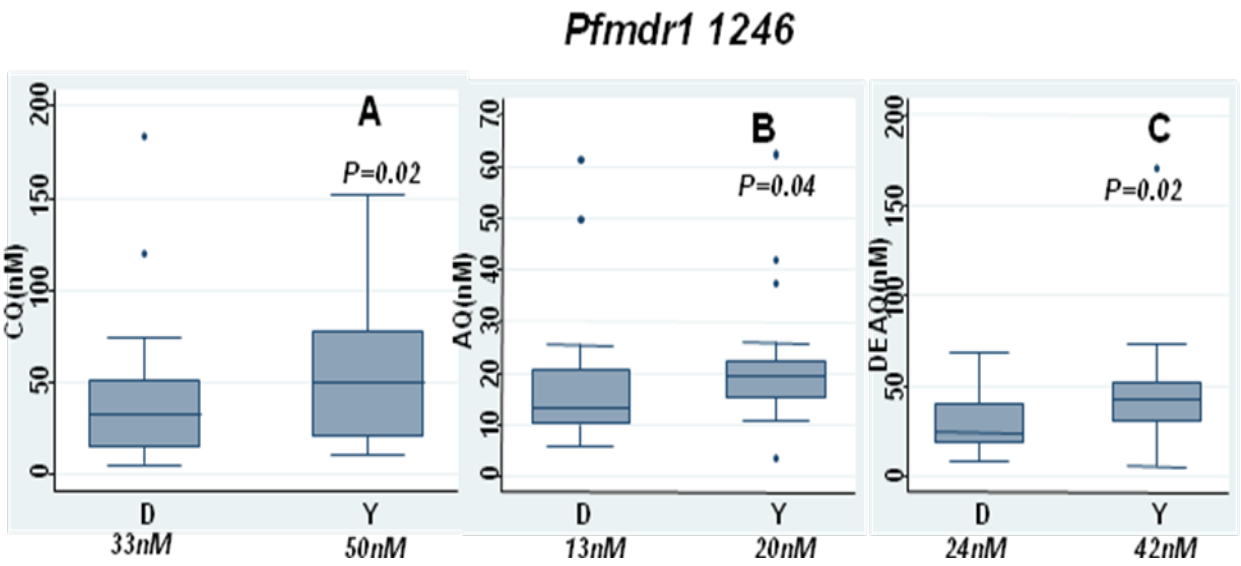


Figure 3-8: Relationship between CQ, AQ and DEAQ IC_{50} (concentration that kills 50% of parasites) and mutation at *pfmdr1 1246*. The figures in italics indicate the corresponding median IC_{50}s. The corresponding numbers were CQ D 49, Y 31; AQ D 31, Y 17; DEAQ D 31, Y 17. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

The *pfmdr1-D1246Y* mutation did not significantly alter susceptibility to PQ (Figure 3-9A), LM (Figure 3-9B) and DHA (Figure 3-9C).

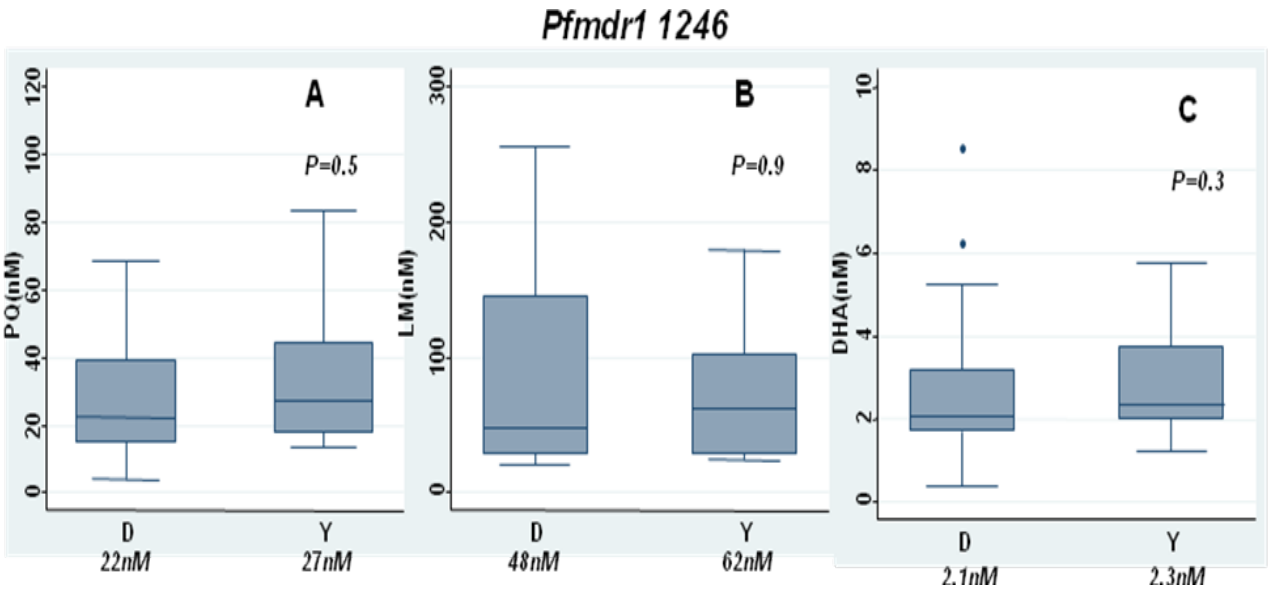


Figure 3-9: Relationship between PQ, LM and DHA IC_{50} (concentration that kills 50% of parasites and mutation at *pfmdr1 1246*. The figures in italics indicate corresponding median IC_{50}s. The corresponding numbers were PQ D 27, D 15 ; LM D 27, Y 15; DHA D 27, Y 15. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

There was no significant association between the *pfmdr1-Y184F* mutation and susceptibility to any of the drugs tested (p>0.05).

3.3.2.3 Combining pfmdr1-86 and pfmdr1-1246 mutations

Two *pfmdr1* mutations (86 and 1246) had a significant influence on antimalarial activity (see sections 3.3.2.1 and 3.3.2.2). To analyse their interaction, I categorised the sequenced isolates into 4 main categories based on their mutation status at *pfmdr1-86* and *pfmdr1-1246* i.e.*pfmdr1-86* wild type and *pfmdr1-1246* wild type (WW), *pfmdr1-86* wild type and *pfmdr1-1246* mutant (WM), *pfmdr1-86* mutant and *pfmdr1-1246* wild type (MW), *pfmdr1-86* mutant and *pfmdr1- 1246* mutant (MM). For this analysis, I excluded isolates with mixed alleles.

Isolates carrying the MM genotype at *pfmdr1-86* and *pfmdr-1246* were the most susceptible to LM (median LM IC_{50} 56nM, IQR 29-84nM) whilst those carrying the WW genotype were the least susceptible (median LM IC_{50} 136nM, IQR 72-178nM), (adjusted p=0.002) (see table S1

in appendix 3). However, this effect was similar to that observed when *pfmdr1-86* was considered alone (see Figure 3-5B), a clear indication that this effect is primarily attributable to *pfmdr1-86* alone, and suggesting that *pfmdr1-1246* may have a neutral effect.

There were no significant differences observed between groups for CQ, PQ, AQ, DEAQ and DHA (adjusted p value>0.05). As *pfmdr1-1246* alone had a significant impact on CQ, AQ and DEAQ activity (see Figure 3-8), the absence of effect on drug activity when *pfmdr1-86* and *pfmdr1-1246* were combined suggests that in the case of CQ, AQ and DEAQ, the presence of *pfmdr1-86* mutation negated the effect of *pfmdr1-1246*.

3.3.3 Combining pfmdr1 and pfcrt mutations.

3.3.3.1 Combining pfcrt-76 and pfmdr-86 mutations

I also investigated the interaction between *pfcrt-76* and *pfmdr1-86* in altering drug susceptibility. After excluding isolates with mixed genotypes, isolates were classified into 4 categories as follows: *pfcrt-76* mutant and *pfmdr1-86* mutant (MM), *pfcrt-76* mutant and *pfmdr1-86* wild type (MW), *pfcrt-76* wild type and *pfmdr1-86* mutant (WM) and *pfcrt-76* wild type and *pfmdr1-86* wild type (WW).

Parasites carrying MM genotype were the least susceptible to CQ (n=48, median CQ IC_{50} 73nM, IQR 49-106nM) whilst those carrying WW genotypes were the most susceptible (n=12, median CQ IC_{50} 11nM, IQR 9-18nM) (Figure 3-10A). Similarly, parasites carrying MM genotypes were less susceptible to DEAQ than all other categories (Figure 3-10B).

In contrast *pfcrt-76* and *pfmdr1-86* genotype had an opposite effect on LM susceptibility; isolates carrying MM genotype were the most susceptible to LM (n=29, median LM IC_{50} 31nM IQR 25-49nM) whilst those carrying the WW genotype were the least susceptible (n=11, median LM IC_{50} 124nM, IQR 93-202nM), adjusted P value<0.05 (see Figure 3-10C).

There was no significant association between combined *pfcrt-76* and *pfmdr1-86* genotypes and PQ, AQ and DHA activity (adjusted p value>0.05, Figure 3-11).

When I included mixed genotypes in this analysis, no significant difference was observed in the results (see supplementary table S2 in appendix 3).

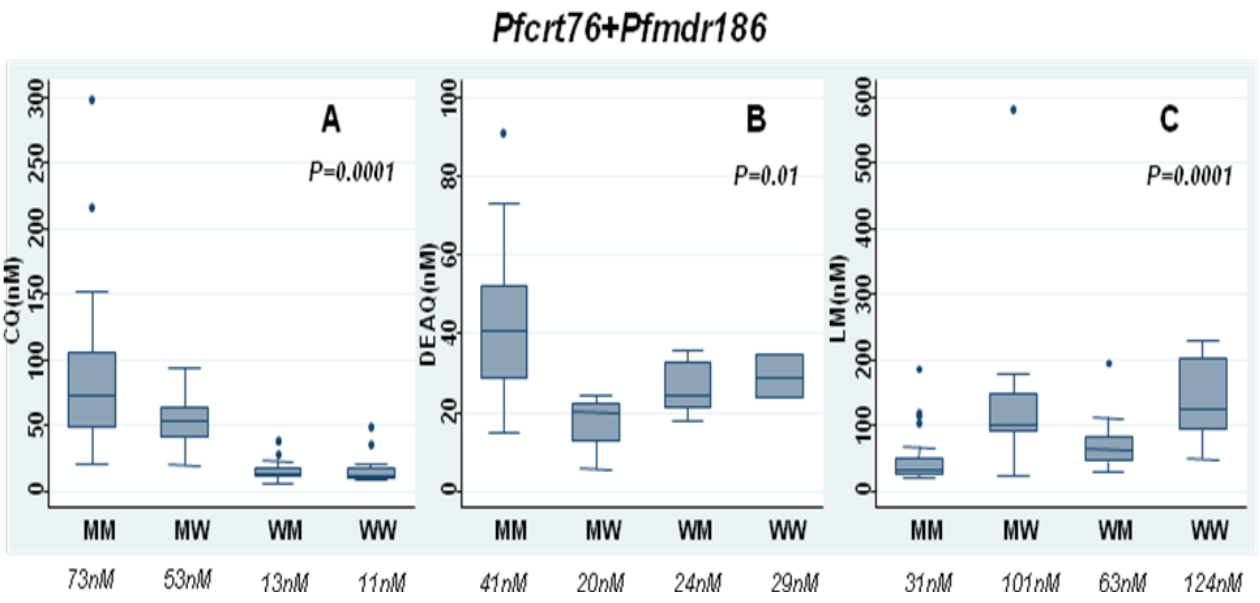


Figure 3-10: Relationship between CQ, DEAQ and LM IC_{50}s (inhibitory concentration that kills 50% of parasites) and *pfcrt-76* and *pfmdr1-86* genotypes. MM, mutant *pfcrt-76* and *pfmdr1-86*; MW, mutant *pfcrt-76* and wild type *pfmdr1-86*, WM, wild type *pfcrt-76* and mutant *pfmdr1-86* and WW, wild-type *pfcrt-76* and *Pfmdr1-86.* The Figures in italics are the corresponding median IC_{50}. The corresponding numbers were CQ MM 48, MW 16, WM 24, WW 12; DEAQ MM 26, MW 4, WM 6, WW 3; LM MM 29, MW 12, WM 20, WW 11. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

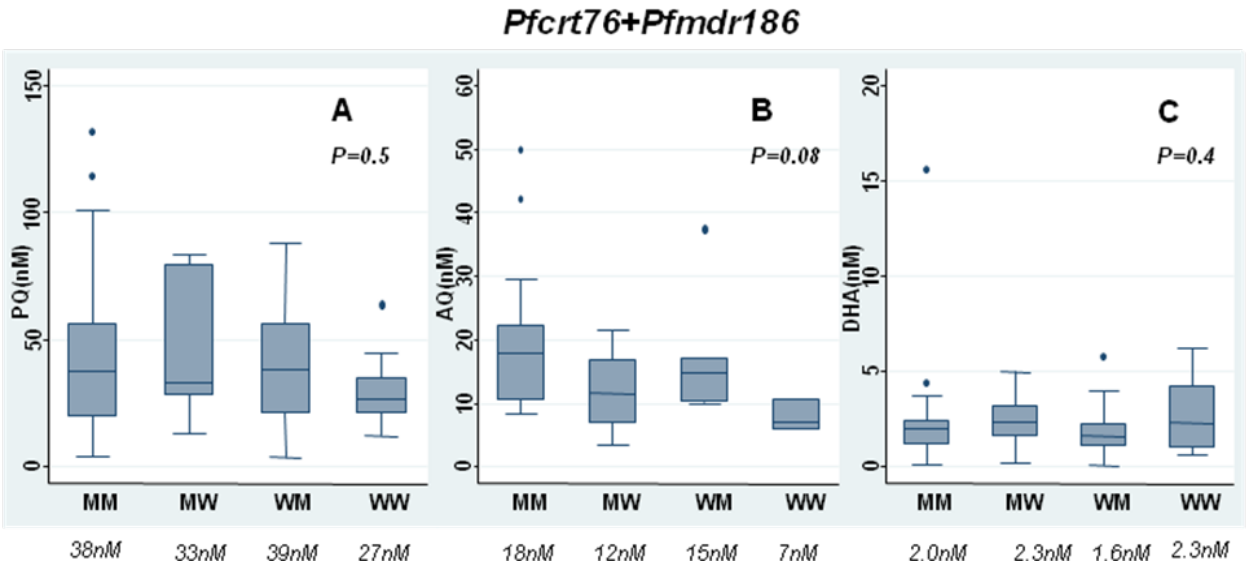


Figure 3-11: Relationship between PQ, AQ and DHA IC_{50}s (inhibitory concentration that kills 50% of parasites) and *pfcrt-76* and *pfmdr1-86* genotypes.MM, mutant *pfcrt76* and *pfmdr186*; MW, mutant *pfcrt-76* and wild type *pfmdr1-86*, WM, wild type *pfcrt-76* and mutant *pfmdr1-86* and WW, wild-type *pfcrt-76* and *pfmdr186.* The Figures in italics are the corresponding median IC_{50}s. Corresponding numbers were PQ MM 29, MW 11, WM 18, WW 11; AQ MM 26, MW 4, WM 6, WW 3; DHA MM 28, MW 12, WM 20, WW 10. Statistical analysis was done using the Kruskal-Wallis non-parametric test.

3.3.3.2 Combining pfmdr1-1246 and pfcrt-76

I also investigated the potential interaction between the *pfmdr1-1246* and *pfcrt-76* mutations in altering drug susceptibility. Isolates were grouped into 4 categories : *pfmdr1-1246* wild type and *pfcrt-76* wild type (WW), *pfmdr1-1246* wild type and *pfcrt-76* mutant (WM), *pfmdr1-1246* mutant and *pfcrt-76* wild type (MW) and *pfmdr1-1246* mutant and *pfcrt-76* mutant (MM).

Parasites carrying the MM genotype at *pfmdr1-1246* and *pfcrt-76* were the least susceptible to CQ (median CQ IC_{50} 63nM, IQR 48-79nM) whilst those carrying the WW genotype were the most susceptible (median CQ IC_{50} 15nM, IQR 11-20nM) and this effect was statistically significant (Adjusted p value=0.0002) (see supplementary table S3 in appendix 3). However, this effect was similar to that observed when *pfcrt-76* was considered alone (see Figure 3-2A), showing that it was most likely attributable to mutation at *pfcrt-76* (with *pfmdr1-1246* having

a neutral effect). There was no significant difference in the susceptibility of the 4 genotypes to PQ, LM, AQ, DEAQ or DHA (adjusted p value >0.05).

When considered on its own, mutation at *pfcrt-76* had a significant impact on LM activity (see Figure 3-2B). The observation that the significance of this effect disappeared when *pfcrt-76* and *pfmdr1-1246* were combined, suggests that the effects of *pfmdr1-1246* and *pfcrt-76* were antagonistic on LM activity.

3.3.4 Estimation of pfmdr1 copy number

Amplification of *Pfmdr1* has previously been associated with altered susceptibility to various antimalarials including LM and MFQ. I estimated the *pfmdr1* copy number of 80 Kilifi isolates using a qPCR relative quantification assay. The results showed that the estimated *pfmdr1* copy number ranged between 0.55 and 1.98 in Kilifi. An isolate was considered to have > 1 *Pfmdr1* copy if the estimated number of *pfmdr1* copies was >1.7. Based on this criterion, only 1 isolate was estimated to have 2 copies (relative *pfmdr1* copy number was 1.98). All other isolates had one copy of *pfmdr1*.

3.4 Discussion

3.4.1 Antimalarial chemo sensitivity profiles

3.4.1.1 Chloroquine

CQ was the mainstay of therapy for uncomplicated malaria for many years until widespread resistance led to its withdrawal. In Kenya, the official withdrawal of CQ was implemented in 1998 [284]. Our data demonstrated that Kilifi isolates had a median IC_{50} of 40 nM, and 168 out of 193 isolates assayed (87%) had CQ IC_{50} <100nM (Figure 3-1). The *in vitro* cut off point previously cited to distinguish CQ sensitive and CQ resistant isolates is 100nM [223,

277, 317-321]. Using the proposed *in vitro* cut off point, most Kilifi isolates would be considered to be CQ sensitive. However, the analysis of mutation at *pfcrt- 76* indicated that i) most parasites (60%) carried the *pfcrt-76T* mutation, ii) these mutants had a significant higher median CQ IC_{50}s (57nM, IQR 45-80nM) than mixed (19nM, IQR 15-34nM) and wild type parasites (13nM, IQR 10-18nM) (see section 3.3.1.1). Based on this *pfcrt-76* genotype data, the cut-off point for CQ resistance in Kilifi could be considered to be 25 nM (see Figure 3-2A), a value considerably lower than the cut-off point of 100nM previously cited [226, 277, 317-322]. Overall CQ resistance is still high in Kilifi, in line with observations from other endemic areas in Africa [122, 277, 323-327].

The *pfmdr-86* mutation on its own did not significantly alter CQ susceptibility in this study (p=0.4) (Figure 3-6A), whilst the *Pfmdr1-D1246Y* mutation was significantly associated with decreased CQ susceptibilty (P=0.02) (Figure 3-8A). Combining either *pfmdr1-86* or *pfmdr1-1246* with *pfcrt-76* had a significant influence on CQ susceptibility (P<0.001) (see section 3.3.3). Our observations are in agreement with previous suggestions that although *pfmdr1* mutations may not have an impact on CQ resistance on their own, they may modulate CQ resistance in a *pfcrt-76* mutant background [175, 226].

3.4.1.2 Lumefantrine

LM *in vitro* activity was high (median IC_{50} 55nM) in the majority of Kilifi isolates (Figure 3-1), although 28 of the 112 (25%) isolates had LM IC_{50} >100nM. To date, there has been no validated clinical resistance to LM-ATM in Africa and there is no established cut-off point to distinguish between LM susceptible and resistant isolates *in vitro* (though an arbitrary cut-off point of 150nM was quoted in one study [325]. The observation that 25% of isolates had decreased susceptibility to LM is of particular concern and highlights the need for monitoring the efficacy of LM-ATM in Kilifi.

Interestingly, the majority of isolates with decreased LM-susceptibility primarily carried wild type *pfcrt-76* and *pfmdr1-86* genotypes associated with CQ susceptibility. Thus the use of the LM-ATM could potentially select for wild type parasites (susceptible to CQ), an observation reported in several clinical studies [233-235, 328-330]. I have confirmed these previous *in vivo* reports by *in vitro* analaysis of clinical isolates. Indeed, parasites with lower CQ activity (high CQ IC_{50}s) were more susceptible to LM and vice versa (see Figure 3-10A &B). This inverse relationship between LM and CQ supports the hypothesis that the use of the LM-ATM would select for parasite susceptible to CQ.

In an analysis of a genetic cross between the reference isolates 3D7 and HB3, decreased susceptibility of the reference isolate 3D7 to LM was attributed to sequence differences at positions 86, 184 and 1042 of *pfmdr1*. Further, previous studies reported the selection of the additional wildtype *pfmdr1* alleles (184F and 1246D) together with the wildtype 86N allele after treatment with LM-ATM *in vivo* [167, 233, 235] .However, these additional alleles were not associated with *in vitro* LM susceptibility in this study (see section 3.3.2). These observations suggest that the role of specific *pfmdr1* mutations in mediating LM resistance may vary according to the genetic background of the parasite.

All these observations suggest that wild type *pfmdr1* and *pfcrt* genotypes account for the inverse relationship between CQ and LM in *P.falciparum.* Therefore, the use of LM-ATM is likely to lead to the selection of CQ susceptible parasites, and *pfcrt-76* and *pfmdr1-86* will be important molecular markers of LM-ATM efficacy in Kilifi. I discuss the return of CQ susceptible parasites in chapter 5.

Interestingly, in isolates from South East Asia, point mutations in *pfcrt* and *pfmdr1* have not been associated with decreased LM susceptibility *in vivo* [166]. Rather, *in vivo* resistance to LM-ATM is associated with *pfmdr1* gene amplification in S.E. Asia [166, 246]. The selection of *pfmdr1* amplification by LM-ATM in Asia occurred against a background of high

prevalence of parasites with increased *pfmdr1* copy number, a phenomenon that was driven by a long history of MFQ use in the Asian setting [242]. *pfmdr1* gene amplification has also been reported to mediate *in vitro* resistance to multiple antimalarial drugs, including LM, MFQ, HLF and ART [245, 247]. My analysis revealed that the frequency of *pfmdr1* gene amplification was 1% in Kilifi (see section 3.3.4), in line with previous observations that *pfmdr1* amplification is a relatively rare event in African isolates [167, 248, 249, 328, 331, 332]. This is probably attributable to the low use of MFQ in Africa. However, it is possible that the use of LM-ATM would also select for parasites with increased *pfmdr1* copy number in African settings. Therefore, monitoring of *pfmdr1* amplification will be important with the continued use of LM-ATM in Kilifi.

3.4.1.3 Amodiaquine and desethyl amodiaquine

AQ and DEAQ belong to the amino quinoline group of antimalrial drugs, similar to CQ [188, 189]. AQ is a short-lived pro drug, rapidly metabolized to DEAQ (the pharmacologically active metabolite with a longer elimination half-life) [333-335]. As expected, AQ had a higher intrinsic activity than DEAQ in Kilifi isolates (Figure 3-1). Further, there were significant positive correlations between the activities of CQ, AQ, and DEAQ, as would be expected based on their structural similarity (Table 3-3).

Arbitrary *in vitro* cut off points of AQ resistance have previously been cited as 30nM for AQ [336] and 60nM for DEAQ [121, 277, 324, 326, 336, 337], and one study proposed an arbitrary a value of 80nM for DEAQ [325]. Based on the proposed cut off point of 30nM for AQ and 60nM for DEAQ, 6/98 of isolates (6%) were resistant to AQ and 15/98 (15%) were resistant to DEAQ. These data are in line with previous work indicating a relatively high prevalence (>85%) of *in vitro* sensitivity to AQ and DEAQ in many African settings [320, 324, 325, 336, 338-340]. However, in spite of this relatively high *in vitro* susceptibility, data from several African sites, including Kilifi, have shown a substantial decrease of AQ efficacy

in vivo [116-119, 295, 324]. Interestingly, in some of these reports, AQ failure was not associated with *in vitro* resistance to AQ and DEAQ when the proposed *in vitro* cut-off-points were applied [295, 320, 324]). Thus, more studies are needed to more clearly define the predictors of AQ and DEAQ failure *in vitro*.

The structural similarity between CQ, AQ and DEAQ and the observed correlation of their activities in this study (see Table 3-3) and other studies [121-123, 320, 326, 337] suggest that these drugs may share some common mechanisms of resistance. Indeed, although *pfcrt-K76T* could not account for AQ or DEAQ reduced activity (P>0.5) (see Figure 3-3), the *pfmdr1-N86Y* mutation was associated with a significant decrease in AQ susceptibility (p=0.04) (see Figure 3-5); for DEAQ, this association was stronger when *pfmdr1-86* and *pfcrt-76* were combined (P=0.01, see Figure 3-10). In addition, the *pfmdr1-D1246Y* mutation was associated with a significant decrease in AQ and DEAQ sensitivity *in vitro* (Figure 3-6), although these effects disappeared when *pfmdr1-86* and *pfmdr1-1246* were considered together (p>0.5) (see 3.3.2.3). These observations suggest that *pfcrt-76* and *pfmdr1-86* are important determinants of AQ and DEAQ susceptibility in Kilifi isolates. However, *pfmdr1-86* negated the effect of *pfmdr1-1246* in mediating AQ and DEAQ resistance, and there was no association between the *Y184F* mutation with *in vitro* AQ or DEAQ activity. This lack of association may be due to the small sample size of this study. The *in vivo* selection of mutant *pfmdr1* (*N86Y*, *Y184F* and *D1246Y)* and mutant *pfcrt* alleles has been demosnstrated in clinical studies after treatment with AQ or AQ combination therapy [128, 235, 249, 341-343]. Together, these observations suggest that the high background of CQ resistance in Kilifi could hamper AQ effectiveness, as shown in previous *in vivo* studies [295]. Thus, *pfcrt-76* and *pfmdr1-86* mutations may be useful molecular markers of AQ efficacy in Kilifi. The decreased susceptibility of Kilifi *P.falciparum* isolates to AQ is in contrast with previous reports that AQ retains activity, even in areas of high CQ resistance [109].

Whereas all wild type parasites carry the CMNK haplotypes at *Pfcrt* codons 72, 74, 75 and 76, those carrying the *Pfcrt-76T* mutation may occur as several haplotypes. The main haplotypes are CIET reported in isolates from Asia and Africa, CIDT from South East Asia, CIKT from the Pacific regions and SMNT from South America, Cambodia and the Pacific [175, 194]. The novel haplotypes SMDT and CMDT were recently reported from the Phillipines [344], whilst SMET was reported from Colombia [345]. All Kilifi isolates carried either the wildtype CMNK or the mutant CIET (also found in Asia) (see section 3.3.1.2)., supporting, at least partly, the hypothesis that CQ resistant parasites in Africa originated from Asia [346]. It is notable that the *pfcrt* SMNT haplotype, which has been associated with decreased DEAQ susceptibility *in vitro* [347] was not detected in any Kilifi isolate, despite the existence of AQ resistance in this setting [295]. This haplotype has been reported in parts of Asia [129, 175], South America [175], Papua New Guinea [126], Angola [132] and interestingly in an area of Tanzania where AQ monotherapy has been used widely due to SP failure [133]. The analysis of *Pfcrt* haplotypes over time will be useful when monitoring the trends of resistance to 4-aminoquinolines in Kilifi.

3.4.1.4 Piperaquine

PQ is an aminoquinoline drug similar to CQ, AQ and DEAQ. The activity of PQ was high in Kilifi isolates (median PQ IC_{50} 31nM, IQR 19-46nM) (see Figure 3-1).Similar PQ activity has been reported in other African sites [321, 348, 349].

Some reports from China [101-103] and more recently from Papua New Guinea [350] suggested that *in vivo* PQ efficacy may be reduced against a background of CQ resistance. These observations were also supported by a laboratory study of genetically modified *P.falciparum* isolates which showed that parasites carrying CQ resistant *pfcrt* haplotypes had decreased sensitivity to PQ *in vitro* [351]. My data clearly show that PQ retains susceptibility against parasites resistant to CQ and those with reduced sensitivity to AQ and DEAQ. (see

Table 3-3). Further, there was no association between *pfcrt* and *pfmdr1* mutations with PQ activity (see Figure 3-3, Figure 3-6, Figure 3-9 Figure 3-11 and Figure 3-11). These observations suggest that CQ resistance does not have a bearing on the activity of PQ. In support of these observations, the efficacy of DHA-PQ combination in Africa is reported to be high, despite the high background of CQ resistance [352-356].

The lack of association between the activity of PQ and other aminoquinolines may be attributable to stereo chemical differences between these drugs. Although structurally similar, PQ (Molecular weight, MW 536) is a larger molecule than AQ (MW 465) and CQ (MW 520). Therefore, owing to its larger size, PQ may use transporters other than *pfcrt* and *pfmdr1*. This hypothesis was supported by a laboratory experiment showing that *D. discoideum* transformants expressing mutant *pfcrt* could expel CQ but not PQ [357].

The high activity of PQ suggests that the combination of DHA-PQ is a promising alternative regimen to LM-ATM in the Kilifi setting. However it is notable that due to inadequate regulation, various PQ formulations are already available in the informal sector in Kenya [358]. This is a matter of concern as the increased and unsupervised use of these regimens may increase the rate of selection of resistance to the valuable DHA-PQ regimen. Therefore increased pharmaceutical regulation will be required to increase the therapeutic lifetime of this and other valuable antimalarials.

3.4.1.5 Dihydroartemisinin

Artemisinin compounds are the most potent antimalarials in clinical use [78]. The activity of DHA was very high in this study with DHA median IC_{50} of 2nM (IQR 2-3nM). This is in agreement with previous reports of high activity of artemisinins against African isolates [320, 325, 327, 349, 359, 360]. In spite of the existing strategies to delay artemisinin resistance, a study that evaluated artemether activity in >500 isolates from three countries with different artemisinin use (Cambodia, Senegal and French Guiana) identified parasites with decreased

susceptibility to artemether in French Guiana and Senegal (IC_{50}s of up to 117nM and 45nM in French Guiana and Senegal respectively) [88]. Recently, *in vivo* tolerance to artemisinins, characterized by prolonged parasite clearance times, was reported in Cambodia [74, 75, 361-364]. This development has raised serious concerns about the future of ACT.

I observed a significant positive correlation between DHA and LM activity (r=0.33, p=0.0005, see Table 3-3), in agreement with some previous studies [316, 321, 359]. It has been suggested that the positive correlation observed between artemisinins and LM activity *in vitro* may be attributable to *pfmdr1* mutations [316]. However, there was no association between *pfmdr1* polymorphisms and artemisinin activity in this study (see section 3.3.2). I propose that the lack of association in this study may be due to a limitation of sample size.

Although the mechanism of artemisinin resistance is still unresolved, it has been proposed that mutations in the *PfATP6* , the SERCA-type Ca^{2+} ATPase in *P. falciparum* may mediate artemisinin resistance [87, 88, 365, 366], although some studies could not confirm this hypothesis [165, 367]. Artemisinin tolerance has been attributed to a quiescence mechanism [74, 165]. Therefore, the precise mechanisms by which parasites become resistant to artemisinins are yet to be determined, but these mechanisms are most likely complex and multigenic.

The WHO recommends the use of artemisinins in combination with long half-life partner drugs to delay the development of resistance [89]. However, this strategy may be undermined by the availability of artemisinin monotherapies in Kenya [358]. Therefore better regulation in the pharmaceutical sector is required to delay the emergence of resistance. The positive correlation between DHA and LM activity (the active components of LM-ATM) is a matter of concern as it suggests that these drugs may have some shared mechanisms of resistance. The identification of isolates with decreased LM susceptibility coupled with the availability of

artemisinin monotherapy highlight the need for the continuous monitoring of LM-ATM efficacy in our setting, and the identification of potential alternative regimens.

3.4.2 Limitations

The majority of *P.falciparum* infections occurring in Kilifi are polyclonal. All the *P.falciparum* isolates analysed in this study were first adapted to *in vitro* culture. However the process of adaptation may alter the complexity of infections, with fewer genotypes appearing post-adaptation [368, 369]. In spite of this limitation, the process of adaptation offered a significant benefit as it allowed the cultivation of parasites isolates with low parasitaemias to thresholds required for *in vitro* assay testing, and the generation of sufficient amounts to allow cryopreservation for future assays.

Due to the challenges encountered during the laboratory handling and processing of clinical isolates, recovery rates were low and only a small subset could be analysed. Therefore, the inability to detect significant differences between groups for some drugs may be attributable to a limitation of statistical power. Statistical pooling of these data with those from similar studies may be one way to resolve this problem.

The cut-off-points previously cited for CQ and AQ resistance *in vitro* from other sites were not useful as predictors of resistance in Kilifi isolates (see section 3.4.1.1 and 3.4.1.3). These discrepancies may be attributable, at least in part to the variability of chemo sensitivity assays across different laboratories; many factors can influence the results of a chemo sensitivity assays including initial parasitaemia, hematocrit content, time of incubation, use of serum substitutes, oxygen tension, presence of mixed infections amongst others [137, 318, 370]. This highlights the need for standardisation and quality assurance of the *in vitro* tests across laboratories, as proposed by the World Wide Antimalrial Resistance Network (WWARN) [137].

The inability to predict CQ resistance based on the cut off points previously cited highlights the limitations of arbitrary cut off points in defining antimalarial resistance. The use of such arbitrary cut off points has previously been questioned [137]. Clearly, there is a need to link *in vitro*, molecular and *in vivo* data when setting thresholds of antimalarial resistance. In addition, my results demonstrate that for the drugs tested in Kilifi, their *in vitro* susceptibilities (IC_{50}s) represent a continous distribution rather than the strict bimodal distribution suggested by application of *in vitro* cut off points. Further, such cut off values often vary from setting to setting and unless continuously validated, they have no meaning in the clinical setting. Therefore, longitudinal studies of *in vitro* parasite susceptibilities within a population, and comparison across sites would be more useful in revealing trends in sensitivity over time, and in identifying any progressive declines in drug sensitivity. Identifying incipent resistance in this way would be cheaper than *in vivo* testing, and could provide the earliest possible warning signs of emerging resistance.

3.4.3 Conclusions

I have presented the baseline activity of CQ, PQ, LM, DEAQ and DHA against *P.falciparum* isolates taken between 2006 and 2008, the period when LM-ATM was introduced in Kilifi. I have also presented the baseline prevalence of *pfmdr1* and *pfcrt* mutations and the relationship between these polymorphisms and drug activity. These data will be important in the continuous monitoring of antimalarial resistance in Kilifi.

I observed a high overall activity of antimalarial drugs in Kilifi. However, I identified a number of isolates with reduced susceptibility to LM, highlighting the need for continuous monitoring of LM-ATM efficacy. The isolates with reduced susceptibility to LM will provide useful tools for further studies on LM resistance.

Parasites carrying wild type genotypes at *pfcrt-76* and *pfmdr1-86* were the least susceptible to LM, demonstrating an inverse relationship between CQ and LM activity. Therefore, the use

of LM-ATM may lead to the selection of CQ-sensitive parasites and *pfmdr1-86* and *pfcrt-76* will be useful molecular markers of LM-ATM efficacy in Kilifi. The return of CQ sensitive parasites is discussed in chapter 5.

The significant association between *pfmdr1-86* and *pfcrt-76* mutation and reduced DEAQ susceptibility showed that the high background of CQ resistance in Kilifi could hamper AQ effectiveness, in agreement with *in vivo* findings [295]. Therefore, AQ containing regimens are not viable treatment options for this setting.

There was no evidence of cross-resistance between CQ and PQ, suggesting that DHA-PQ is a potentially useful alternative regimen in Africa, despite the high background of CQ resistance. In addition, the decrease in LM activity was not associated with any corresponding decrease in PQ activity. Therefore, DHA-PQ is potentially a good alternative to LM-ATM.

CHAPTER FOUR

4 Is selective pressure for resistance to DHA-PQ, LM-ATM and AQ exerted by PQ, LM and DEAQ in vivo?

In this chapter, I provide an introduction highlighting the role of drug elimination half-life in the selection of antimalarial drug resistance. I present results of a study I conducted as part of my DPhil project to explore whether resistance to LM-ATM, DHA-PQ and AQ is exerted by the long half-life partner drugs LM, PQ and DEAQ respectively. I have outlined the methodology used to generate these data in sections 2.5 and 2.6.

4.1 Introduction

LM, PQ and DEAQ (the active metabolite of AQ *in vivo*) are eliminated slowly from the body, with a terminal half-life > 4 days for LM [97], 33 days for PQ [105] and 1-3 weeks for DEAQ [110]. On the other hand, artemisinin compounds are short acting, with elimination half-lives of less than 2 hours [371]. Therefore, LM, PQ and DEAQ persist in the body below effective concentrations following treatment with LM-ATM, DHA-PQ and AQ regimens.

Recent evidence suggests that drug tolerance is an important intermediate step in the evolution of antimalarial drug resistance [106] (see a detailed overview of drug tolerance in section 1.6). Therefore, the duration when LM, PQ and DEAQ persist below effective concentration is a critical period in the evolution of resistance as during this period, parasites are exposed to sub therapeutic drug levels, promoting the selection of drug tolerant parasites. Such selective pressure is not expected with the short acting artemisinin partner drugs.

In this study, I used the DHA-PQ, LM-ATM and AQ clinical studies to assess the selective pressure exerted by PQ, LM and DEAQ respectively. I hypothesized that parasites collected

up to 28 days after treatment with AQ and up to 84 days after treatment with DHA-PQ or LM-ATM would be less susceptible to DEAQ, PQ and LM while their level of susceptibility to DHA and AQ would not change.

Evidence from Africa shows that treatment with LM-ATM is associated with the selection of wild type *pfcrt* and *pfmdr1* genotypes [233-235, 328-330], whilst treatment with AQ regimens is associated with the selection of *pfcrt* and *pfmdr1* mutant genotypes [128, 235, 249, 341-343]. Reports from China [101-103] and more recently from Papua New guinea [350] suggested that cross-resistance may exist between CQ and PQ. Based on this evidence, I hypothesised that *pfmdr1* and *pfcrt* genotypes (which are associated with CQ resistance, see chapter 3) would vary significantly before and after treatment with LM-ATM, DHA-PQ and AQ.

4.2 Results

4.2.1 DHA-PQ treatment group

An overview of the DHA-PQ vs LM-ATM clinical trial is given in section 3.2.1.1. I assessed whether PQ exerted selective pressure for resistance to DHA-PQ by comparing the activity of PQ in isolates taken at day 0 (before administration of DHA-PQ) and those taken after treatment. I also analysed the activity of LM, DHA and CQ (as a comparator) against the same isolates (Table 4-1).

Table 4-1: Comparison of IC_{50} (nM) in isolates taken before and after treatment with DHA-PQ

Drug	Day 0 (N=16) Median IC_{50} (IQR)	After treatment (N=16) Median IC_{50} (IQR)	*P value* Mann-Whitney
CQ	39 (17-69)	48 (15-72)	0.87
PQ	22 (13-38)	31 (18-51)	0.09
LM	48 (29-101)	65 (35-106)	0.46
DHA	2 (2-3)	2 (2-3)	0.39

Following DHA-PQ treatment, median PQ IC_{50}s were slightly higher for isolates taken after treatment (31nM) compared to those taken at day 0 (22nM), although this was not statistically significant (p=0.09). Although there was no evidence that the use of DHA-PQ promoted the selection of isolates with decreased susceptibility to PQ in this study, further analysis with larger sample sizes is recommended to confirm this observation.

As expected, the activities of CQ, LM and DHA did not vary before and after treatment, showing that the use of DHA-PQ did not select for isolates with reduced LM, DHA and CQ susceptibilities.

4.2.1.1 Comparison of pfcrt and pfmdr1 genotypes before and after treatment

I analaysed the frequencies of *pfcrt-76*, *pfmdr1-86, pfmdr1-184* and *pfmdr1-1246* before and after treatment with DHA-PQ. The results show that frequencies of wild type and mutant genotypes did not vary significantly before and after treatment (P>0.05, Figure 4-1 and supplementary table S5 in appendix 4), an indication that the use of DHA-PQ did not select for isolates with decreased CQ susceptibility. This is in line with the data on PQ *in vitro* activity (section 4.2.1).

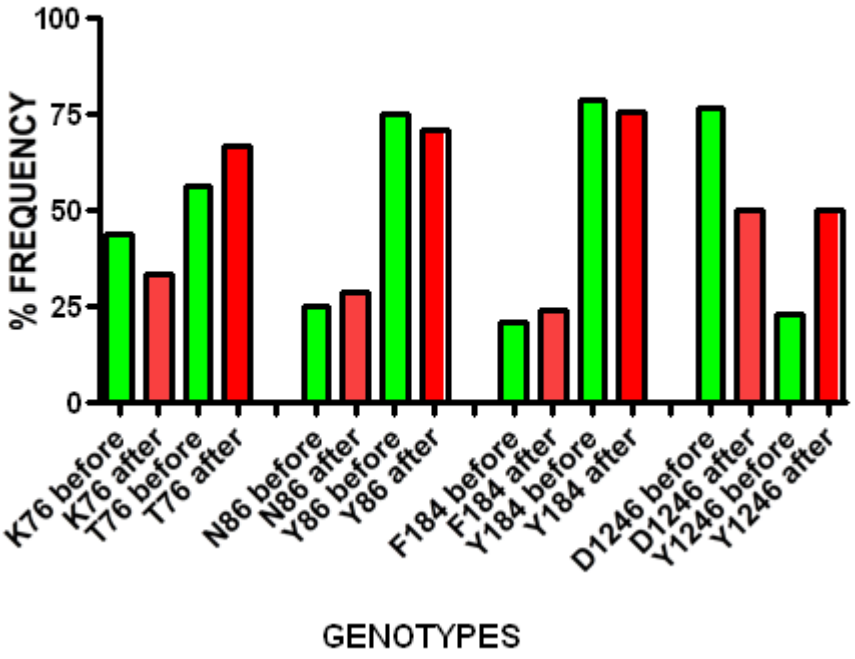


Figure 4-1: The frequency of *pfcrt* and *pfmdr1* genotypes before and after treatment with DHA-PQ in Kilifi. None of the observed variation was statistically significant.

4.2.2 LM-ATM treatment group

4.2.2.1 Comparison of drug activities in isolates taken before and after treatment

I also compared the activity of LM in isolates taken at day 0 (before treatment with LM-ATM) and those taken up to 84 days after treatment. For comparison, I analysed DHA, PQ and CQ activity in a similar manner (See Table 4-2).

Table 4-2 : Comparison of drug activities (nM) in isolates taken before and after treatment with LM-ATM. Significant changes are shown in bold.

Drug	Median IC_{50} (IQR) Day 0 (N=32)	Median IC_{50} (IQR) After treatment (N=32)	*P value* Mann-Whitney
CQ	**66 (43-105)**	**20 (13-51)**	**0.01**
PQ	38 (18-56)	31 (22-64)	0.76
LM	29 (29-95)	57 (48-94)	0.06
DHA	2 (1-3)	2 (1-3)	0.55

My observations suggested that the use of LM-ATM was associated with the selection of isolates with decreased susceptibility to LM. Indeed, median LM IC_{50}s were higher in isolates taken after treatment with LM-ATM (57nM) than at day 0 (29nM); these differences were of borderline statistical significance (p=0.06). This provides some evidence, though weak, that LM may exert the main selective pressure for resistance to LM-ATM *in vivo*.

In addition, my data show that the use of LM promoted the selection of isolates with increased CQ susceptibility. Indeed, median CQ IC_{50}s were significantly lower in isolates taken after treatment with LM-ATM (20nM) than at day 0 (66nM) (p=0.01). I did not observe any significant change in the activities of PQ and DHA after treatment with LM-ATM. These data support previous observation of the existence of an inverse relationship between the activity of LM and CQ, and that the use of LM-ATM may lead to the selection of CQ sensitive parasites (see chapter 3).

4.2.2.2 Comparison of pfcrt and pfmdr1 genotypes before and after treatment

I compared the frequencies of *pfmdr1* and *pfcrt* genotypes in parasites collected before and after treatment with LM-ATM, as reported for PQ-DHA (section 4.2.1.1.)

The frequency of *pfcrt-76T* mutant genotypes decreased significantly from 9/10(90%) to 6/13 (46%) after treatment with LM-ATM (p=0.03), while that of wild type *pfmdr1-86N* genotypes increased significantly from 1/11(9%) to 4/15(27%) (p=0.002) (See Figure 4-2 and supplementary table S5 in appendix 4).

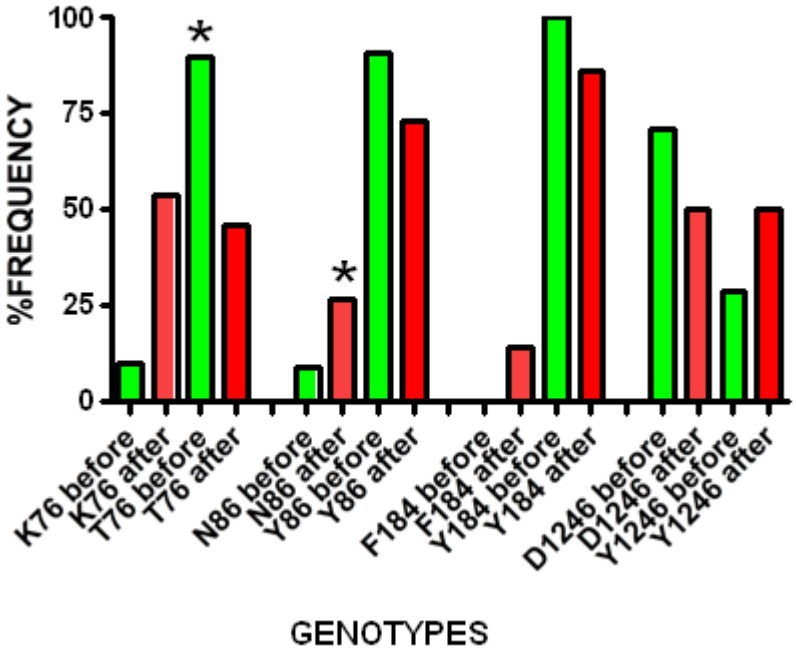


Figure 4-2: The frequency of *pfcrt* and *pfmdr1* genotypes before and after treatment with LM-ATM. The genotypes that were significantly different before and after treatment are indicated by an * mark.

Therefore, treatment with LM-ATM led to the selection of parasites carrying CQ sensitive genotypes, in agreement with the observations in section 4.2.2.1 and chapter 3. There was no significant difference in the frequencies of *pfmdr1-184* and *pfmdr1-1246* genotypes before and after treatment.

4.2.3 AQ treatment group

Similar to the analysis done in the DHA-PQ and LM-ATM treatment groups (sections 4.2.1 and 4.2.2 above), I also assessed the selective pressure associated with the use of AQ by analysing the activity of AQ and DEAQ in isolates collected before and after treatment.

4.2.3.1 Comparison of drug activities in isolates taken before and after treatment

I compared the activities of DEAQ, AQ and CQ (used as control) in isolates taken before treatment with AQ and within 28 days following treatment with AQ.

Table 4-3: Comparison of drug activities (nM) in isolates taken before and after treatments with AQ. Significant changes are shown in bold.

Drug	**Median IC_{50} (IQR)** **Day 0 (N=68)**	**Median IC_{50} (IQR)** **After treatment (N=19)**	***P value*** **Mann-Whitney**
CQ	**32 (17-68)**	**52 (31-108)**	**0.02**
AQ	17 (11-24)	15 (9-21)	0.19
DEAQ	28 (21-57)	30(20-46)	0.61

As shown in Table 4-3, the difference in AQ and DEAQ IC_{50} before and after treatment with AQ was not significant, suggesting that the use of AQ is not associated with a selective pressure for AQ and DEAQ resistance.

However, isolates taken after AQ treatment were less susceptible to CQ (median CQ IC_{50} 52nM) than those taken at day 0 (median CQ IC_{50} 32nM, p=0.02), indicating that treatment with AQ led to the selection of parasites with decreased CQ susceptibility.

4.2.3.2 Comparison of pfcrt and pfmdr1 genotypes before and after treatment

The genotyping of *pfcrt* showed hat the use of AQ led to a significant increase in mutant *pfcrt-76T alleles*, from 20/31 (65%) to 18/18 (100%). This increase in mutants, as expected was associated with the dissapearance of wild type alleles from 11/31(35 %) to 0/18(0%) (See Figure 4-3 and supplementary table S5 in appendix 4). These data are in line with *in vitro* observations (section 4.2.3.1) showing that AQ exerts a strong selective pressure for CQ resistance.

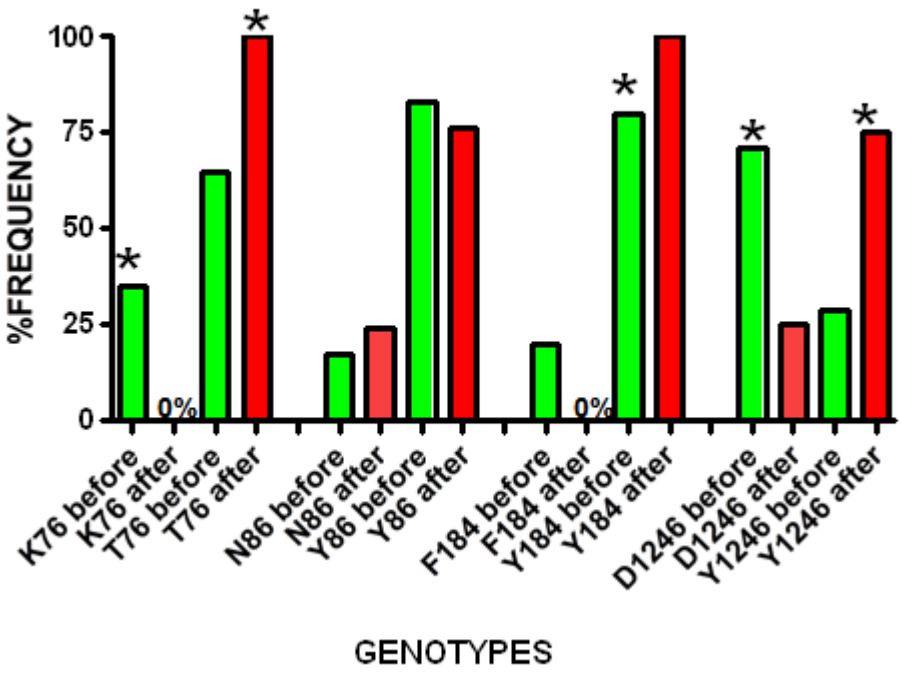


Figure 4-3: The frequency of *pfcrt* and *pfmdr1* genotypes before and after treatment with AQ.The genotypes which were significantly different before and after treatment are indicated by an * mark.

The frequency of mutant *pfmdr1-184Y* genotypes also increased from 25/30 (83%) to 6/6 (100%) after treatment with AQ (P=0.02). This was accompanied by a significant decrease in wild type *pfmdr1-1246D* genotypes from 22/32 (69%) to 2/8 (25%) (p=0.02) and a significant increase in *pfmdr1-1246Y* mutants from 10/32(31%) to 6/8(75%), p=0.02. Therefore,

treatment with AQ selected for *pfcrt-76*T, *pfmdr1-84Y* and *pfmdr1-1246Y* mutant genotypes but did not have a significant effect on *pfmdr1-86* genotypes.

4.3 Discussion

4.3.1 LM-ATM treatment arm

LM-ATM is the current first line treatment for uncomplicated malaria in Kenya. In this study, isolates taken after LM-ATM treatment were less susceptible to LM than those taken at day 0 (see Table 4-2 and section 4.2.2), an indication that LM may exert selective pressure for resistance to LM-ATM. Interestingly, there was an inverse relationship between LM and CQ. LM-ATM selected for isolates that were less susceptible to LM, and these parasites were more susceptible to CQ. Further, there was a significant selection of CQ sensitive *pfmdr1-86N* genotypes and a corresponding decrease in CQ resistant *pfcrt-76T* genotypes (see section 4.2.2.2). These findings are in agreement with previous reports from *in vivo* studies in Tanzania [167, 234, 328, 372], Burkina Faso [329, 330], Mali [373] and Uganda [233] showing the selection of wild type (CQ sensitive) *pfcrt and pfmdr1* genotypes after treatment with LM-ATM. Therefore, the use of LM-ATM may lead to the selection of CQ susceptible parasites in Africa. I have discussed the return of CQ susceptible parasites and its possible implications in chapter 5.

Of interest, selection of CQ sensitive *pfmdr1* and *pfcrt* alleles after treatment with LM-ATM has not been reported outside Africa. In South East Asia where LM-ATM has been used widely, LM selective pressure *in vivo* has been associated with *pfmdr1* gene amplification rather than mutation [166, 246]. As I discussed in chapter 3 this phenomenon arose following the the long-term use of MFQ in Asia. The prevalence of *pfmdr1* amplification was low (1%) in Kilifi (see chapter 3, section 3.3.4). It will be important to monitor the frequencies of *pfmdr1 and pfcrt* mutations and amplified *pfmdr1* with the continued use of LM-ATM in Kilifi

4.3.2 DHA-PQ treatment group

DHA-PQ has been undergoing clinical evaluation in Africa, and evidence indicates that it is a suitable alternative to LM-ATM, the current first line drug in Kenya [374]. Therefore, it is important to understand the mechanisms of resistance to this valuable antimalarial drug.

In this study, there was no evidence that the long acting PQ exerted selective pressure for DHA-PQ resistance *in vivo* based on the comparison of PQ activity at baseline and up to 84 days after treatment (section 4.2.1). Further, there was no significant selection of *pfcrt* and *pfmdr1* genotypes after treatment with DHA-PQ, in agreement with previous reports [375]. PQ activity was high in all Kilifi isolates (median IC_{50}s at baseline and after treatment <50nM).

The observation that *pfcrt* and *pfmdr1* genotypes do not have significant impact on PQ activity suggests that although structurally related, PQ and CQ may have different mechanisms of resistance. In agreement with this, *pfmdr1* and *pfcrt* genotypes did not have a significant impact on PQ activity, and PQ and CQ activities were not correlated *in vitro* (see chapter 3). These results support previous findings in Africa showing that DHA-PQ is highly effective when compared with other drugs (including LM-ATM, AS-MFQ, AQ-SP and AS-AQ) [352-356]. However, this contrasts with initial evidence from China [101-103] and more recently from Papua New Guinea [350], suggesting that PQ activity may be reduced in areas with a high background of CQ resistance. The higher activities of PQ reported in Africa may be attributable to the lower levels of CQ resistance in Africa compared to Asia.

Interestingly, although I identified some isolates with decreased LM susceptibility in Kilifi (Figure 3-1), the activity of PQ was not correspondingly lower in these isolates. This suggests that the continued use of LM-ATM would not compromise the efficacy of PQ.

The high activity of PQ despite a high background of CQ resistance in Africa and the lack of evidence for selective pressure suggest that DHA-PQ is a promising alternative treatment regimen in this setting. However, the availability of PQ formulations in the informal sector in Kenya [358] is a matter of concern, since this could lead to a quicker selection of resistance and a shortening of the drug's useful therapeutic lifetime. Therefore, it will be important to improve the regulatory environment in the region in order to protect this and other valuable antimalarial regimens.

4.3.3 AQ treatment arm

My findings indicate that the use of AQ is not associated with a pressure for resistance to DEAQ after AQ *in vivo* (see section 4.2.3). However, recurrent isolates were significantly less susceptible to CQ than day 0 isolates, suggesting that treatment with AQ led to the selection of CQ resistant parasites. Further, the use of AQ led to a significant increase in the frequency of mutant *pfcrt-76T, pfmdr1-184Y* and *pfmdr1-1246Y* genotypes (see Figure 4-3). These findings support previous observations of the selection of CQ resistant genotypes after treatment with AQ containing regimens in Tanzania [235], Kenya [128, 343, 376], Sudan [343], Nigeria [377], Burkina Faso [341] and Mali [373]. Of interest, I did not observe significant selection of *pfmdr-186* genotypes after treatment with AQ ($p>0.5$) although this has been linked with AQ treatment failure in some of the previous studies [341, 373, 376, 377].

The observed cross-resistance between CQ and AQ may hamper AQ effectiveness in Kilifi. This is in agreement with previous observations of decreased *in vivo* efficacy of AQ in African settings where CQ resistance is high, such as Kenya [295, 376], Rwanda [116], Tanzania [118] and Gabon [119], though a few reports indicted good efficacy of AQ in areas where CQ resistance is high [108, 109]. Therefore, AQ containing drugs are not suitable treatment alternatives in areas with a high background of CQ resistance.

4.3.4 Limitations

Based on my analysis of mutations at *pfmdr1-86* and *pfcrt-76*, the prevalence of mixed genotypes in Kilifi was 10-15%. In my final analysis, I excluded these mixed *pfmdr1* and *pfcrt* alleles when evaluating the selection of molecular markers after drug treatment. Due to their low prevalence in this study, the exclusion of these genotypes did not significantly alter my results. However, mixed infections may indeed influence the selection of drug resistance at a population level (see 1.6.2.2). Estimating the number of distinct clones carried by each individual contributing to this analysis would complement these data, by providing a better estimate of the relative contribution of mutant and wild type alleles within mixed infections. In addition, complementary studies to estimate the drug levels post treatment for each individual would have helped to better understand the association between declining drug levels and the risk of infection with less susceptible parasites.

Due to technical challenges encountered during the processing of clinical isolates in the laboratory, only a small subset of clinical isolates were successfully adapted *in vitro*. Therefore, this resulted in small sample sizes within groups. Clearly, inadequate statistical power may have limited the ability of my study to detect some significant and potentially important effects. Nevertheless, my analysis gives important insights into the selection of drug resistant parasites following treatment with LM-ATM, DHA-PQ and AQ. Bigger studies are however needed to confirm my observations.

4.4 Conclusions

I have shown that treatment with LM-ATM promotes the selection of parasites that are less susceptible to LM but more susceptible to CQ *in vitro*. Therefore, this work provides some evidence that LM may exert selective pressure for resistance to LM-ATM *in vivo*, and that it may lead to the restoration of CQ susceptibility in Kilifi.

I have shown that treatment with AQ promotes the selection of CQ resistant parasites, Therefore, AQ regimens may not be suitable alternatives for malaria treatment in Kilifi and other sites with a high background of CQ resistance.

I did not observe any evidence that PQ exerts selective pressure for resistance to DHA-PQ *in vivo* in this study and the selection of isolates with decreased LM susceptibility was not associated with decreased PQ susceptibility, suggesting that the use of LM_ATM may not have an impact on the efficacy of PQ. Therefore, DHA-PQ may be a promising alternative treatment for uncomplicated malaria in Kilifi.

CHAPTER FIVE

5 Chloroquine resistance before and after its withdrawal in Kenya

5.1 Introduction

In this chapter, I provide an introduction highlighting the importance of monitoring the impact of withdrawing failing antimalarial drugs in endemic settings. I have assessed the prevalence of CQ resistance in Kenya from 1993 (5 years before its withdrawal) to 2006 (6 years after its withdrawal), and compared my data with that reported in a similar study in Malawi. The data presented in this chapter have already been published [307] (see copy of published paper in appendix 6). I have outlined the methods used in this analysis in section 2.7.

5.1.1 The effects of withdrawing CQ from clinical use

It has been suggested that mutations that confer drug resistance in an organism come with a fitness cost, and that removal of drug pressure may lead to the return of drug susceptible parasites (see detailed overview in section 1.10). In the field of malaria chemotherapy, this has been supported by observations in Malawi whereby the change in antimalarial drug policy from CQ to SP in 1993 led to the return of CQ efficacy both *in vitro* [270, 271, 273] and *in vivo* [272]. However, although this phenomenon has been reported in a few other endemic sites [275-278], the changes observed have been less dramatic than those of Malawi. This suggests that additional factors may significantly influence the effects of withdrawing antimalarial drugs from endemic areas. The return of CQ efficacy in Malawi has raised the interesting hypothesis that it may be possible to reintroduce antimalarial drugs that had

previously failed. Therefore, monitoring the effects of withdrawing antimalarial drugs in endemic settings is an important component of antimalarial drug resistance studies.

In Kenya, the official change of policy from CQ to SP was implemented in 1998 [284] (see chapter two section 2.2.1 for a detailed overview of drug use in Kilifi). In this chapter, I present the trends in CQ resistance based on the analyses of the prevalence of CQ resistant genotypes (*pfcrt-76* and *pfmdr1-86*) in *P.falciparum* isolates collected in Kilifi from 1993, prior to the withdrawal of CQ to 2006, 6 years after the withdrawal of this drug. As a control, I have also analyzed the changes that have occurred in *dhfr* (the main gene associated with resistance to SP) over the same period, which was the time SP was used in Kenya. I compared these trends with those observed in Malawi. I discuss how the use of PQ, AQ and LM may influence the drug resistance trends observed in Kilifi.

5.2 Results

In Kilifi, samples were collected during clinical trials of SP, CPG-DAP, SP-AS, AQ, LM-ATM or DHA-PQ [93, 295, 296, 304-306], and were taken either before or after treatment : 406, 240 and 323 isolates collected between 1993 and 2006 were genotyped at *pfcrt-76*, *pfmdr1-86* and *dhfr* respectively. Of these, 41, 28 and 58 carried mixed alleles and were not used in the analysis. Data on *pfcrt* genotypes from patients that had been treated with AQ (samples from 2006) were also excluded because post-treatment samples harboured a significantly higher frequency of the *pfcrt-76* mutant (19/19) than pre-treatment samples, (19/31. P=0.001) (see also chapter 4). To analyse the prevalence of genotypes over time, I randomly selected 25-30 samples per year.

Comparative data from Malawi was obtained from samples obtained from children with uncomplicated malaria as previously published [243]. In this Malawian study, 206, 265 and

153 parasites, collected between 1992 and 2000, were genotyped at *pfcrt-76*, *pfmdr1-86* and *dhfr* respectively. [270].

5.2.1 pfcrt-76

I compared the prevalence of the *pfcrt-76T* mutation (the main marker of CQ resistance) over time in Kilifi and Malawi .In Kilifi, the frequency of the mutant decreased significantly from around 95% to 63% over a period of 13 years (see Figure 5-1).

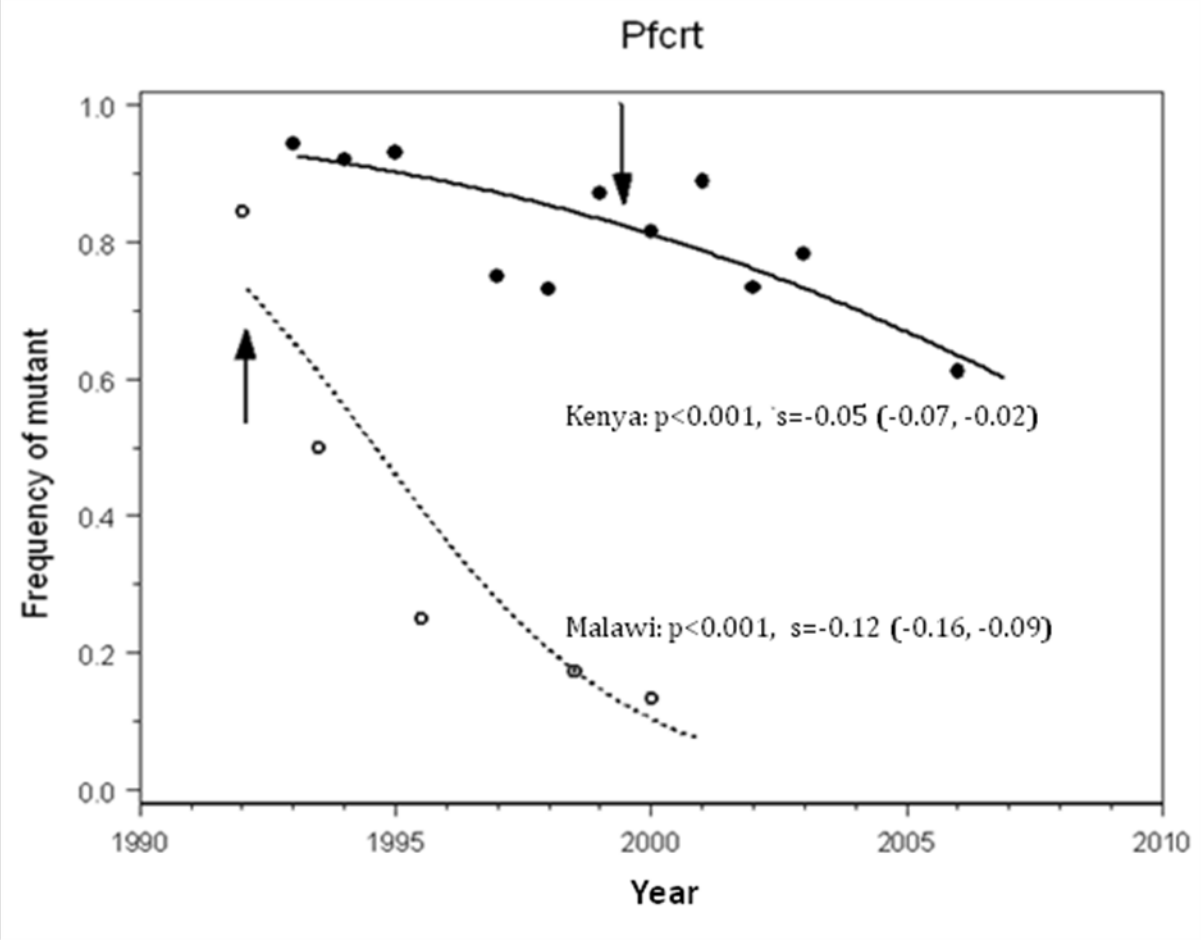


Figure 5-1 : Frequencies of *pfcrt-76* mutant alleles over time in Kenya and Malawi. Kenya (solid line, closed symbols) and Malawi (dashed line, open symbols). The arrow mark indicates the year in which CQ was officially withdrawn.The *P*-value indicates whether the slope of the predicted line is significantly different from zero. The selection coefficient (*s*) of the resistant allele relative to the wild-type allele is shown for each country with its 95% confidence intervals. Adapted from Ref. [307].

Interestingly, the decline in *pfcrt-76T* mutants was occurring before the official withdrawal of CQ in 1998. There was no detectable difference in the rate of decline before and after this

time (P>0.9 by test for an interaction between the slope and time period in the regression model). The estimated fitness of this resistant mutant was 5% lower than that of the wild type (s=–0.05).

In Malawi, CQ resistance declined from 85% to 13% in 9 years after CQ withdrawal. The estimated fitness of resistant mutants in the Malawi study was 12% lower than that of the wild type (s=–0.12). The rate of decline in Malawi was significantly higher than that of Kilifi (P<0.001 by Student's t-test of the regression slopes).

5.2.2 pfmdr1-86

I also compared the prevalence of the *pfmdr1-86* mutation (another gene associated with CQ resistance) in Kenya and Malawi. *pfmdr1-86 mutants* did not decline significantly in the Kilifi population (see Figure 5-2). In Malawi on the other hand, the frequency of the *pfmdr-86* mutants decreased slightly (P = 0.05) with an estimated selection coefficient of –3%.

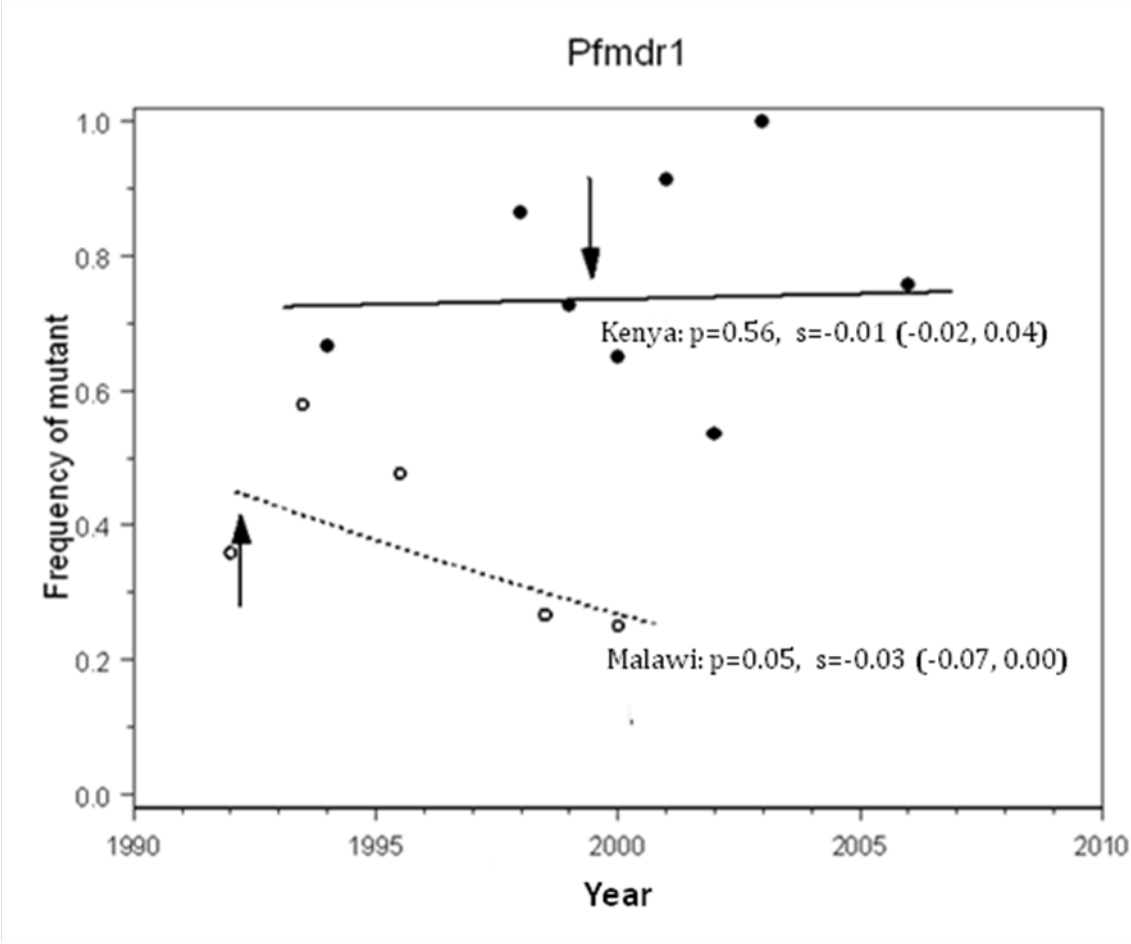


Figure 5-2: Frequencies of *pfmdr1-86* mutant alleles over time in Kenya and Malawi. Kenya (solid line, closed symbols) and Malawi (dashed line, open symbols).The arrow marks indicate the time that CQ was officially withdrawn. The *P*-value indicates whether the slope of the predicted line is significantly different from zero. The selection coefficient (*s*) of the resistant allele relative to the wild-type allele is shown for each country with its 95% confidence intervals. Adapted from Ref.[307].

5.2.3 dhfr

As a control, I analysed the prevalence of *dhfr* mutations (the main molecular markers of SP resistance) in Kenya and Malawi after CQ withdrawal (corresponding to the time SP was in use). In Kilifi, no isolate carried the *dhfr-164* mutation. Relative to the wild type allele, the selection coefficients of the *dhfr51-108* double mutant, *dhfr59-108* double mutant and *dhfr51-59-108* triple mutant were 6%, 7% and 7% respectively (Figure 5-3).

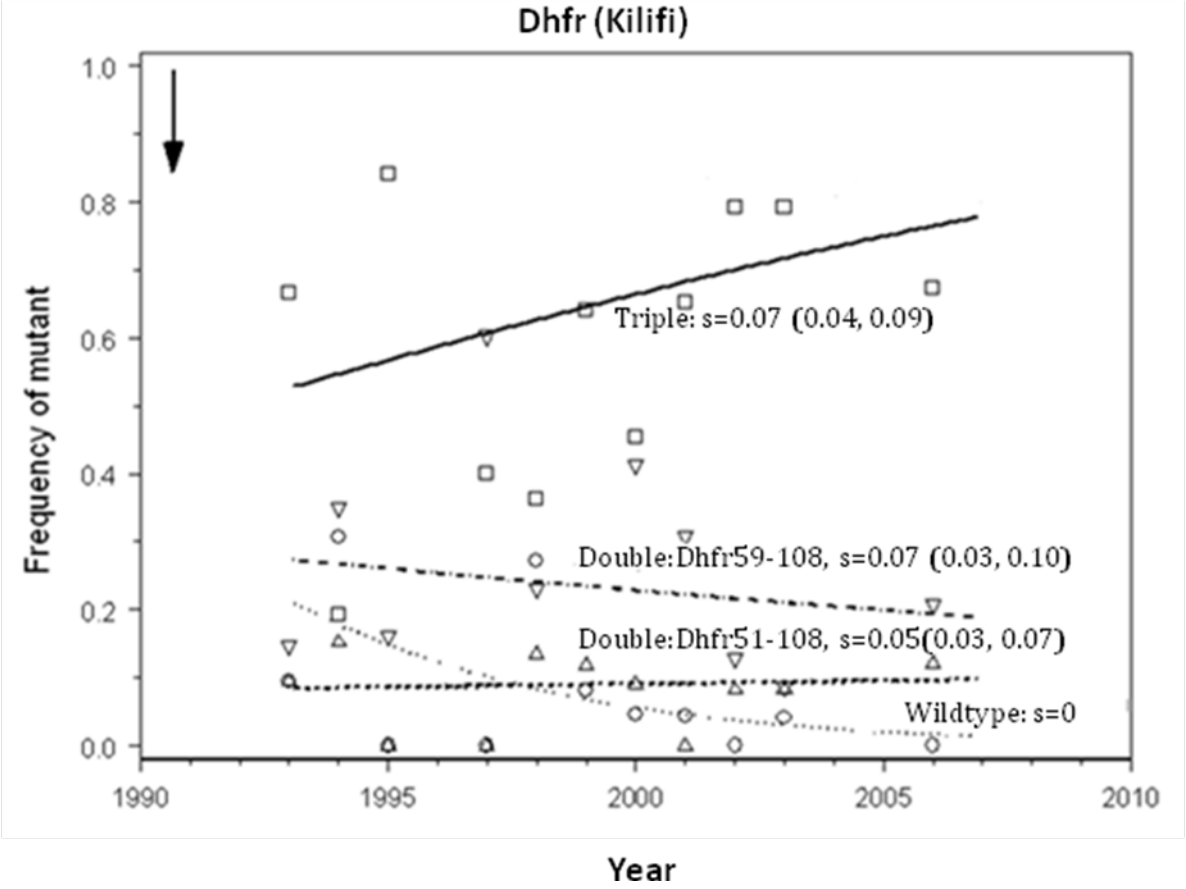


Figure 5-3: Frequencies of *dhfr* alleles over time in Kenya. The arrow mark indicates the time that SP officially replaced CQ as the first line drug. The P-value indicates whether the slope of the predicted line is significantly different from zero. The selection coefficient (s) of the resistant allele relative to the wild-type allele is shown with its 95% confidence intervals. Adapted from Ref.[307].

Interestingly, the frequencies of the double mutants stayed approximately the same through time, possibly because as they were gaining ground to the wild type and single mutants, they were losing ground to the triple mutant. Since the selection coefficients of double and triple mutants were comparable, I used the double and triple mutants combined to estimate the selection coefficient (s).

Notably, the frequency of the *dhfr* double and triple mutants combined was already high (around 80%) in Kilifi 5 years before SP was introduced as the first line treatment for uncomplicated malaria (see Figure 5-4). Thereafter, the frequency of the double and triple mutants alleles increased significantly ($P<0.001$) to above 95% with the continued use of SP.

Relative to the wild type and single mutants, the triple and double mutants combined had an estimated selection coefficient of 7%.

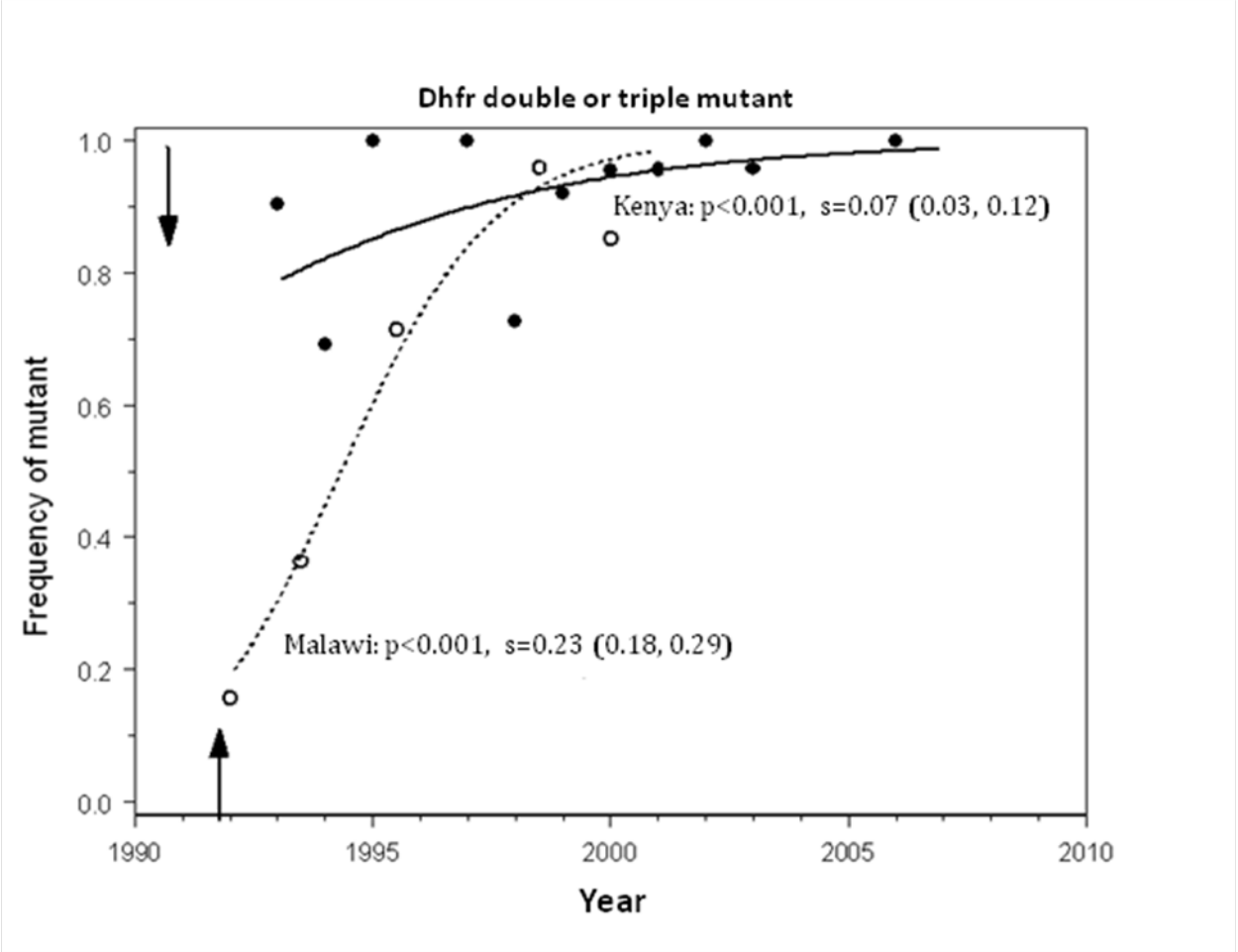


Figure 5-4: Frequencies of *dhfr* mutant alleles (double and triple mutants combined) over time in Kenya and Malawi.Kenya (solid line, closed symbols) and Malawi (dashed line, open symbols).The arrow marks indicate the time that SP officially replaced CQ as the first line drug. The *P*-value indicates whether the slope of the predicted line is significantly different from zero. The selection coefficient (*s*) of the resistant allele relative to the wild-type allele is shown for each country with its 95% confidence intervals. Adapted from Ref. [307].

In contrast, the rise in frequency of *dhfr* mutants was faster in Malawi (from 15% to above 80% within 9 years) with a selection coefficient of 23% for the double and triple mutants combined. This rate was significantly higher than that observed for Kilifi ($P<0.001$ by Student's *t*-test of the regression slopes) (see Figure 5-4).

5.3 Discussion

5.3.1 The effects of the CQ to SP policy change on CQ and SP resistance in Kenya and Malawi.

CQ resistance declined steadily in Kilifi between the early 1990s to 2006 as evidenced by the declining prevalence of *pfcrt-76T* mutants (95% to 63% over a 13 year period, section 5.2.1 and Figure 5-1). CQ was officially withdrawn in Kenya in 1998 [284], the withdrawal becoming effective the following year in health facilities. Between 1998 and 2001 in Kilifi, there was an educational programme training rural drug retailers on the appropriate use of antimalarials. A survey carried out after this training programme reported that the proportion of drug users purchasing adequate doses of CQ from drug retailers rose from 8% to 33% between 1998 and 1999. Between 2000 and 2001, with the introduction of SP, the proportion of drug users receiving an adequate dose of the new first-line drug, SP rose to 64% [293] and this represented 90% of all OTC antimalarial drug sold [293]. Thus, this evidence suggests that owing to the success of the training programme and the change in drug policy, CQ was largely discontinued in Kilifi, and replaced by SP. This discontinuation presumably underlies the steady decline of CQ resistance that I observed in Kilifi.

However, the rate of decline of *pfcrt-76* mutants in Kilifi was much lower than that of Malawi (see section 5.2.1). As drug-resistant isolates in Africa are derived from a common origin in South-East Asia [151, 378-380], it is unlikely that genetic differences between Kenyan and Malawian parasites can account for the differences observed. It is likely that the observed differences in the rates of decline of CQ resistance in Kilifi and Malawi may be attributable to higher efficiency in withdrawing CQ from circulation in Malawi as compared to Kilifi. This is supported by observations that CQ continued to be available at the community level as a drug for self-medication in Kenya, up to 4 years after its official withdrawal [285]. This informal use of CQ may have contributed to a substantial level of CQ pressure, explaining why the rate

of decline of mutants in Kilifi was less dramatic than in Malawi. Alternatively, this may be attributable to different usage rates of AQ (structurally related to CQ) in the Kilifi and Malawi settings. In Kilifi, this drug was used as secondline treatment to SP. Data from this study (chapter 3) and other studies [128, 235, 249, 341-343] have demonstrated that the use of AQ or AQ combination therapy can select for CQ resistant genotypes. These observations demonstrate that prevailing drug use patterns and the efficiency which drug policy is implemented can significantly influence the outcome of withdrawing an antimalarial drug from use.

In Malawi, a significant decrease in the frequency of *Pfmdr1* mutants after CQ withdrawal was observed. However, this was not the case in Kilifi (see section 5.2.2 and Figure 5-2). This difference in the rates of decline of *pfmdr1* mutants may be attributable to the faster decline in CQ resistance that occured in Malawi.

The frequency of *dhfr* combined double and triple mutants was already high (about 80%) in Kilifi by 1993 (5 years before the official introduction of SP) (see section 5.2.3. and Figure 5-3). This may be due to the fact that although SP was not the official first line treatment, SP may have been used for treatment of malaria due to widespread CQ failure. This view is supported by reports that significant levels of CQ resistance had already been recorded in the early 90s in Kenya [284] (also see Figure 5-1). Therefore, the subsequent high usage of SP well before it became official policy may explain why the decline in *pfcrt* mutants was occurring even before the official withdrawal of CQ

5.3.2 How will the use of LM, AQ or PQ affect the trends observed in Kilifi.

Based on the rates observed, it can be predicted that, if drug pressure remained unchanged, the return to CQ susceptibility in Kilifi isolates (i.e. less than 10% frequency of the *pfcrt-76T* mutants) will be achieved in 2018, almost 20 years after the official removal of the drug in Kenya. The rate of *pfmdr1-86* mutants may decline significantly too as the rates of *pfcrt-76*

decline further, as was observed in Malawi. As AQ resistance is mediated by *pfcrt* and *pfmdr1* mutations (see chapter 3), the widespread use of AQ would promote the selection of *pfcrt-76* and *pfmdr1-86* mutants, thus slowing down the predicted decline in their prevalence. PQ resistance does not appear to be mediated by *pfcrt* or *pfmdr1* mutations (see chapter 3); therefore, the use of PQ containing regimens is not expected to affect the observed rates in the decline of *pfcrt-76* and *pfmdr1-86* mutants.

Although SP has officially been withdrawn from use as first line antimalarial, this drug is recommended for use in intermittent preventive treatment of malaria [381]. The antifolate antibiotic co-trimoxazole (sulphamethoxazole-trimethoprim combination, SFZ-TM, structurally related to SP) is recommended for chemoprophylaxis in HIV positive patients [382]. Although it is thought that mutations in *dhfr* are mainly driven by the use of SP, it has been shown that cross-resistance exists between SFZ-TM and SP, and that *dhfr* mutations are associated with decreased SFZ-TM activity [383-385]. Therefore, the prevalence of *dhfr* mutations would be expected to remain high.

Of interest, data from this project (see chapter 3) and from other studies [233, 234, 328-330] show that the use of LM-ATM (which is the current official first-line treatment) selects for *pfcrt* and *pfmdr1* wild type genotypes. These observations suggest that with the continued use of LM-ATM in Kilifi, the return of these wild type genotypes (CQ susceptible parasites) in Kilifi may be faster than predicted. These wild type genotypes would be associated with increased resistance to AL but increased sensitivity to CQ and AQ. If this is confirmed, CQ could potentially be re-introduced after AL fails in Kilifi. For instance, the return of CQ susceptible parasites in Malawi (12 years after its withdrawal) led to a clinical evaluation of CQ [272] ;the findings of this *in vivo* study clearly showed that CQ had regained clinical efficacy in Malawi, supporting the concept of reintroducing CQ.

However, there are concerns that CQ resistance may re-emerge relatively quickly, as the mechanism of CQ resistance is likely to be multigenic (with *pfcrt* being the most important gene) (150, 363). Therefore, the return to wild type *pfcrt* may not necessarily indicate that all genes that are involved in resistance have returned to wild type status. Consequently, the re-introduction of CQ could quickly select for resistance.

One way of overcoming this shortfall would be to use CQ in combination therapy. This approach is being currently evaluated in Malawi with CQ in combination with artesunate, azithromycin or atovaquone-proguanil (ClinicalTrials.gov NCT0037982). Results of this investigation will provide interesting information on the potential of re-introducing CQ in combination with other antimalarials.

It is of interest that in Guinea Bissau, CQ is used at relatively higher (and reportedly safe) doses of 76mg/kg compared to 25mg/kg used in other settings, and the prevalence of *in vivo* and *in vitro* CQ resistance in Guinea Bissau is reported to be low despite the continued use of CQ [386-390]. Therefore, it has been suggested that using CQ at higher therapeutic doses may be safe, and can delay the development of CQ resistance. In support of this, pharmacodynamic modelling studies predicted that higher doses of CQ can indeed eliminate CQ resistant malaria parasites [391]. The observations in Guinea Bissau suggest that increasing the doses of CQ previously used may be considered as a strategy to delay the spread of CQ resistance if CQ is re-introduced. However, it will be important to systematically evaluate the safety and tolerability of such higher doses in other endemic settings, and the feasibility of using such higher doses in combination therapy.

In summary, the observations in Kilifi and Malawi have provided renewed hopes of the potential to re-use cheap antimalarial drugs that have previously failed, such as CQ. However, several factors that may limit the applicability of re-introcuing CQ must be carefully evaluated.

5.3.3 Limitations

I chose a traditional population genetics approach to model changes in allele frequency over the alternative approach of estimating the prevalence of resistance within an infected population that was applied elsewhere [160, 270]. My choice of approach was based on the consideration that measuring prevalence may have provided an overestimate of allele frequencies in the population, bearing in mind that 10-15 % of samples were composed of mixed infections. A precise estimate of the relative contribution of resistant and sensitive genotypes within this group of samples would have necessitated an additional analysis of the number of clones per infection for each individual contributing to the analysis. I chose to eliminate mixed infections from the analysis as when mixed infections were eliminated, the remaining data provided sufficient power to detect time trends. However, despite the fact that mixed genotypes were eliminated, the results of this analysis still provide a precise estimate of the trends in drug resistant allele frequencies over time in Kilifi.

5.4 Conclusion

These results show that CQ resistance has been declining steadily in Kilifi since the early 90s, the time that this drug was still the first line of the treatment of malaria, up to 2006, several yeas after its withdrawal. However, this decline was much slower than that reported in Malawi. It is likely that the different rates of disappearance of drug resistant parasites in Kilifi and Malawi can be attributed to the differences in application of drug policy between the two settings. Data from this study and previous studies (section 3.4.1.2 and 4.2.2) showed that the use of LM-ATM is likely to select for CQ susceptible parasite at a quicker pace, raising the hope that CQ could be re-introduced when LM-ATM fails.

CHAPTER SIX

6 The role of genes other than *pfcrt* and *pfmdr1* in mediating the response of *P.falciparum* to Lumefantrine

6.1 Introduction

In this chapter, I provide an introduction highlighting the important role of *in vitro* selection of drug resistance and whole genome approaches in understanding antimalarial drug resistance. I present results on the selection of LM resistance *in vitro* and the use of microarray and qPCR techniques to identify genes that may mediate altered response to LM. I co-authored a published review on the selection of *P.falciparum* drug resistance *in vitro*; a copy of this review is provided in appendix 6. The methods used in the generation of this data are outlined in section 2.8.

6.1.1 The Need to understand LM resistance in P.falciparum

The combination of LM-ATM is the first line treatment for malaria in many African countries including Kenya. LM belongs to the quinoline-methanol (or aryl-amino alcohol) group of antimalarials and is structurally similar to MFQ, QN and HLF (see section 1.5.2.2 for a detailed overview of LM). Although ACT was hoped to reduce the pace of selection of drug-resistant parasites, there is growing concern that in the case LM-ATM, resistance may occur quickly with LM providing the main selective pressure for resistance, due to its extended elimination half-life when compared to that of the fast-acting ART[100].

Therefore, studies clarifying the mechanisms by which parasites acquire resistance to LM can aid in the development of strategies to prevent or track resistance. As part of my Dphil

studies, I investigated the mechanisms by which parasites acquire LM resistance.To this end, I used LM resistant parasites selected *in vitro* by drug pressure.

6.1.2 In vitro selection of antimalarial resistance

In vitro selection of resistance is a potentially useful technique for studying the mechanisms of antimalarial drug resistance (see sections 1.6 and 1.9.2 for a detailed overview of selection of drug resistance). In particular, this method can be used to generate drug resistant phenotypes, which can be used to study the mechanisms of resistance before clinical resistance emerges.

However, the utility of *in vitro* selection has been limited by the difficulty in obtaining stable resistant phenotypes. The majority of *P. falciparum in vitro* selection studies suggest that the selection of drug-resistant parasites occurs in two stages: first the selection of an unstable, drug-tolerant population, then the development of a stable phenotype. The initial phase may arise relatively quickly, and is associated with gene amplification/deletion, or the differential expression of various genes [256]. The time required for establishment of stable resistance is dependent upon several factors, and is highly variable (see introduction section 1.6.2 for a detailed overview of acquisition of antimalarial drug resistance).

Notably, evidence suggests that the efficiency of selection of *in vitro* resistance may be enhanced in various ways [260]. For example, parasites are more prone to generate resistant lines if they are exposed to a drug for longer periods and if higher initial parasitaemias are used during *in vitro* culture under drug pressure. Further, parasites with multi-drug resistant backgrounds have been shown to acquire resistance at 10-1000 fold higher frequencies than sensitive strains, a phenomenon refereed to as accelerated resistance to multidrug (ARMD) [260]. Therefore, to initiate the selection of LM resistance *in vitro*, I cultured the multidrug resistant reference strain V1S under LM pressure for 16 months.

6.1.3 Whole genome approaches to study antimalarial drug responses

Studies in yeast and bacteria have demonstrated that whole genome approaches such as gene expression profiling can provide valuable insight into the key mechanisms underlying drug action and resistance, by allowing the identification of potentially important but unrecognized genes [392-394]. Interestingly, this whole genome approach was recently used to identify a novel amino-alcohol resistance locus PF10_0355, whose role in mediating drug resistance has subsequently been confirmed by functional testing [395].

Gene expression profiling in antimalarial drug resistance studies has not been fully exploited to date, mainly because the changes observed in mRNA levels after exposure of *P.falciparum* to drugs are often of low amplitude [179, 180]. However recent studies have shown that despite their low amplitudes, these changes are dose dependent, highly reproducible and involve functionally related genes, demonstrating that they could provide biologically meaningful information [261]. Therefore, the analysis of *P.falciparum* gene expression in response to antimalarial treatment could provide useful insight in the mechanisms of drug resistance

6.1.4 The role of transporters in LM resistance

Importantly, studies in various pathogens, including *P.falciparum* have shown that transporters are important mediators of drug resistance. For example, the two main genes involved in resistance to quinoline antimalarials in *P.falciparum* (*pfcrt and pfmdr1*) encode transporters (see introduction section 1.7 for a detailed overview of transporters).

The analysis of *pfcrt* and *pmdr1* polymorphisms in Kilifi isolates showed that parasites carrying wild type alleles at *pfcrt-76* and *pfmdr1-86* were the least susceptible to LM but displayed a wide range of responses to the drug (interquartile range, IQR 93-202 nM) (see section 3.3.3.1). These variable responses suggest that LM resistance is not attributable to a

single molecular event, but most likely involves various functionally related genes. Therefore, transporters other than *pfcrt* and *pfmdr1* may be important in mediating LM resistance.

Using the PfSANGER affymetrix array (a 25-mer custom designed array, covering both strands of the *P. falciparum* 3D7 strain), I compared the expression profiles of V1S (the parent isolate) and $V1S_{LM}$ (an isolate derived from culturing V1S for 16 months under LM pressure). I hypothesized that genes that encode for transporters would be amongst those differentially expressed (DE) in $V1S_{LM}$.

6.2 Results

6.2.1 Selection of LM resistance in vitro

LM resistance was selected against the multidrug resistant reference strain V1S (resistant to CQ and antifolate antimalarials but sensitive to LM [396]. Resistance was selected as described in section 2.8.1 and as illustrated in Figure 6-1. The LM-sensitive isolate displayed an initial half-maximal inhibitory concentration, (IC_{50}) of 24±14nM. After 16 months (approximately 166 *P. falciparum* cycles) of continuous culture with varying LM concentrations (Figure 6-2), the selected parasite line $V1S_{LM}$ could grow in LM concentration >378 nM, a concentration 15 times higher than the initial IC_{50}. I demonstrated that the intraerythrocytic development cycle rates between the parental line (V1S) and the selected line ($V1S_{LM}$) were similar using the statistical likelihood based approach previously described[397] (FigureS2, appendix 5).

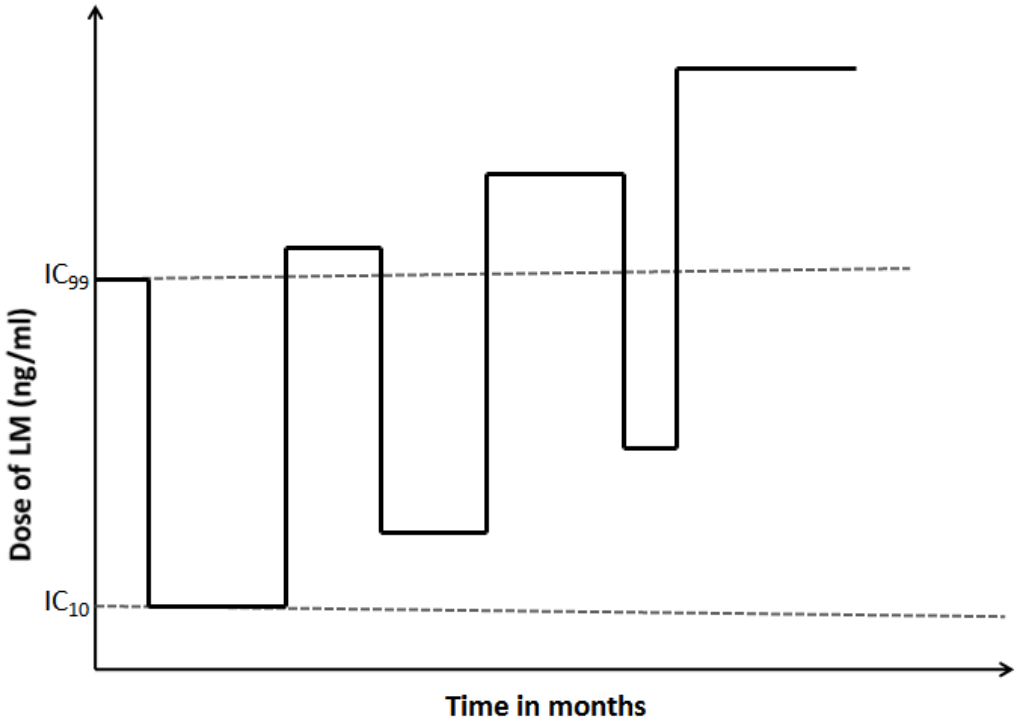


Figure 6-1: Selection of V1S$_{LM}$ under varying concentrations of LM. The broken lines indicate the concentrations that kill 10% and 99% of parasites (IC$_{10}$ and IC$_{99}$). The solid line represents the typical selection regimen consisting of periods under high drug pressure (above or equal to the IC$_{99}$) followed by periods under low drug pressure (ranging between IC$_{10}$ and IC$_{99}$) .As the selection process progressed, the parasite was better adapted to high drug pressure and the length of the periods under low drug pressure decreased.

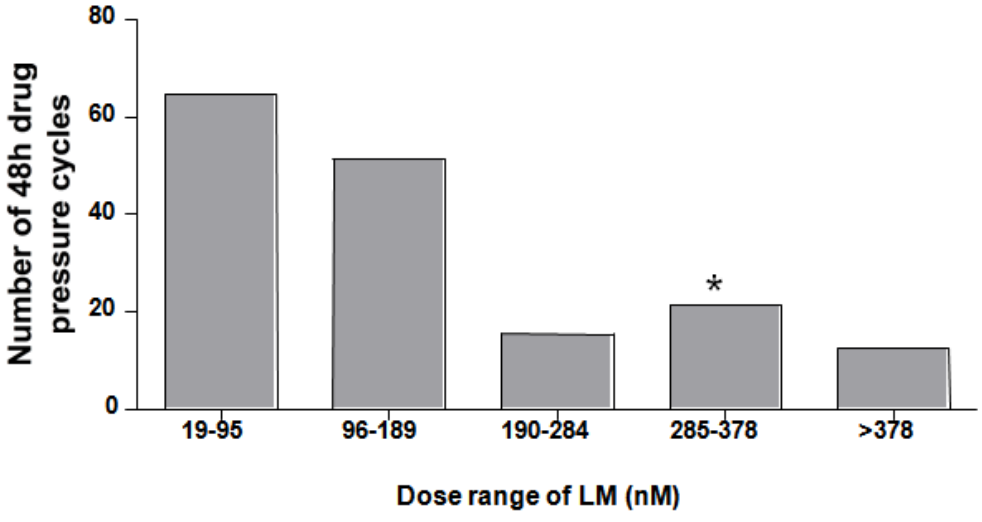


Figure 6-2: A figure showing the estimated duration of culturing V1S$_{LM}$ under varying concentrations of LM.V1S$_{LM}$ was maintained under LM pressure for an estimated 166 *P. falciparum* cycles over a 16-month period. The * indicates the maximum concentration of 378nM at which V1S$_{LM}$ could grow steadily in LM.

However, when cultured in drug-free medium, $V1S_{LM}$ IC_{50} returned to that of the initial, parental strain V1S within 2 weeks ($V1S_{LM}$ IC_{50} =32±7 nM), showing the transient nature of resistance.

6.2.2 Global gene expression profiles

I performed a global transcriptional profiling of $V1S_{LM}$ compared to V1S by microarray analysis using the PfSANGER Affymetrix pseudo-tiling array. To this end, RNA samples from 3 biological replicates of V1S and $V1S_{LM}$ cultures were collected at 4 time points following synchronization with sorbitol: 0, 12, 24 and 36 hours. These parasite samples (24 in total) correspond to ring, early and mature trophozoite, and schizont forms respectively. Out of the initial 24 samples (12 for each line) used in microarray hybridization 19 were selected for final analysis, as three showed signs of RNA degradation (see supplementary figures in appendix 5). A linear modelling approach was used to compare the expression of V1S and $V1S_{LM}$ at each time point (see section 6.2.2.1). A supplementary analysis using EDGE [313] was used to identify any additional and potentially useful transporter genes that were differentially expressed across the entire time course

6.2.2.1 Linear modelling

In the linear modelling approach, I compared the expression of V1S and $V1S_{LM}$ isolates pair wise at each time point (0h, 12h, 24h, and 36h). An overall F test of variance identified 589 genes out of the 5778 genes represented on the array to be differentially expressed with $P < 0.05$ (see Figure 6-3); this corresponds to 10% of the total number of predicted *P. falciparum* genes.

Sixty three percent of genes (371/589) displayed a minimum log 2 expression level of 4 when averaged across all time points and both culturing conditions. Two hundred and sixty six genes were DE with a minimum fold change of 1.5 (see gene list in appendix 5), while 184

were DE between $V1S_{LM}$ and V1S in at least one time point (Bayesian B>0); 5189 genes remained unchanged. The analysis revealed a subset of transcripts with opposite expression patterns i.e. transcripts that were gradually switched off across time (all subtelomeric and included 1 *stevor*, 19 *rifins* and 19 *pfemp1* genes) and transcripts that were gradually turned on across time (encoding ATP and GTP-binding proteins, cell cycle regulators, kinases and phosphatase and 4 transporters;these are marked by lateral blue and red bars in Figure 6-3. While the first cluster may not be directly attributable to the drug pressure and may simply be associated with *Plasmodium's* asexual development [398], the latter cluster is particularly interesting as it highlights the up-regulation of transporter proteins under drug pressure.

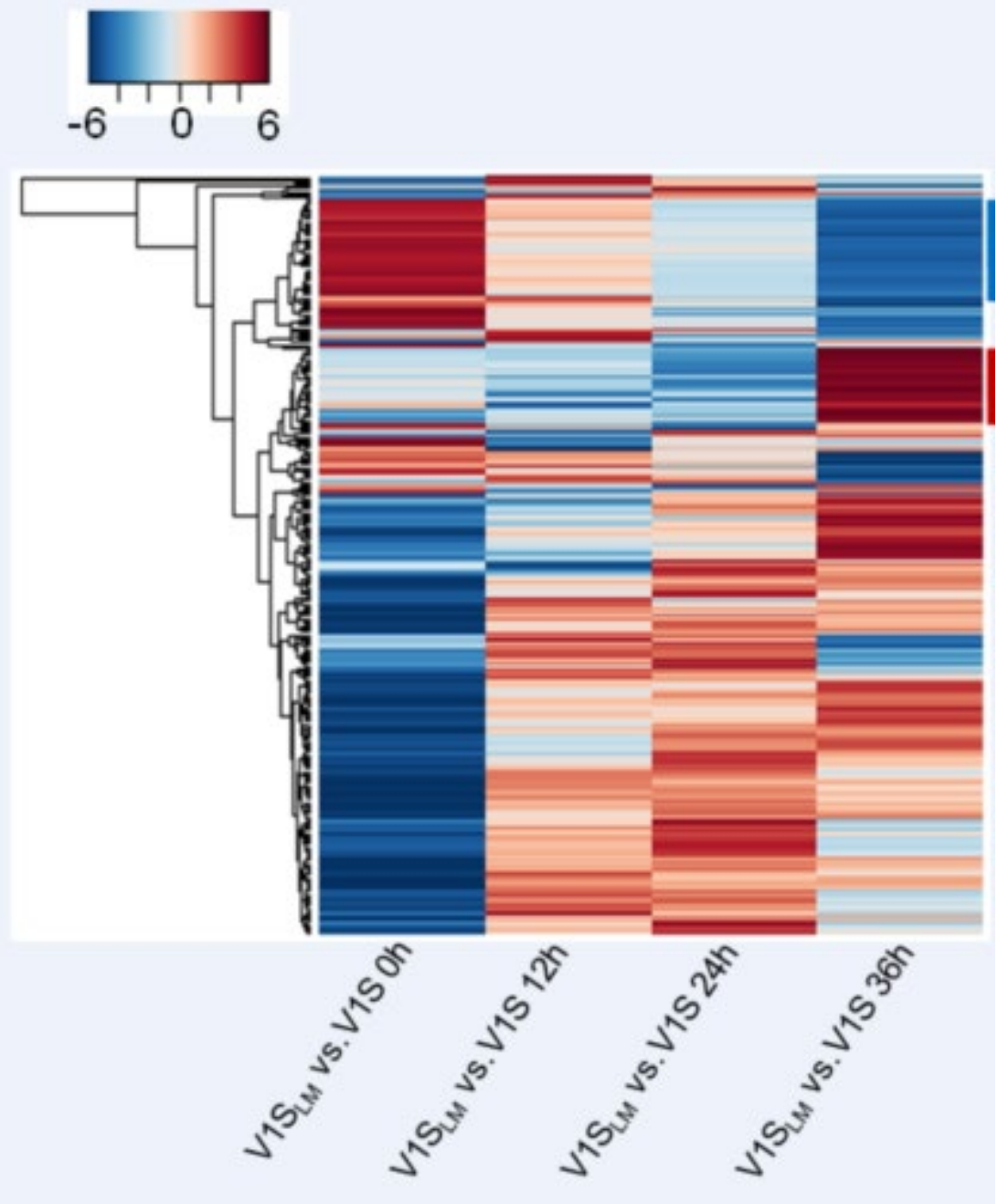


Figure 6-3: A heatmap showing differentially expressed genes between $V1S_{LM}$ vs. V1S. Five hundred and eighty nine genes were differentially expressed in at least one time point (F adj pvalue<0.05). Log_2 ratio of $V1S_{LM}$ vs V1S differential expression is indicated by the colour key ranging from -6 (blue, under-expression) to +6 (red, over-expression). The 2 clusters highlighted with blue and red bars on the right hand side of the heat map correspond to subtelomeric genes gradually switched off in the presence of LM and transporters and cell cycle regulators gradually switched on in the presence of LM, respectively.

Three quarters (133/184) of all DE genes appeared at (0h), 13 at 12h, 12 at 24h, and 43 at 36h (see Figure 6-4).

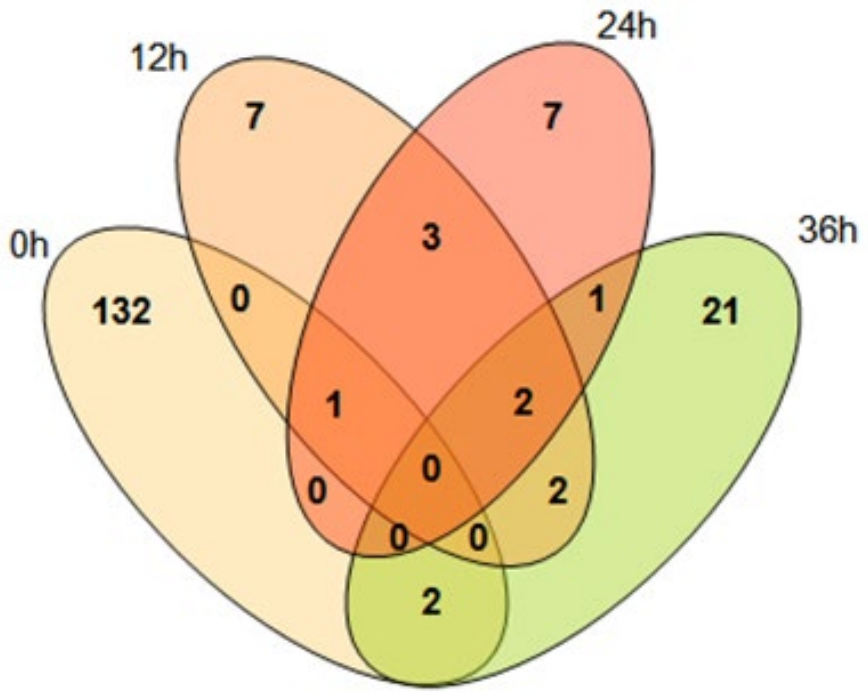


Figure 6-4: Venn diagram showing 184 genes differentially expressed following LM pressure. Different colours indicate the genes corresponding to the various time points.

The majority of DE genes were down regulated at ring stage (101/144) while up-regulation was mainly observed in later time points (14/24 at12h, 12/14 at 24h and 38/56 at 36h). Amongst the most highly DE genes, were those involved in antigenic variation (34, mainly at ring stages), transcription/translation (23), fatty acid biosynthesis (7) protein binding/folding (12), proteolysis (7), and a function in transport (14; whether annotated as such or considering the number of trans-membrane domains (TM) >7). A gene ontology (GO) analysis (*P. falciparum* annotations downloaded in March 2010 at (http://www.geneontology.org/) revealed that 104 out of 184 genes that were DE (see Figure 6-4) had a corresponding GO annotation. GO enrichment analysis of the 104 genes revealed significant ($P<0.05$) over-representation of genes involved in transport activity (both by molecular function [MF] and

Biological Process [BP]), phosphatidyl-inositol metabolic processes (BP), ubiquination (BP), and transferase activity (MF) in ring stages (0h). At 12h, genes involved in DNA polymerase activity were significantly over-represented whilst from 12-36 hours most of DE genes were those involved in fatty acid metabolic processes (BP) (Figure 6-5).

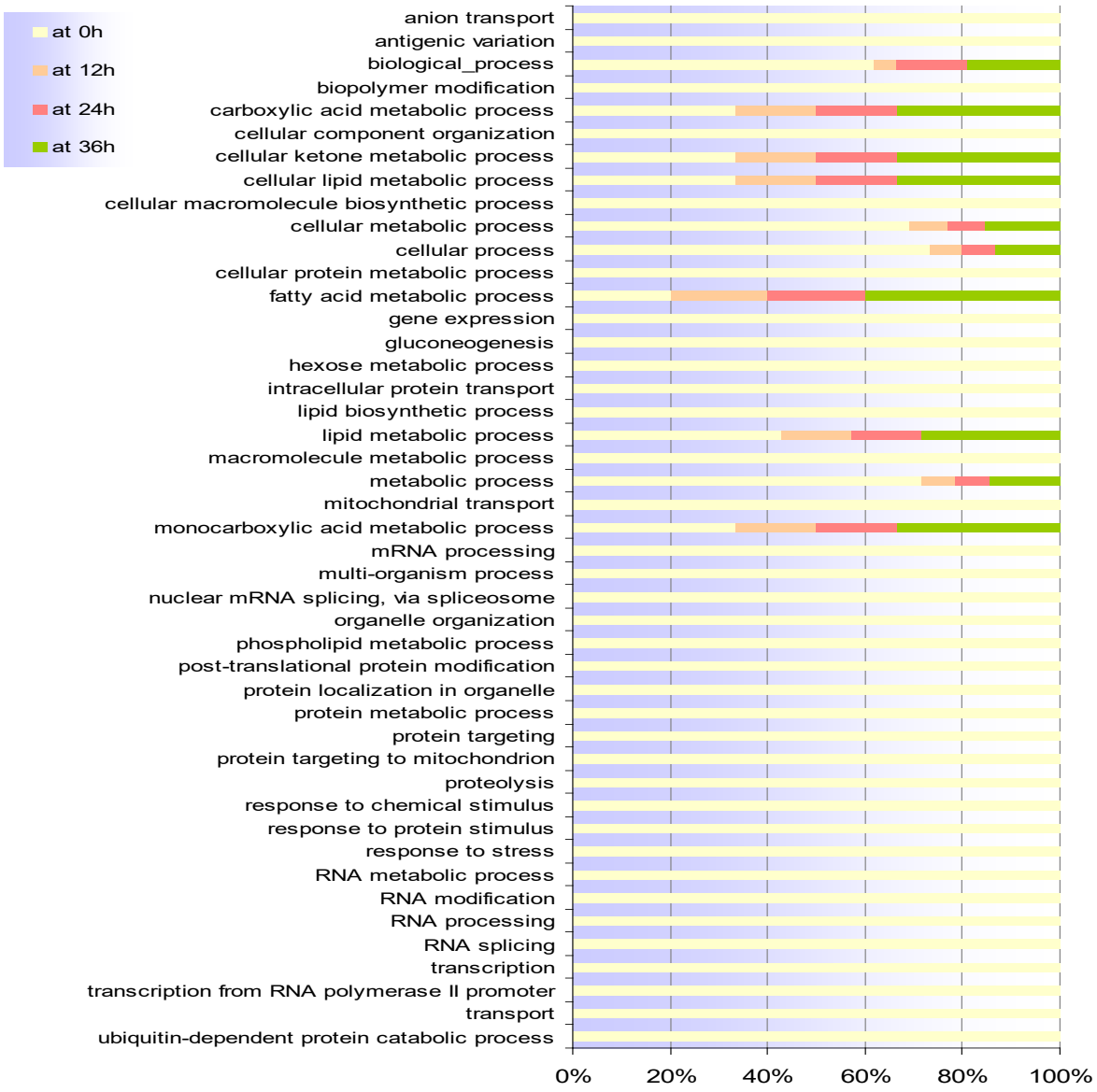


Figure 6-5: The Gene ontology (GO) enrichment analysis of 104 differentially expressed genes that had a GO annotation.The corresponding time-points are colour coded (time-point colour legend indicated on the top left hand corner of the bar plot). The annotated GO categories are shown on the Y-axis. The relative contribution of the genes identified at each time point to each GO enrichment category is indicated as stacked percentages on the X-axis.

6.2.3 Identification of candidate transporters

Transporters are important mediators of *P.falciparum* drug resistance (see sections 1.7 and 6.1.4 of this thesis for an overview). Notably, GO analysis of the microarray data (when analysed by linear modelling) revealed a significant over-representation of genes encoding transporters in response to LM (see section 6.2.2).

Previous work suggested that the majority of genes that encode for transporters in *P.falciparum* are predicted to encode a protein with 7 or more transmembrane (TM) domains, with only few transporters having <7TM [186]. Therefore, in this study, I considered that a gene was likely to encode for a transporter if it was predicted to contain seven or more TM domains or if it was annotated as having a transporter function in www.plasmodb.org.

Globally, I dentified 18 candidate tranporters (see.Table 6-1). Out of the candidate transporters identified by linear modelling, 8 were down-regulated in $V1S_{LM}$ at ring stage (including PfSR25, MAL7P1.64, which have been previously reported to be constitutively expressed in blood stages (374), 1 up-regulated at late trophozoite stage and 4 and 1 were up and down-regulated in schizonts, respectively. After text mining and GO annotation analysis, several of the identified transporter genes were of special interest. The genes encoding the V-type K+ independent H+ translocating pyrophosphatase, PfVP2 (PFL1700c), the folate/biopterin transporter (PF11_0172), the sugar transporter (PFI0785c), the P-type ATPase (PFC0840w) and PfMRP1 (PFA0590w) were all over-expressed in late time points, whilst PFI0720w (a putative transporter of the MFS family) was down regulated at 36h. *pfvp2*, sugar transporter gene PFI0785c, the P-type ATPase gene PFC0840w, and *pfmrp1* displayed a gradual increase in expression during the intra-erythrocytic cycle in $V1S_{LM}$ as compared to V1S (red marked cluster in Figure 6-3).

Table 6-1 : Candidate transporters identified by microarray analysis to play a role in LM *in vitro* tolerance

ID	Product description	F test Adj P value	EDGE P.value
MAL13P1.271	V-type Atpase, putative	0.023	0.172
MAL7P1.64	Serpentine receptor, putative (PfSR25)	0.008	0.669
PF08_0031	Oxogluarate/malate translocator protein, putative	0.028	0.000
PF10_0366	ADP/ATP transporter on adenylate translocase	0.023	0.111
PF11_0172	Folate/biopterin transporter, putative	0.023	0.013
PF13_0300	Mitochondrial inner membrane translocase, putative	0.036	0.099
PF13_0358	Mitochondrial import inner membrane translocase, putative	0.025	0.161
PFA0590w	ABC transporter, CT family, putative, PFMRP1	0.027	0.021
PFC0725c	Formate nitrate transporter, putative	0.035	0.543
PFC0840w	P-type Atpase, putative, PfATPASE7	0.038	0.016
PFE1340w	Conserved Plasmodium protein, unknown function	0.025	0.880
PFl0720w	Transporter (MFS family), putative	0.042	0.023
PFl0785c	Sugar transporter, putative	0.011	0.024
PFL1700c	V-type K+ independent H+ translocating inorganic pyrophosphatase	0.030	0.004
PFE0825w	Metabolite/drug transporter, putative	0.097	0.038
PFE1455w	Sugar transporter, putative	0.119	0.008
PFE1525w	Conserved Plasmodium membrane protein, unknown function	0.357	0.003
PFL0220c	Conserved Plasmodiun membrane protein, unknown function	0.037	0.619

I also identified 5 genes which although were predicted to have<7TM, were annotated to have a transport function. These included an ADP/ATP transporter on adenylate translocase

(PF10_0366), 2 putative mitochondrial inner membrane translocases (PF13_0300 and PF13_0358) and an oxoglutarate/ malate translocator protein (PF08_0031).

The EDGE analysis underlined 4 more genes annotated with at least 7 TM: PFE1525w and PFL0220c (both conserved Plasmodium membrane proteins with unknown function), PFE0825w (a putative metabolite/drug transporter) and the putative sugar transporter PFE1455w.

6.2.4 Confirmation of differential expression of transporters results by qPCR

From the 18 candidate transporter genes identified above, 7 were randomly chosen for confirmation of microarray analysis by qPCR. In addition, *pfmdr1* (PFE1150w), known to be associated with LM resistance, was also assayed by qPCR and showed over-expression at 12h. Although not significantly (F-test, p=0.14), this transcript was over-expressed at 24h in $V1S_{LM}$ by microarray analysis. There was a high correlation between microarray and qPCR analysis (Figure 6-6).

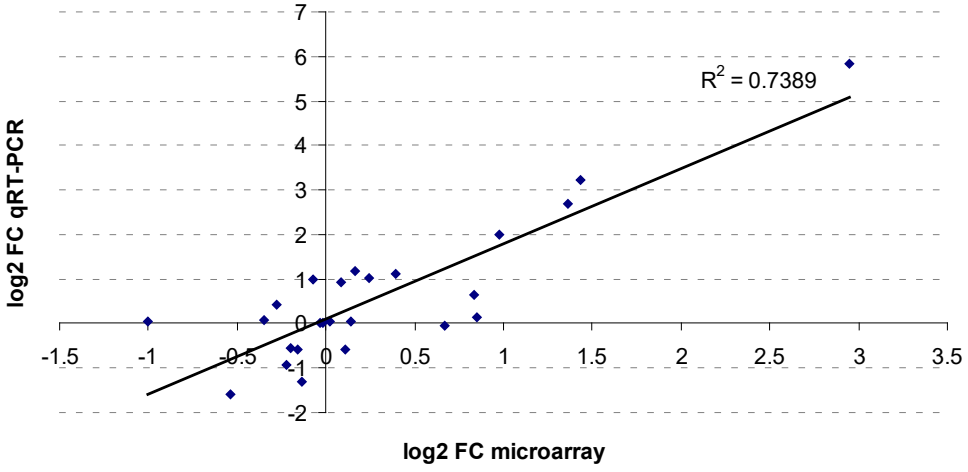


Figure 6-6: Correlation of microarray and q PCR results. The y and x axis indicate corresponding log_2 fold changes (FC). The linear regression is indicated on the plot (R^2=0.7389). Expression was measured for 8 genes at 4 timepoints.

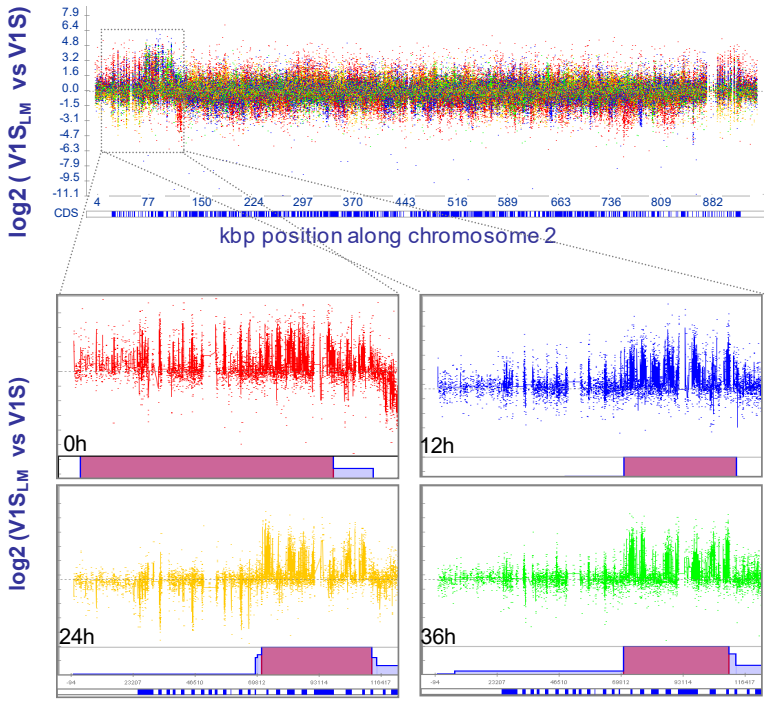
Out of the genes chosen for confirmation by qPCR, all those identified by linear modelling were confirmed to be DE in at least one time point by qPCR, and only 1 out of 10 genes (PFE1525w) which was identified by EDGE analysis did not show differential expression by qPCR. Therefore, there was good agreement between microarray and qPCR analysis.

6.2.5 Differential expression of genes involved in fatty acid metabolism.

GO annotation analysis revealed a significant over representation of genes involved in fatty acid metabolism amongst those DE in $V1S_{LM}$ (see section 6.2.2 and Figure 6-5). Of interest, two of the transporter genes already identified above (PFC0840w and PF08_0031) (see section 6.2.3) are predicted to be involved in phospsholipid and dicarboxylic acid transport (BP and MF) respectively. Other genes identified include PF13_0128 (a beta-hydroxyacyl-ACP dehydratase precursor), PF13_0285 (an inositol-polyphosphate 5-phosphatase), MAL13P1.82 (a phosphatidylinositol synthase), PF10_0016 (an acyl CoA binding protein, isoform 2, ACBP2) and PF10_0015 (an acyl CoA binding protein, isoform 1, ACBP1)

6.2.6 Gene deletion on chromosome 2 and 10

Interestingly, several consecutive genes on the left arms of chromosomes 2 and 10 were consistently up-regulated at all time points in $V1S_{LM}$ when compared to the parental isolate V1S; This observation was statistically significant for 5 and 7 of the genes in chromosome 2 and 10 respectively (see Figure 6-7 and Figure 6-8).



21 over-expressed CDS in V1S$_{LM}$- vs. V1S:

PFB0010w, PfEMP1
PFB0015c, Rifin
PFB0020c, PfEMP1-exon2
PFB0025c, Stevor
PFB0030c, Rifin
PFB0035c, Rifin
PFB0040c, Rifin
PFB0045c*, PfEMP1-truncated
PFB0050c, Stevor pseudogene
PFB0055c, Rifin
PFB0056c, hypothetical
PFB0060w, Rifin
PFB0065w, Stevor
PFB0070w, hyp10
PFB0075c, hyp9
PFB0080c, PHISTb
PFB0085c*, DNAJ protein
PFB0090c*, RESA-like
PFB0095c, PfEMP3
PFB0100c*, KAHRP
PFB0105c*, PHISTc

Figure 6-7: Overexpression of the left arm of chromosome 2 in LM treated parasites.

Amplification signals have been enlarged at each time-point. Each coloured dot corresponds to a 25mer probe with redrepresenting expression at 0h, blue 12h, green 24h and yellow 36h.Underneath every enlarged chromosomal arm are pink bars indicating 100% robustness of signal amplification at p<0.01 (using SnoopCGH program with Smith–Waterman algorithm implementation). A normal distribution of the log ratios (y-axis) around the zero horizontal line is expected if the expression levels are the same along the chromosome (indicated as kilo base pair-kbp). The CDS (represented under each chromosome by blue rectangles) are contained within the amplified regions and are indicated on the right with their appropriate annotation (www.genedb.org). The genes marked with an * were significantly expressed at B>0 in the pairwise comparisons of the microarray data in at least one time point, while the expression of the underlined genes was confirmed by qRT-PCR.

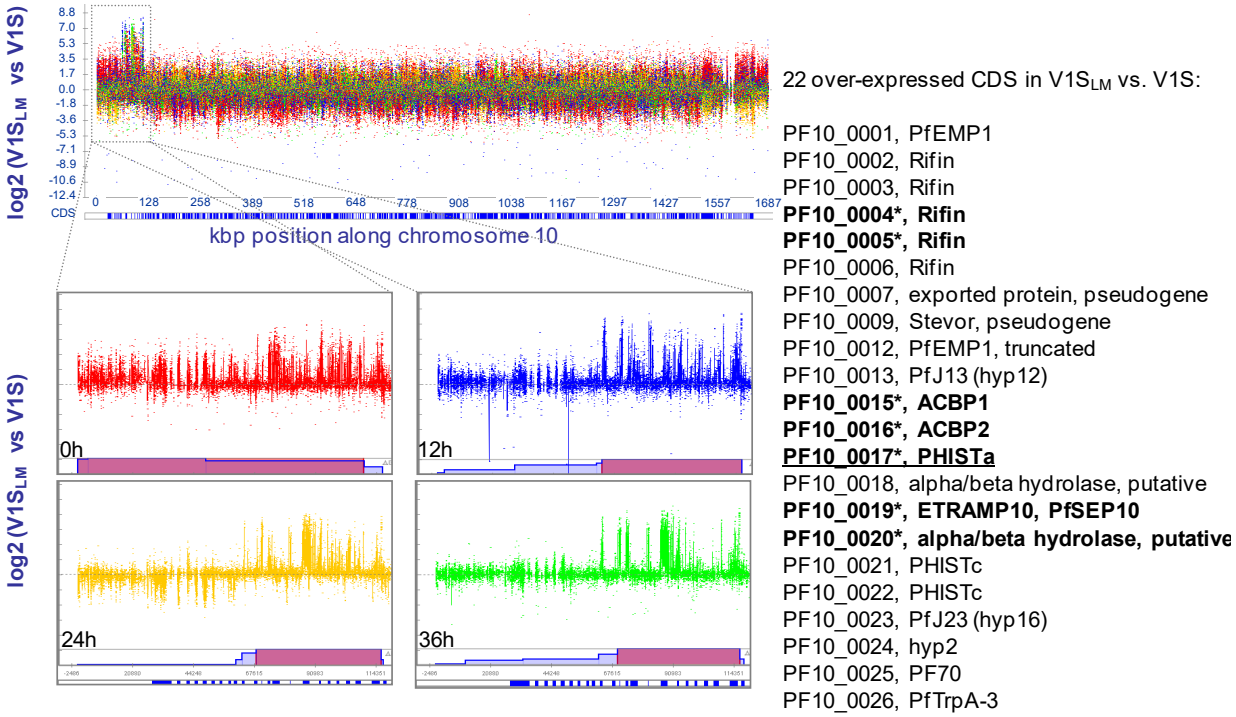


Figure 6-8: Overexpression of the left arm of Chromosome 10 in LM treated parasites. Amplification signals have been enlarged at each time point. Each coloured dot corresponds to a 25mer probe with red representing expression at 0h, blue 12h, green 24h and yellow 36h.Underneath every enlarged chromosomal arm are pink bars indicating 100% robustness of signal amplification at p<0.01 (using SnoopCGH program with Smith–Waterman algorithm implementation). A normal distribution of the log ratios (y-axis) around the zero horizontal line is expected if the expression levels are the same along the chromosome (indicated as kilo base pair-kbp). The CDS (represented under each chromosome by blue rectangles) are contained within the amplified regions and are indicated on the right with their appropriate annotation (www.genedb.org). The genes marked with an * were significantly expressed at B>0 in the pairwise comparisons of the microarray data in at least one time point, while the expression of the underlined genes was confirmed by qRT-PCR.

As *P. falciparum* gene expression is thought to be monocistronic [399], I hypothesized that the observed DE was more likely due to the loss of these two chromosomal arms. A segmentation algorithm, run at the probe level, confirmed that chromosomes 2 and 10 left arms were amplified in V1S$_{LM}$ compared to V1S (P<0.01), suggesting that the V1S (parental line) line lost these portions of the chromosomes after long term *in vitro* culture(Figure 6-7 and Figure 6-8). Therefore I included PFB0105c (chromosome 2) and PF10_0017 (chromosome 10) to confirm the observed under-expression and possible deletion of the left arm of chromosomes 2 and 10 in V1S. Quantitative PCR using cDNA and genomic DNA

isolated from V1S and $V1S_{LM}$ at the end of the 16-month culture period showed a signal for PF10_0017 in the drug-resistant isolate while no signal was found in the parental line V1S. PFB0105c was 4-21 times more highly expressed in $V1S_{LM}$ than in V1S. (see table Table 6-2) There was a good degree of agreement between qPCR and microarray analysis (R^2=0.74, Figure 6-6).

Table 6-2: qPCR analysis of PF10_0017 and PFB0105c using cDNA and genomic DNA templates. cDNA samples were taken at the 36h timepoint. All data were normalised to PF10_0210

Gene	cDNA template			gDNA template		
	Relative expression V1S	Relative expression $V1S_{LM}$	Ratio of expression $V1S_{LM}$/V1S	Estimated quantity V1S Mean (±SD)	Estimated quantity $V1S_{LM}$ Mean (±SD)	Ratio of quantity $V1S_{LM}$/V1S
PF10_0017	0.02 (±0.01)	1.47 (±0.59)	73.5	0.11 (±0.08)	0.65 (±0.33)	5.90
PFB0105c	1.04 (±0.19)	6.73 (±2.32)	6.47	0.34 (±0.18)	0.61 (±0.18)	1.8

6.3 Discussion

6.3.1 Instability of the LM resistant phenotype selected in vitro

One of the main limitations of the selection of *in vitro* resistance by drug pressure has been the difficulty in obtaining a stable resistant phenotype. I cultured the V1S line under LM pressure for 16 months, achieving up to 15 times higher the initial LM IC_{50}. However, after removing drug pressure, the parasites retained their initial IC_{50}s range, typical of a transient drug resistant phenotype. Such phenotypes have been reported previously after *in vitro* selection of antimalarial drug resistance. For instance, the selection of high levels of MFQ resistance against the W2mef line generated a parasite line that could grow in presence MFQ concentrations 148 times higher than the initial IC_{50}; however the resulting resistant phenotype was unstable [241]. Likewise, recently, a transient resistant phenotype was obtained when the F32-Tanzania strain was cutured in presence of increasing concentrations

of ART. In the case of ART, this phenomenon was attributed to changes in the metabolism of young ring forms, which became quiescent under drug pressure [165]. However, as the growth rate of LM selected parasites was comparable to that of the parent line, this mechanism may be different from that observed for artemisinin resistance in the cited study.

Importantly, the availability of technologies that allow for the analysis of the whole Plasmodium genome (such as DNA microarrays) can now allow the investigation of mechanisms underlying resistance in such transient resistant phenotypes. Some of the mechanisms involved in transient resistance arising *in vitro* may be similar to those that result in stable resistance. For example, treatment with CQ and ART was associated with altered expression of *pfmdr1*, one of the main genes implicated in stable resistance to these drugs [179, 236, 259] (also see section 6.3.3). In this study, I used the $V1S_{LM}$ strain to investigate the mechanism of LM resistance using DNA microarrays.

6.3.2 Differential expression of genes involved in transport and fatty acid metabolism in response to LM

I analysed the expression pattern of $V1S_{LM}$ (selected under LM pressure for 16 months) using microarrays. Of the 184 genes identified by linear modelling analysis (see section 6.2.2.1), GO analysis revealed significant over-representation of transporter genes and genes associated with fatty acid metabolic processes (14 and 7 genes respectively) (see section 6.2.2). I discuss these two groups of genes below.

6.3.2.1 Genes involved in transport

Of the 14 transporter proteins identified by linear modelling, five were up regulated at mature stages (24h-36h). This is in agreement with the proposed mechanism of LM action (thought to exert its antimalarial effects by interacting/interfering with heme polymerization), which peaks in mature parasites [40, 188]. Of the candidate transporter genes identified, 11 are

annotated as either transporters or translocators (http://www.Plasmodb.org), and some of these have previously been associated with drug resistance. The localization and/or identification as part of the permeome of the identified candidate transporters [186] suggests that they may have a role in modulating drug response. I provide an overview of previous evidence linking some of these genes with resistance below.

In a previous study where the expression profiles of parasites treated with sublethal CQ were analysed, the putative P-type ATPase gene (*pfatpase7,* PFC0840w) and the V type-H+ pyrophosphatase gene (p*fvp2*, PFL1700c) were DE [400]. PfVP2 belongs to a novel class of plant and protozoan H+ pumps [401] and is localized to the digestive vacuole as well as the parasite plasma membrane [402], although it was also found on the RBC membrane [403]. Previous studies demonstrated that the pH across the membrane of the digestive vacuole was an important determinant of CQ activity [184]. Further, CQ activity was shown to be sensitive to a specific inhibitor of vacoular ATPases [183]. It can therefore be speculated that PfVP2 modulates LM or CQ response by altering cytosolic pH balance, most likely working together with other transporters such as PfCRT

The ABC transporter gene *pfmrp1* (PFA0590w) was similarly up-regulated in mature parasites (24h time point) consistent with its wild type transcriptional profile as measured by RNAseq [404]. The protein is homologous to the human multidrug associated protein 2 (MRP2) [405], a major cause of cancer chemotherapy failure [170], and has been localized to the plasma membrane and vesicles of trophozoite, ring and merozoite stages [177, 405]. Twenty seven SNPs in *pfmrp1* have been identified in field isolates; a significant selection of parasites carrying the 876V allele was observed *in vivo* after treatment with LM-ATM [406], while selection of the 1466K allele was observed after treatment with SP in Tanzania [407]. Mutation in *pfmrp1* was also linked with altered susceptibility to CQ in a genome wide association study [178]. Interestingly, *pfmrp1* disruption in the CQ-resistant parasite W2 resulted in reduced parasite fitness *in vitro*, increased accumulation of radioactive glutathione

and sensitivity to several antimalarials including quinine, CQ, piperaquine, mefloquine and artemisinin [177]. These effects were attributed to an impaired ability to transport drugs and toxic metabolites, as parasites in which *pfmrp1* was disrupted accumulated higher levels of CQ and quinine than wild type parasites[177]. Therefore, *pfmrp1* may be an important mediator of drug resistance in malaria parasites. The small changes in IC_{50} (two fold) observed after gene disruption in the cited study suggest that *pfmrp1* does not act alone but together with other transporters to modulate drug resistance.

I also observed the over-expression of *pfmdr1* (PFE1150w) in the LM selected line $V1S_{LM}$ both by microarray (albeit not statistically significant) and qPCR. *pfmdr1* copy number changes [166, 245-247] and point mutations [232-235] are associated with LM resistance. Increased *pfmdr1* expression has also been linked to quinoline-based drugs [236, 259, 408] and artemisinin [259], but not to the antifolate pyrimethamine; up-regulation of *pfmdr1* may therefore be specific to only a certain group of antimalarials. The observed over expression of *pfmdr1* in this study confirms its already established role in LM resistance.

The association of PFa0590w *(pfmrp1),* PFL1700c *(pvp2),* PFC0840w *(pfatpase7*) and PFE1150w *(pfmdr1)* with LM response in this study in agreement with previous reports of their role in drug response and strongly supports the hypothesis that the *P.falciparum* response to LM is multigenic. Interestingly the expression of several transporter genes (*pfmrp1, pfatpase7, pfvp2* and PFI0785c) was observed to gradually increase as the erythrocytic cycle progressed from 0 to 36h in $V1S_{LM}$ (LM treated parasites) compared to V1S (control parasites), further suggesting that the differential expression observed was indeed a consequence of LM pressure. Our microarray analysis also showed that 5 of the 18 transporters identified were up-regulated at mature stages (24h-36h). This agrees with the proposed mode of action of LM which like other quinoline antimalarials, is thought to interfere with heme polymerization which peaks, in mature parasites [188, 190].

6.3.2.2 Genes involved in fatty acid metabolism

Previously, the altered expression of several fatty acid metabolism genes (including PF10_0016 identified here) has been reported after exposure of malaria parasites to CQ [181] and ART [259]. The increased expression of genes involved in fatty acid metabolism after exposure to LM is supported by observations that quinoline methanols MFQ and QN (similar to LM) bind with high affinity to membrane and purified phospholipids, suggesting that these molecules may be their biological targets [68-72]. As LM, QN and MFQ are structurally similar, they are proposed to have similar modes of action and resistance. It has been suggested that these drugs exert their antimalarial effects by impairing membrane recycling within the endolysosomal sytem of the intracellular parasite [40, 47, 48] and that this impairment ultimately blocks heme degradation, leading to death. Thus, it can be speculated that $V1S_{LM}$ increased the synthesis of fatty acids in order to compensate for the impaired membrane recycling induced by LM pressure, in agreement with the proposed mode of action of LM. This observation further supports the view that analysis of the expression profile after drug exposure can provide biologically meaningful information about the response of malaria parasites to drug.

6.3.3 Using the Plasmodium transcriptome to study antimalarial drug response

The utility of using transcriptome analysis in studying antimalarial drug action and resistance is controversial. For example, several studies reported low amplitude mRNA changes after *in vitro* exposure of parasites to CQ and antifolates with no apparent link to the drugs' presumed mode of action [179, 180, 409], while others reported biologically relevant transcriptional responses after exposure to CQ [400], tetracyclines [410] and artesunate [259]. Importantly, recent evidence has shown that despite the low amplitudes, such transcriptional changes resulting from *P.falciparum* exposure to drug exposure are dose-dependent and highly

reproducible, supporting the view that they represent physiologically relevant processes involving functionally related genes [261].

In this study, I observed relatively small, 2-4 fold changes (FCs) after LM treatment. The relatively small amplitudes of change observed here are similar to those reported previously for *P.falciparum* [179, 180, 261], demonstrating relatively significant differences of the *P.falciparum* reponse to drug exposure when compared to other organisms such as yeasts and bacteria. This may simply reflect particular mechanisms of gene regulation in malaria parasites. Alternatively, these small fold changes can be attributed to the fact that that there is no single adaptive response to LM and could indicate that several functionally related genes are involved.

The marked bias in the differential expression of transporter and fatty acid metabolism genes by GO analysis (see section 6.3.2 below) is the most salient observation of this study. Of interest, a number of the genes identified have already been linked to drug responses in previous studies, strengthening the hypothesis that the majority of genes identified in my study are indeed involved in LM tolerance. Although not all studies are directly comparable due to different methodologies, the repeated identification of similar genes strongly suggest that such transcriptional adaptations are specific and biologically relevant. Therefore, these observations provide useful insights in the understanding of the *P.falciparum* response to LM and possibly other antimalarial drugs.

6.3.4 LM selects for parasites that do not carry deletions on chromosome 2 and 10

These microarray data showed the over-expression of several consecutive genes on the left arms of chromosomes 2 and 10. Deletion of approximately 100kb of the left arm of chromosome 2 is known to happen spontaneously in laboratory and field isolates after short term culture [411-415]; the DE segment on chromosome 2 coincides with this particular

region. I confirmed these findings by qPCR using V1S and $V1S_{LM}$ genomic DNA and cDNA as template. My analysis strongly suggested that the majority of V1S parasites lost this segment of chromosome 2 in continuous laboratory culture, while a small sub population of parasites retained this segment. Long-term LM exposure subsequently resulted in the selection of the sub population of parasites carrying this locus in $V1S_{LM}$ .This suggests that parasites carrying this region of chromosome 2 are fitter under LM pressure. Therefore, this region of chromosome 2 may be important in mediating the *P.falciparum* response to LM.

The same phenomenon was observed for consecutive genes along ~130kb on the left arm of chromosome 10 in response to LM pressure. Copy number variations (CNV) of 3 genes within this segment (PF10_0013, PF10_0014 and PF10_0023) have previously been reported in lab isolates [413, 414], while CNV of PF10_0016 was reported in field isolates after short term culture [414]. Interestingly, four of the other over-expressed genes identified here (PF10_0015, PF10_0016, PF10_0019 and PF10_0021) were deleted in parasites treated with sub lethal concentrations of CQ [181].

My qPCR experiments detected no signal for PF10_0017 in the V1S parental line but clear amplification in $V1S_{LM}$ from both cDNA and gDNA templates. Interestingly, an inverse relationship has been described previously between CQ and the amino alcohols group of antimalarials (which include LM and MFQ) [239-241, 314]; this inverse relationship has previously been attributed to polymorphism in *pfmdr1* (for MFQ)[245] or *pfmdr1* and *pfcrt* (for LM) [245, 314]. The inverse relationship that has been described previously between CQ and amino alcohols such as LM [239-241, 314] may explain the opposite effects observed in this chromosome 10 region whereby CQ pressure led to selection of parasites carrying the deleted genes in the study cited study [181], whilst LM pressure led to selection of parasites carrying this region in this study. Both segments of chromosomes 2 and 10 identified carry the Plasmodium exported proteins (PHIST). Moreover, 5 other PHIST transcripts were found significantly DE in my analysis. Therefore, it is tempting to speculate that these genes, which

constitute part of the Plamodium exportome, may play a role in regulating the transport of substances and possibly drugs such as LM in the malaria parasite. Interstingly, a novel candiadate gene PF10_0355 was recently demonstrated to modulate resistance to LM and structurally related antimalarials in *in vitro*, reinforcing the notion that novel genes may indeed may indeed play a crucial role in antimalarial resistance[395]. However further studies are needed to confirm whether indeed the gene deletions on chromosome 2 and 10 have any role in mediating LM resistance.

6.3.5 Possible Limitations of this study

I performed microarray analysis using the PfSanger array which was designed based on the genome of the CQ sensitive reference parasite line 3D7. I chose V1S (a multidrug resistant line sensitive to LM) as the parent isolate based on previous observations that *in vitro* multi-drug resistance isolates can acquire resistance faster than sensitive ones [146]. It is known that the genetic background of parasites influences the acquisition of drug resistance [147, 178, 228], and it is possible that the LM adaptations observed here could vary in lines with different genetic backgrounds. Although the resultant $V1S_{LM}$ resistant population grew steadily in high concentrations of LM, the IC_{50} was only marginally increased and the phenotype ultimately transient. Such unstable tolerant phenotypes have already been reported in MFQ [241] and ART [74, 165] studies.

The comparison of these data with those obtained from tolerant/resistant isolates of different genetic backgrounds may shed some light on how acquisition of LM resistance varies according to genetic background of parasites. The cloning of parasites during the process of drug selection could be explored in future experiments. Aleternatively beginning selection with a diverse mixture of parasites may increase the chances of selecting a parasite line with a genetic background against which stable resistance can develop.

In this experiment, the expression of V1S was compared to that of the drug-selected line $V1S_{LM}$ whilst growing under sub lethal concentrations of LM. The comparison of the expression data obtained here with that of a third phenotype ($V1S_{LM}$ growing in the absence of drug) would provide further insight into the direct transcriptional effects in response to LM as opposed to more long-term effects resulting from drug selection.

6.4 Conclusion

This study has identified 18 candidate transporter genes (other than *pfcrt* and *Pfmdr1*) that are potentially useful in modulating responses to LM in *P. falciparum*. It also identified the over-representation of various genes involved in fatty acid metabolism in response to LM pressure, supporting the prevailing hypothesis that LM exerts its antimalarial action by interfering with membrane phospholipids. I also observed the over-expression of consecutive genes on the left arms of chromosome 2 and 10 (some of which were previously observed to be deleted in laboratory and cultured field isolates), suggesting that these genes may have a role, even if indirect, in mediating antimalarial drug resistance. Interestingly, I documented an opposite effect of CQ and LM on several genes on the left arm of chromosome 10.

This study demonstrates that the analysis of the expression profile of malaria parasites under drug pressure may provide important insights on drug response. More functional and field association studies are required to clarify the exact function of the transport proteins, fatty acid metabolism genes and chromosome 2 and 10 deletions in mediating resistance to LM and other antimalarials.

CHAPTER SEVEN

7 Summary and Recommendations

In this chapter, I provide an overview of the main objectives of this DPhil project and present a summary of the main findings. I also provide a conclusion and recommendations for future work.

7.1 Introduction

This DPhil project had five main objectives:-

a) To establish the baseline activities of CQ, PQ, LM, AQ, DEAQ and DHA in *P.falciparum* isolates from Kilifi (results presented in chapter 3).

b) To investigate the impact of *pfcrt* and *pfmdr1* polymorphisms in mediating resistance to CQ, PQ, LM, AQ, DEAQ and DHA in Kilifi *P.falciparum* isolates. I used CQ as a reference (results presented in chapter 3).

c) To investigate if selective pressure to LM-ATM, DHA-PQ and AQ resistance *in vivo* is exerted by the long acting components LM, PQ and DEAQ *in vivo* (results presented in chapter 4).

d) To investigate the effects of withdrawing CQ drug pressure from clinical use in Kilifi, and how the use of LM-ATM, DHA-PQ or AQ might influence the observed trends in drug resistance (results presented in chapter 5).

e) To select LM resistance *in vitro* and to identify novel genes that may mediate the *P.falciparum* response to LM using DNA microarrays (results presented in chapter 6).

The overall goal was to understand the mechanisms by which *P.falciparum* acquires resistance to LM, PQ and DEAQ (all are long elimination-half-life artemisinin partner drugs), and to identify molecular markers that may be used to monitor resistance to LM-ATM, DHA-PQ and AQ in the field. I have highlighted the main findings of this project below.

7.2 Summary of studies

7.2.1 The baseline activity of CQ, PQ, LM, AQ, DEAQ and DHA in Kilifi

I have presented the baseline activities of CQ, PQ, LM, AQ, DEAQ and DHA in chapter 3. This study demonstrated that the baseline activity of these drugs in Kilifi is high; median DHA IC_{50} was <5nM, AQ, PQ, CQ, DEAQ median IC_{50}s were <50nM whilst LM median IC_{50} was 55nM. Of interest, >80% of isolates tested had CQ IC_{50} <100nM (the cut-off point previously quoted to distinguish CQ sensitive and resistant parasites *in vitro*).

However, I identified 28/112(25%) of isolates with decreased susceptibility to LM. This is a matter of concern, and highlights the need for monitoring of LM activity. The trends in LM activity will be an important indicator of LM-ATM efficacy.

7.2.2 The Impact of pfcrt and pfmdr1 polymorphisms on the activity of PQ, LM, AQ, DEAQ and DHA.

I analysed the impact of polymorphisms in *pfcrt* and *pfmdr1* (the main genes associated with quinoline resistance) on the activity of PQ, LM, AQ, DEAQ and DHA. I used CQ as a reference.

The frequency of *pfcrt76* mutants (the main molecular marker of CQ resistance) was 65% in baseline isolates, indicating that CQ resistance was still high in Kilifi. However the 100nM cut-off point previously proposed as the *in vitro* cut off point for CQ resistance was not useful

as a predictor of CQ resistance when mutation at *pfcrt-76* was analysed, showing that this cut-off point is not applicable in our setting (see chapter 3). Analysis of *pfcrt-76T* mutation in Kilifi showed that the *in vitro* cut-off point for CQ resistance was 25nM, highlighting that *in vitro* cut off points may vary and are not generalisable across laboratories.

Mutation at *pfcrt-76* and *pfmdr1-86* combined was significantly associated with decreased susceptibility to CQ and DEAQ. Therefore, *pfcrt-76* and *pfmdr1-86* may be used as molecular markers of AQ efficacy in our setting. In contrast, CQ and LM had opposite mechanisms of resistance; whilst wild type isolates at *pfcrt-76* and *pfmdr1-86* were the most susceptible to CQ, these were the least susceptible to LM *in vitro.* This confirms previous observations in *vivo*, that the use of LM-ATM promotes the selection of CQ susceptible parasites. Therefore, *pfcrt-76* and *pfmdr1-86* may be used as molecular markers to monitor LM activity in our setting.

In China, the use of PQ monotherapy against a background of CQ resistance led to rapid selection of resistance [101-103]. Surprisingly, there was no association between PQ activity and *pfcrt* and *pfmdr1* polymorphisms in Kilifi isolates, even though this would have been expected based on its structural similarity with CQ. Further, there was no correlation between PQ activity and that of the amonoquinolines CQ, AQ and DEAQ (see chapter 3). This suggests that PQ resistance is not likely to be selected against a background of CQ resistance in Kilifi isolates, making DHA-PQ a promising alternative antimalarial regimen for this setting. Interestingly the activity of PQ was high in all Kilifi isolates, and was not decreased in isolates with decreased LM susceptibility. This showed that the efficacy of DHA-PQ is not likely to be compromised by the continued use of LM-ATM (the current first-line drug).

7.2.3 Do LM, PQ and DEAQ exert selective pressure for resistance to LM-ATM, DHA-PQ and AQ respectively?

PQ, LM and DEAQ have long elimination half-lives and are used in combination with short acting artemisinin derivatives in ACT (see section 1.5.2 for an overview of ACT). Evidence suggests that such a mismatch in pharmacokinetics may promote the selection of drug resistant parasites, with the long acting partner drug providing the main selective pressure for resistance.

I analysed whether PQ, LM and DEAQ exert selective pressure for resistance to DHA-PQ, LM-ATM and AQ *in vivo* respectively.

I observed that treatment with LM-ATM promoted the selection of isolates that were less susceptible to LM but more susceptible to CQ, further demonstrating the opposite mechanisms of resistance between CQ and LM. This shows that the use of LM is likely to lead to the selection of CQ susceptible parasites.

Treatment with AQ led to the selection of isolates that were less susceptible to CQ, and this effect was most likely mediated by DEAQ, the long acting active metabolite of AQ *in vivo*. This shows that the use of AQ is likely to promote the selection of CQ resistance.

There was no evidence that PQ exerted selective pressure to DHA-PQ *in vivo,* emphasizing its importance as an alternative antimalarial regimen in our setting. Due to the limitation of sample size in this study, It is recommended that the findings be confirmed in studies with larger sample sizes, or in a pooled analysis.

7.2.4 CQ resistance before and after its withdrawal in Kilifi

The withdrawal of drug pressure can lead to the return of drug susceptible parasites. In the case of malaria treatment, this was previously documented in Malawi, whereby the

withdrawal of CQ from clinical use led to the return of CQ efficacy *in vitro* and *in vivo* (see section 1.10 for an overview).

I analysed the prevalence of CQ resistance since its withdrawal in Kenya and compared the observations of Kilifi to those of Malawi. I observed that CQ resistance had been declining in Kilifi since the early 90s after its withdrawal from clinical use, although the rate of this decline was much slower than that observed in Malawi. I propose that the differences between the rates in Kenya and Malawi are attributable to differences in implementation of the CQ to SP policy change and in the AQ use patterns between the two settings (see chapter 5 for a detailed description). This shows that the application of drug policy may greatly influence the outcome of withdrawing a failing antimalarial drug.

I predicted that at the current rates of decline, CQ susceptibility of more than 90% would be achieved in Kilifi in 2018. However, the observation that the use of LM-ATM promotes the selection of CQ susceptible parasites suggests that these rates of CQ susceptibility will be achieved even faster. This presents the interesting possibility of re-introducing CQ in Kilifi, once LM-ATM fails.

7.2.5 Using DNA microarrays to identify genes that mediate the P.falciparum response to LM.

Although *pfmdr1-86* and *pfcrt-76* wild type genotypes were significantly associated with decreased susceptibility to LM, I observed a wide variability of LM responses (see chapter 3). I therefore hypothesized that additional genes were responsible for the variable responses of *P.falciparum* to LM. Evidence suggests that transporters are important mediators of drug resistance (see section 1.7 for an overview). I therefore hypothesized that transporter genes other than *pfcrt* and *pfmdr1* would be important in mediating LM tolerance and/or resistance.

I compared the expression profiles of V1S (a multidrug resistant reference isolate) and $V1S_{LM}$ (a V1S line grown in LM for 16 months and could grow steadily in the presence of up to 15 times the original LM IC_{50} *in vitro*) to identify genes that were responsible for the altered response of LM in $V1S_{LM}$ (chapter 6). I identified 18 candidate transporters (other than *pfcrt* and *pfmdr1)* that were differentially expressed in the LM selected line $V1S_{LM}$.

GO analysis showed the over-representation of transporter genes and of genes associated with fatty acid metabolism amongst those DE. The over-representation of transporter genes is in agreement with my hypothesis that transporters other than *pfcrt* and *pfmdr1* may be important mediators of resistance to LM. Interestingly, some of the candidate transporters identified had been linked to antimalarial drug resistance in previous studies. Further, this data supports the hypothesis that fatty acids may be important biological targets of LM (see section 1.5.1 for an overview of the role of fatty acids in quinoline resistance). Interestingly, I also observed the over expression of certain genes on chromomes 2 and 10 in response to LM. Further analysis suggested that these regions had been deleted in the majority of V1S parasites and were subsequently selected for by LM pressure. These data demonstrate that the *P.falciparum* response to LM is multigenic.

Therefore, a global analysis of the trancriptome can provide useful insights into the response of *P.falciparum* to antimalarial drugs. Further functional studies and studies in field isolates are needed to confirm the exact roles of the identified genes in altering response to LM and other antimalarials.

7.3 Conclusion and recommendations

7.3.1 Need for Monitoring of antimalarial drug resistance

The continued monitoring for resistance to CQ, PQ, LM, AQ, DEAQ and DHA in Kilifi and other malaria endemic areas is important. To this end, I have provided the baseline activity of

the activities of these drugs and the baseline frequencies of the *pfcrt* and *pfmdr1* genotypes in Kilifi.

Although clinical failure to LM-ATM has not emerged yet in our setting, the identification of isolates with decreased susceptibility to LM further highlights the need for continued monitoring of resistance to LM-ATM. My analysis shows that *pfcrt-76* and *pfmdr1-86* will be useful molecular markers of LM-ATM resistance in Kilifi. Although rare in Kilifi isolates, continued monitoring of *pfmdr1* amplification will be essential, as this polymorphism is associated with LM resistance in South East Asia.

7.3.2 Need for preparedness and for identification of alternative antimalarials

Although clinical failure to LM-ATM has not yet occurred in our setting, the identification of isolates with decreased susceptibility to LM is an indicator that LM-ATM may eventually fail. Therefore, the identification of alternatives is required. The high activity of DHA-PQ *in vivo* and *in vitro* shows that it is a promising alternative for Kilifi, if LM-ATM fails.

Importantly, the development of new antimalarial regimens, preferably containing at least two antimalarial drugs with long and comparable half-lives is recommended. Such combinations would be optimal for providing a prophylactic effect after treatment whilst at the same time delaying the emergence of resistance.

7.3.3 Need for standardisation of methods

The discrepancy regarding the *in vitro* cut off point for CQ resistance in Kilifi highlights the need for standardization of the methods used across laboratories, and the linking of *in vitro* chemo sensitivity, molecular and *in vivo* data in order to better define resistance. These efforts

are already being spearheaded by the World Wide Antimalarial Resistance Network (WWARN) [416].

7.3.4 Further studies needed to understand quinoline drug resistance

Although quinoline and quinoline methanol group of antimalarials are thought to kill malaria parasites in a similar manner (by interfering with heme degradation), my analysis showed that LM (a quinoline methanol) and CQ (a 4-aminoquinoline) had opposite mechanisms of resistance. Interestingly, this opposite effect was not observed between LM and the other 4-aminoquinolines AQ, DEAQ and PQ. Further, despite structural similarity, the activity of PQ was not correlated with that of the other quinoline drugs (CQ, AQ, DEAQ and LM), and *pfcrt* and *pfmdr1* polymorphisms did not have any impact on PQ activity.These findings show that the mechanisms of resistance are not similar across all quinoline antimalarial**s.**

Therefore, further studies are required to understand the mechanisms of action and resistance to quinoline antimalarials. The new genes identified globally as part of this DPhil project (see section 7.2.5) are good candidates for further analysis.

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9 Appendices