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ASPECTS OF MOLECULAR MARKERS IN DRUG RESISTANT MALARIA

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Cover illustration

By Irina Jovel and Engels Banegas
Scheme of a *Plasmodium* trophozoites
Sequence alignment of *pvdhfr* in Honduran samples

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Aspects of molecular markers in drug resistant malaria

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To Max

ABSTRACT

BACKGROUND: There were an estimated 207 million cases of malaria in 2012 of which 91% were due to *Plasmodium falciparum*. Antimalarial drug resistance constitutes a major problem in the efforts to control malaria. Drug resistance typically arises by the gradual accumulation of genetic changes that enable parasites to tolerate gradually increasing drug concentrations until fully resistant parasites develop. The aim of this thesis was to determine temporal and geographic frequencies of genetic polymorphisms linked to *P. falciparum* and *P. vivax* resistance after exposure to various antimalarial drugs.

METHODS: Polymorphisms in *P. falciparum* and *P. vivax* multidrug resistance 1 (*pfmdr1* and *pvmdr1*), dihydrofolate reductase (*pfdhfr* and *pvdhfr*) genes, *P. falciparum* chloroquine resistance transporter (*pfcrt*), dihydropteroate synthase (*pfdhps*), V-type H+ pyrophosphatase (*pfvp2*), Na+/H+ exchanger-1 (*pfnhe1*) and multidrug-resistant protein 1 (*pfmrp1*) genes were determined in field samples. *P. falciparum* and *P. vivax* samples from Honduras, where drug resistant malaria has not been detected, were analysed for paper I. Samples from Honduras, Colombia, Liberia, Guinea-Bissau, Tanzania, Iran, Thailand and Vanuatu that represented varying origins and frequency of chloroquine (CQ) resistant *P. falciparum* malaria were analysed for paper II. Samples from 4 villages in Liberia in 1978 after children were administered village specific treatments of CQ, pyrimethamine (PYR), chlorproguanil or placebo monthly for 2 years were used for paper III. Samples (n=1806) from virtually all children aged <15 years with uncomplicated malaria between 2003 and 2012 in Guinea-Bissau were analysed for paper IV. Artemether-lumefantrine replaced an effective high dose CQ treatment in Guinea-Bissau in 2008 but quinine was prescribed to 43% of the children between 2010 and 2012.

RESULTS and CONCLUSIONS:

Paper I: No genetic polymorphisms associated with CQ or sulphadoxine-pyrimethamine (SP) resistance were found indicating that *P. falciparum* remains CQ sensitive in Honduras. CQ resistance associated *pvmdr1* 976F (7/37) and SP resistance associated *pvdhfr* 57L+58R (2/57) were detected suggesting a degree of tolerance to CQ and SP in *P. vivax*.

Paper II: *Pfvp2* SNPs were found in only 20/367 patient samples. Nevertheless, the V405 + K582 + P711 haplotype was associated with *pfcrt* 76T (P=0.007) in line with previous *in vitro* data. However, the limited variation suggests that the results should be interpreted with caution.

Paper III: *Pfcrt* 72-76 CVIET without other downstream resistance associated SNPs occurred in 1/178 samples from the village using CQ in Liberia. CVIET might have developed in Liberia in 1978 but the haplotype was not detected in 1981 suggesting that it probably did not provide a selective advantage in this area. *Pfdhfr* 108N was significantly lower (1/46, P=0.001) in the Liberian village using PYR compared with other villages (64/123). *Pfdhfr* 108N probably occurred as a wild type allele prior to the introduction of antifolates in Liberia. Decreased *pfdhfr* 108N frequency concurrently with the development of in vivo PYR resistance suggests a resistant mechanism other than *pfdhfr* point mutations.

Paper IV: Between 2003 and 2007 *pfcrt* K76T and *pfmdr1* N86Y frequencies were stable in Guinea-Bissau. After 2007, mean annual *pfcrt* 76T (24→57%) and *pfmdr1* 86N (72→83%) frequencies increased (p<0.001). Increased *pfcrt* 76T was probably driven by quinine that was as commonly used artemether-lumefantrine. *Pfmdr1* 86+184 NF frequency increased (39→66%) between 2003 and 2011 (p=0.004), possibly driven by artemether-lumefantrine.

LA PROBLEMÁTICA DE LA MALARIA RESISTENTE AL TRATAMIENTO

La malaria o paludismo es una enfermedad producida por un parásito llamado *Plasmodium* y es una de las enfermedades infecciosas más importantes a nivel mundial. En los últimos quince años los enormes esfuerzos para controlar la malaria han permitido que el número de casos y muertes se hayan reducido significativamente. A pesar de ello, la malaria representa una amenaza constante para la vida y salud del 40% de la población mundial que vive en los 90 países donde se transmite la malaria. Actualmente mueren alrededor de 600 000 personas al año debido a la malaria, la mayoría son niños menores de 5 años y mujeres embarazadas.

Uno de los pilares fundamentales en el control de la malaria ha sido el acceso a un tratamiento efectivo. Desde 1890 la quinina constituyó el tratamiento principal para tratar la malaria hasta el descubrimiento de la cloroquina en 1947. A partir de entonces la cloroquina se usó extensamente para prevenir y tratar la malaria. La cloroquina es segura, barata y se considera que ha salvado incontables millones de vidas, convirtiéndola en uno de los tratamientos más exitosos jamás producidos. Lamentablemente a finales de los años 50 los parásitos se volvieron resistentes al tratamiento, es decir, la cloroquina empezó a dejar de funcionar. Este fenómeno ocurrió simultáneamente en Colombia, Venezuela, en la frontera de Tailandia con Cambodia y en algunas regiones del Pacífico. Para los años 80 este fenómeno de resistencia se había difundido en todas las áreas con casos de malaria de América del Sur, Asia y el este de África. Hacia los años 90 la resistencia a la cloroquina constituyó un serio problema en muchos de los países del oeste de África. Curiosamente, al norte del Canal de Panamá la cloroquina todavía es efectiva para tratar la malaria. Debido a este gran problema de resistencia la Organización Mundial de la Salud ahora recomienda el uso de terapias combinadas con artemisina (TCA).

Una definición de resistencia es “la habilidad de un parásito de sobrevivir o multiplicarse a pesar de tomar el tratamiento siguiendo las debidas recomendaciones”. Pero la resistencia no ocurre en un solo paso, probablemente ocurre gradualmente cuando el parásito debe de ajustar su funcionamiento al estar bajo la presión del tratamiento. Estos ajustes ocurren por lo general en el ADN, pequeños cambios o mutaciones que poco a poco se van acumulando haciendo que el parásito empiece a tolerar el tratamiento hasta que eventualmente se convierte en resistente al tratamiento. Este proceso puede tomar de uno a doce años, como ha ocurrido en algunos de los parásitos que causan la malaria.

En este trabajo estudiamos mutaciones de los parásitos de la malaria que están asociados a resistencia al tratamiento y que han ocurrido desde 1978 hasta el 2012 en ocho diferentes países del mundo. Para este trabajo se tomó pequeñas muestras de sangre a personas con malaria en los siguientes países e intervalos de tiempo: Honduras (2004-2006 y 2009), Colombia (2001-2005), Liberia (1978 y 1981), Guinea Bissau (2003-2012), Tanzania (2008), Irán (2001-2002), Tailandia (2002-2008) y Vanuatu (2002); países que han usado diferentes tratamientos para combatir la malaria. En Honduras, Irán, y Vanuatu el tratamiento que se usó fue la cloroquina. En Colombia se usó amodiaquina combinada con sulfadoxina-pirimetamina. En Liberia se utilizaron tres diferentes tratamientos: cloroquina, pirimetamina

y clorproguanil. En Guinea-Bissau se utilizó una dosis inusualmente alta de cloroquina hasta que en el 2008 se introdujo TCA y del 2010 al 2012 además de las TCA se usó quinina. En Tanzania se utilizó como tratamiento TCA. Con la sangre obtenida de las personas con malaria se obtuvo el ADN de los parásitos y se realizaron diferentes metodologías de laboratorio que permitieron identificar las diferentes mutaciones.

Los resultados más importantes de este trabajo se detallan a continuación. En la mayoría de los parásitos que provenían de Honduras no se encontraron mutaciones que están relacionados con resistencia al tratamiento, por lo tanto el tratamiento que se utiliza en el país es adecuado. En Liberia, a pesar de que se observó un caso resistente a la cloroquina en 1978 no se encontraron más casos en 1981, lo que significa que los parásitos resistentes a la cloroquina no lograron establecerse en el área estudiada. Sin embargo, si se encontraron parásitos resistentes a la pirimetamina. Lo que nos llamó la atención con respecto a estos parásitos resistentes a la pirimetamina es que las mutaciones de los parásitos fueron diferentes a los que anteriormente se han encontrado en parásitos de otros países. Esto nos hace pensar que los parásitos en esta área encontraron una forma alternativa para volverse resistentes al tratamiento. En Guinea Bissau, hasta el 2008 la cantidad de parásitos resistentes a la cloroquina fue mucho menor a la cantidad observada en los años posteriores. Lo anterior fue probablemente debido al empleo inusual de una dosis alta de cloroquina. Sin embargo, el aumento de parásitos resistentes observados a partir del 2008 se debió probablemente a las dosis inadecuadas de quinina empleadas para tratar a las personas con malaria. La quinina y la cloroquina son tratamientos muy parecidos y cuando uno de estos se usa inadecuadamente, por ejemplo en dosis bajas, los parásitos no desaparecen completamente del cuerpo de las personas. Los parásitos que quedan y sobreviven en estas dosis bajas de tratamiento son los que eventualmente cambian hasta convertirse en resistentes.

LIST OF SCIENTIFIC PAPERS

I. **Irina Tatiana Jovel**, Rosa Elena Mejía, Engels Banegas, Rita Piedade, Jackeline Alger, Gustavo Fontecha, Pedro Eduardo Ferreira, Maria Isabel Veiga, Irma Gloria Enamorado, Anders Björkman, Johan Ursing. Drug resistance associated genetic polymorphisms in *Plasmodium falciparum* and *Plasmodium vivax* collected in Honduras, Central America. *Malar J*, 2011. 10(1): p. 376.

II. **Irina Tatiana Jovel**, Pedro Eduardo Ferreira, Maria Isabel Veiga, Maja Malmberg, Andreas Mårtenson, Akira Kaneko, Sedigheh Zakeri, Claribel Murillo Francois Nostenh, Anders Björkman, Johan Ursing. Single nucleotide polymorphisms in *Plasmodium falciparum* V type H+ pyrophosphatase gene (*pfvp2*) and their associations with *pfcrt* and *pfmdr1* polymorphisms. *Infect Genet Evol*. 2014; 24:111-5.

III. **Irina Tatiana. Jovel**, Anders Björkman, Andreas Mårtensson, Cally Roper Johan Ursing. Selection of genetic polymorphisms in previously treatment naïve *Plasmodium falciparum* after monthly presumptive antimalarial administrations in Liberia 1976-78. *Submitted*

IV. **Irina Tatiana Jovel**, Poul-Erik Kofoed, Lars Rombo, Amabelia Rodrigues, Johan Ursing. Temporal and seasonal changes of genetic polymorphisms associated with altered drug susceptibility to chloroquine, lumefantrine and quinine in Guinea-Bissau between 2003 and 2012. *Submitted*

CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin based combinations
CQ	Chloroquine
CPGN	Chlorproguanil
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
G6PD	Glucose-6-dehydrogenase deficiency
IRS	Indoor residual spraying
LLINs	Long-lasting insecticidal nets
PCR	Polymerase chain reaction
Pfcrt	*P. falciparum* CQ resistance transporter
Pfdhfr	*P. falciparum* dihydrofolate reductase
Pfdhps	*P. falciparum* dihydropteroate synthase
Pfmdr1	*P. falciparum* multidrug resistance 1
Pfmrp1	*P. falciparum* multidrug resistance-associated protein
Pfnhe1	*P. falciparum* Na^+H^+ exchanger 1
Pfvp2	*P. falciparum* V-type H^+ pyrophosphatase
Pvcrt-o	*P. vivax* chloroquine resistance transporter
Pvdhfr	*P. vivax* dihydrofolate reductase
Pvdhps	*P. vivax* dihydropteroate synthase
Pvmdr1	*P. vivax* multidrug resistance 1
PYR	Pyrimethamine
QN	Quinine
RDT	Rapid diagnostic tests
RFLP	Restriction Fragment Length Polymorphism
SNP	Single nucleotide polymorphism
SP	Sulphadoxine-pyrimethamine
WHO	World Health Organization

1 BACKGROUND

1.1 The *Plasmodium* parasite

Malaria is an infectious disease caused by a parasite of the genus *Plasmodium*. This protozoan is transmitted to humans by the bite of blood feeding female anopheline mosquitoes. There are more than 140 of *plasmodium* species that can infect birds, reptiles and mammals. Only five species have been shown to infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* [1]. The parasite was discovered in human blood by Alphonse Laveran in 1880. Later, in 1898, the parasite was observed in the mosquito by Ronald Ross who completed a description of the life cycle.

1.1.1 Malaria Vectors

There are over 500 hundred species of *Anopheles* mosquitoes. Worldwide ~40 anopheline species have been documented to transmit parasites to humans with varying efficiency [1]. The female anopheles needs a blood meal for egg production. Some vectors prefer blood meals from humans (anthropophilic) whilst others prefer to feed from animals (zoophilic). Some of the vectors have the tendency to enter and rest inside houses (endophilic) and other rest outside (exophilic) after taking a blood meal. Most vectors feed at night but habits vary with species [2]. *Anopheles* species with the greatest capacity to transmit *Plasmodium* are found in the Amazon (*An. darlingi*) and Africa (the species complex of *An. gambiae*). In Southeast Asia a highly efficient vector is *An. dirus* [1].

1.1.2 Life cycle

The female *Anopheles* mosquito injects sporozoites (8-15, up to 100 in some cases) that are present in the saliva of the insect [3, 4]. Sporozoites infect the liver cells where they may remain dormant (hypnozoites) or produce schizonts and merozoites. The liver cycle takes 5-15 days (up to 3 years if hypnozoites from *P. vivax* or *P. ovale* exist). When liver cells rupture, ~10 000 – 30 000 merozoites from each schizont are released into blood and infect the erythrocytes. In the erythrocytes, the young rings mature into trophozoites. These trophozoites develop via schizonts into merozoites in erythrocytes which ultimately burst releasing 6-36 merozoites per schizont. The released merozoites immediately re-invade new red blood cells and start a new asexual cycle. The infection expands logarithmically at approximately 10-fold per cycle [5]. This expansion is determined by the sub-population of erythrocytes invaded. *P. ovale* and *P. vivax* infect immature erythrocytes whereas *P. malariae* infects mature erythrocytes. *P. falciparum* infects both. The erythrocytic cycle takes 48 hours

or 72 hours (*P. malariae*). Some of the merozoites transform into male and female gametocytes.

Gametocytes are ingested by the female mosquito taking a blood meal. Female gametocytes transform into ookinetes that are fertilized forming oocysts in the gut. Oocytes produce sporozoites that migrate to the salivary gland, ready to infect another host. Malaria can also be transmitted by transfusion and transplacentally.

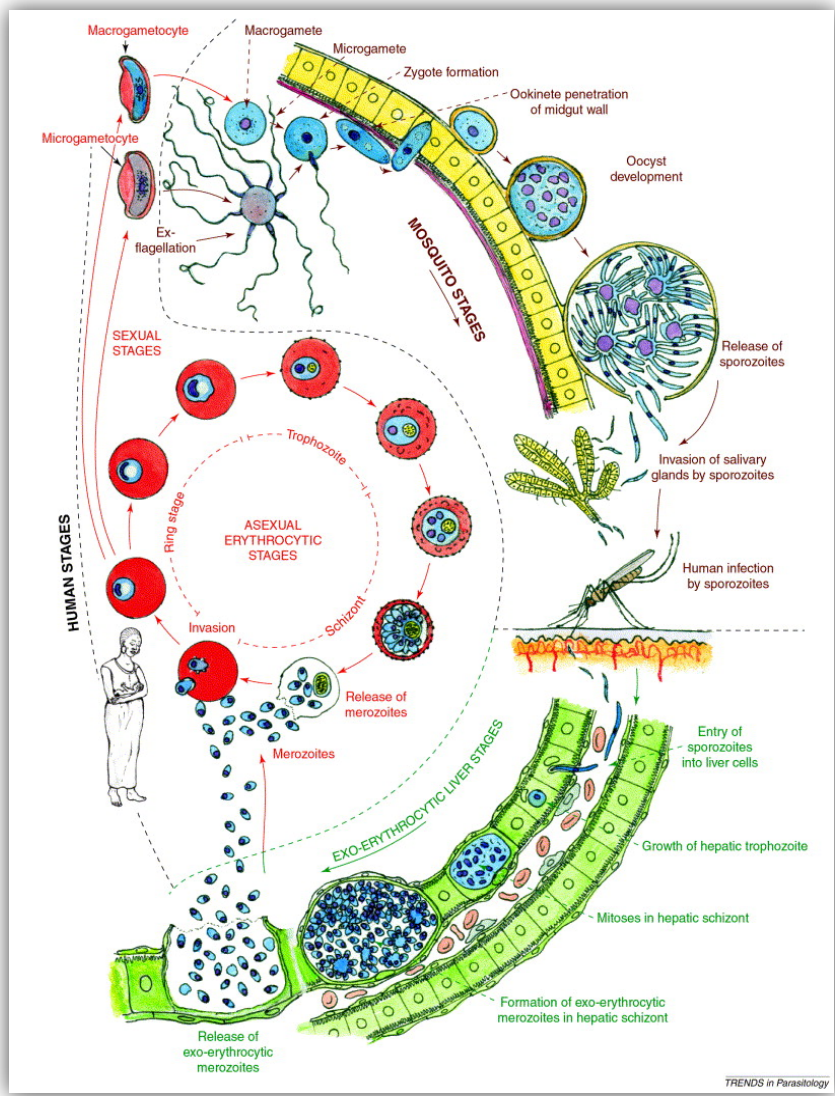

Figure 1 - The life-cycle of *Plasmodium falciparum*. The main phases in the liver and in the red blood cells (asexual and sexual erythrocytic stages) of the human host, and in the gut and in the salivary glands of the mosquito host are depicted. Reprinted from Trends in Parasitology [6], with permission from Elsevier.

1.2 Clinical presentation of malaria

In clinical practice, malaria is defined as uncomplicated or severe. The first symptoms of malaria are nonspecific: a lack of a sense of well-being, headache, fatigue, abdominal discomfort and muscle aches are followed by fever. The classic malarial paroxysms, in which fever spikes, chills, and rigors occur at regular intervals, are unusual and suggest infection with *P. vivax* or *P. ovale*. Most patients with uncomplicated infections have few abnormal physical findings other than fever, anaemia, and in some cases palpable spleenomegaly [1]. Anaemia is common among young children living in areas with stable transmission, particularly where there is resistance to available antimalarials [7].

Severe malaria is an acute life threatening form of malaria with high (~ 10%) mortality in young children [8]. Severe malaria is typically considered to be a feature of *P. falciparum* though *P. vivax* can also result in severe disease and death. The characteristics of severe *P. vivax* are similar to those of severe *P. falciparum* malaria [9-11]. Studies from Indonesia, Papua New Guinea, Thailand and India where both species coexist, showed that 20-40% of malaria admissions were due to *P. vivax* mono-infections. In addition, a review showed that mortality was 0.8-1.6% and 1.6-2.2% due to mono-infections of *P. vivax* and *P. falciparum* respectively [12]. The underlying mechanisms of severe manifestations in *P. vivax* are not fully understood.

1.3 Global Malaria Burden

Malaria generally occurs in tropical and subtropical areas, is commonly associated with poverty and represents a major burden to economic and social development, costing an estimated sum greater than US$ 2.5 billion for the year 2013 [9, 13].

In 2012 it was estimated that 3.4 billion people were at risk of the disease. There were an estimated 207 million cases of malaria worldwide of which 91% were due to *P. falciparum*. Though *P. falciparum* is the most common malaria species, *P. vivax* is the most widespread. *P. ovale, P. malariae* and *P. knowlesi* are less common. The African region contributes the majority of malaria cases (81%) [9, 14]. Worldwide, an estimated 627 000 deaths were attributed to malaria during 2012. Approximately 77% of malaria associated mortality occurred in children under 5 years of age and 91% of the deaths were in Africa [9].

In 2010 it was estimated that 2.49 billion people were at risk of *P. vivax* malaria infection. In 2012 it was estimated that from the 207 million malaria cases 9% were due to *P. vivax* [9].

The majority of the cases are concentrated in Southeast Asia, Middle East and the Pacific [15]. In the Americas, *P. vivax* accounts for around 70% of malaria cases [9].

1.4 Malaria control

Progress in reducing malaria worldwide has been remarkable. There are 79 countries that have eliminated malaria since 1954 and the proportion of the world's population who live in malaria-endemic regions has decreased from 70% to 50% [16]. Malaria eradication was first undertaken by the WHO between 1955 and 1969. The malaria eradication programme used vector control and effective treatment as primary tools to manage malaria. Despite many gains made during the program, the eradication effort was terminated in 1969. Among the reasons for stopping the program were widespread resistance to available insecticides, wars, massive population movements, difficult to obtain funding and finally the emergence of CQ resistance in the 1960s. [17]

In the last 15 years there has been renewed interest and action to support malaria research, control, and eradication. The current malaria control strategy was launched in the early 2000s and included drug use, vector control, confirmation of malaria diagnosis, environmental modification and political and social mobilization [9, 18]. The strategy appears to have been successful. It is estimated that between 2000 and 2012, malaria mortality rates fell by 42% in all age groups and by 48% in children under 5 years [9].

1.4.1 Vector control

The goals of malaria vector control include protection of individual people against infective malaria mosquito bites and reduction of the intensity of local malaria transmission at community level. This should be achieved by reducing the longevity and density of the vector and human-vector contact. The two most powerful and most broadly applied interventions are long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [9]. LLINs have been shown to decrease morbidity and mortality in various malaria transmission settings thereby having a major impact on the malaria burden [19-22]. IRS involves application of insecticides to the inner surfaces of dwellings, where many vector species of *Anopheles* mosquito tend to rest after taking a blood meal. This strategy has also proved to effectively reduce malaria transmission in areas with low and variable/seasonal transmission [23-25]. Worryingly, recent studies in Senegal, Benin and Zambia showed that the prevalence of insecticide tolerant mosquitoes and *P. falciparum* incidence increased after the introduction of LLINs [26-28].

1.4.2 Diagnosis of malaria

Prompt parasitological confirmation by microscopy or rapid diagnostic tests (RDTs) is recommended before treatment is started. In settings with limited health facility access, diagnosis and treatment should be provided at community level through a programme of community case management [29].

1.4.3 Treatment of uncomplicated malaria.

P. falciparum has developed resistance to CQ, followed by SP and then by mefloquine when used as monotherapy. The WHO therefore recommends artemisinin based combination therapy (ACT) to treat uncomplicated *P. falciparum* malaria [30, 31]. In pre-elimination or elimination programs a single dose of primaquine is also recommended [29]

The choice of ACT should be based on the efficacy of the combination in the country or area of intended use. Artemisinin and its derivatives should not be used as oral monotherapies for the treatment of uncomplicated malaria as poor adherence to the required 7 days of treatment results in partial clearance of malaria parasites which will promote resistance to this critically important class of antimalarials [29].

P. vivax malaria should be treated with CQ in areas where CQ is effective. When CQ resistance is present, an appropriate ACT should be used. In order to prevent relapses a 14 day course of primaquine should be used in combination with both CQ and ACT [29].

1.4.4 Treatment of severe malaria

Severe malaria should be treated with a parenteral artemisinin derivatives (artesunate and artemether) followed by a complete course of an effective ACT as soon as the patient can take oral medications. If artemisinin derivatives are not available parenteral quinine (QN) or quinidine can be used as an alternative. Where complete parenteral treatment of severe malaria is not possible patients should be given pre-referral treatment and immediately be referred to an appropriate facility for further treatment. Options available for pre-referral treatment are: Rectal or intramuscular artesunate, intramuscular QN or artemether [29].

1.5 Antimalarial drugs discussed in this thesis

1.5.1 Quinolines and related compounds

The first effective chemotherapy for treatment of malaria was extracts from the Cinchona bark tree. The extract was imported from South America to Europe in the mid 17th century where it became popular for the treatment of fevers including malaria. In 1820 pure QN was isolated from the Cinchona bark and replaced the extract in the treatment of malaria [30]. The basic quinoline ring structure has provided a group of synthetic antimalarials (CQ, amodiaquine, piperaquine, mefloquine and primaquine) collectively named quinolines. Based on more loosely related ring systems the antimalarials halofantrine and lumefantrine have also been synthesized [32].

QN acts on mature trophozoites and does not prevent sequestration or further development of ring stages of *P. falciparum* [29]. It does kill sexual stages of *P. vivax*, *P. malariae* and *P. ovale* but not mature gametocytes of *P. falciparum* [29]. QN is usually used to treat severe malaria. In combination with antibiotics it is used as second line treatment of uncomplicated malaria and it is also used during the first trimester of pregnancy [29]. Though a combination of QN and an antibiotic is recommended, QN is commonly used as monotherapy in most African countries [33].

CQ has been used extensively for the treatment and prevention of malaria since 1947. It is safe, cheap and is estimated to have saved countless millions of lives. As such it is one of the most successful drugs ever produced [34]. Widespread resistance has now rendered it virtually useless against *P. falciparum* infections in most parts of the world but not north of the Panama Canal. It is still efficacious for the treatment of *P. vivax, P. ovale* and *P. malariae* infections in most parts of the world.

Amodiaquine (AQ) is a potent blood schizonticide that was developed in the late 1940s and has been used for the treatment of uncomplicated malaria particularly in Africa [35]. When used for malaria prophylaxes AQ can cause neutropenia and mainly for that reason it was not used for many years. However, it has been revived as part of an ACT.

Mefloquine is effective against all forms of malaria [36]. Mefloquine was introduced to treat patients with CQ resistant parasites. It was initially used as monotherapy in areas of low malaria transmission [37]. Due to resistance it is now principally used in combination with artesunate to treat *P. falciparum* in Southeast Asia (Cambodia, Malaysia, Myanmar, Thailand and Vietnam) and South America (Bolivia, Brazil, Colombia, Peru and Venezuela) [9]

Lumefantrine is a blood schizonticide that was first synthesized in China and first mentioned in scientific literature outside China in 1990. It is only available as an oral preparation co-formulated with artemether. This ACT is highly effective against *P. falciparum* and a corner stone of ACT in Africa [29].

The quinolines do not eradicate hypnozoites unlike primaquine that was developed during the Second World War. Primaquine is effective against intrahepatic forms of all types of malaria parasite. It is also gametocytocidal against *P. falciparum* and has significant blood stages activity against *P. vivax* (and some against asexual stages of *P. falciparum*). The haemolytic effect in patients with glucose-6-dehydrogenase deficiency (G6PD) and gastrointestinal intolerance limits its use.

1.5.1.1 Quinoline mechanism of action

CQ has been the most thoroughly studied antimalarial with the aim of understanding its parasiticidal effect and the mechanisms of CQ resistance in malaria. CQ is a week base that moves rapidly across cell membranes and accumulates in the acidic environment of the digestive vacuole because it becomes protonated there [38]. In the digestive vacuole CQ disrupts the detoxification of haem when haemoglobin is digested by the parasite [39]. This results in haem complexes that are lethal to the parasite [40]. The mechanism of CQ activity against blood stages of *P. vivax* remains unknown [41]. QN, amodiaquine and piperaquine are believed to have similar modes of action to that of CQ [42]. Mefloquine, halofantrine and lumefantrine have also been shown to inhibit the detoxification of haem but they also appear to target other process in the parasite [43, 44].

1.5.2 Antifolates

The antifolates are compounds that bind to enzymes necessary for parasite folate biosynthesis. The principal antifolate drugs used against malaria are sulphadoxine and pyrimethamine (SP).

Sulphadoxine is a structural analogue and competitive antagonists of p-aminobenzoic acid. It potentiates the schizontocidal effect and improves clinical response of pyrimethamine (PYR) when treating *P. falciparum* infection [45-47]. In 1951 PYR was shown to be effective for the treatment of *P. falciparum* [48, 49]. It inhibits dihydrofolate reductase (*pfdhfr*) thus indirectly blocking the synthesis of nucleic acids in the malaria parasite. It is a slow-acting blood schizontocide and is possibly active against pre-erythrocytic forms. Furthermore, it inhibits sporozoite development in the mosquito vector. During the 1950–1960s, PYR was mainly

used for prophylaxis against *P. falciparum* infection or for mass drug administration because CQ was effective in all endemic regions [50-52]. The antifolate combination of sulphadoxine and pyrimethamine (SP) was first introduced in Thailand in the 1960's where the frequency of CQ-resistant *P. falciparum* infections had reached an unacceptable level.

Chlorproguanil (CPGN) is a chloro- derivative of proguanil that is metabolised to chlorcycloguanil. CPGN works by blocking the synthesis of tetrahydrofolate, essential in DNA synthesis. CPGN has recently gained more interest, mainly in combination with dapsone. CPGN-dapsone has been shown to be effective for the treatment of SP resistant parasites [29, 53, 54].

1.5.3 Artemisinin

Artemisinin, also known as qinghaosu, is a semisynthetic drug extracted from the leaves of *Artemisia annua* (sweet wormwood). It has been used in China for the treatment of fever for over a thousand years and artemisinin derivatives are now the cornerstone of antimalarial combination therapy. It is a potent and rapidly acting blood schizontocide and is active against all *Plasmodium* species. It has an unusually broad activity against asexual parasites. Furthermore, it kills *P. falciparum* gametocytes [55]. The mechanism of action of artemisinin is not fully understood [56, 57]. Commonly used artemisinin derivatives are dihydroartemisinin, artemether, and artesunate. The two latter derivatives are *in vivo* converted back to dihydroartemisinin. The 5 ACTs currently recommended are artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + sulphadoxine-pyrimethamine and dihydroartemisinin + piperaquine [29].

1.6 Antimalarial drug resistance

One commonly used definition of clinical drug resistance is "the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of antimalarial drug in the dose normally recommended." Antimalarial drug resistance is not necessarily the same as malaria "treatment failure", which is a failure to clear malarial parasitaemia and/or resolve clinical symptoms despite the administration of an antimalarial. So while drug resistance may lead to treatment failure, not all treatment failures are caused by drug resistance [58].

Drug resistance in malaria does probably not arise in a single step, but as a long process during which parasites become gradually more and more tolerant to the drug in question. This is believed to be the result of a gradual accumulation of genetic changes as discussed below.

The changes alter the natural function of a specific protein that in turn may require additional compensatory genetic changes. Eventually a fully resistant and fit parasite emerges. For *P. falciparum* this process typically takes 1-12 years [59].

The greatest problem with drug resistance occurs with *P. falciparum* but CQ resistant *P. vivax* is a developing problem [60]. There are very few reports on drug resistant *P. malariae* and *P. ovale* although there have also been very few studies. Of greatest concern at the moment are recent reports of *P. falciparum* that are resistant / tolerant to artemisinin and to ACT [61-67].

1.6.1.1 Development and spread of quinine resistance

QN resistance was observed nearly 100 years ago when recrudescences were observed in Brazil [68]. Since the 1980's there have been more reports of QN resistance or reduced susceptibility *in vitro* and *in vivo* in Southeast Asia [69-79] and South America [75, 76, 80-84]. Since the 1990's *in vivo* and *in vitro* resistance and reduced susceptibility has also been reported from Africa [76, 82, 85-97].

1.6.1.2 Development and spread of chloroquine resistance

CQ resistant *P. falciparum* took a long time to develop (>10 years) [31] and only appears to have arisen 5 times [59]. In the late 1950s, CQ resistant *P. falciparum* was identified at the Thai-Cambodian border and simultaneously in two different locations in South America (Colombia and Venezuela) [30]. In Pacific regions CQ resistance was first reported 1959–1961 [98]. Resistance then spread to eastern parts of the Pacific region between 1976 and 1980. By the mid 1970s CQ resistance was spread throughout Southeast Asia. All endemic areas in South America were affected by 1980 and almost all of Asia and the Pacific by 1989 [31]. In Africa CQ resistance first appeared on the east coast in 1978 [99, 100]. During the early 1980s it spread throughout East Africa and by the early 1990s, CQ resistant *P. falciparum* became a serious emerging problem in many West African countries [59]. CQ resistance has been reported from wherever falciparum malaria is endemic, except Central America [101, 102].

P. vivax is still generally sensitive to CQ although sensitivity is decreasing in some areas. CQ resistant *P. vivax* was first described in 1989 when it was reported from Papua New Guinea [103]. Subsequently other reports from Indonesia confirmed those findings [104-106]. By 2002 there were reports from Malaysia, Myanmar, Vietnam, India and Iran [107-111] and by 2009 from Turkey and South Korea [112, 113]. In South America CQ resistance was first

reported in 1996 from French Guyana [114]. By 2003 Brazil, Colombia and Peru had also reported cases of CQ resistant *P. vivax* [115-117]. The first reports from Africa were from Ethiopia and Madagascar in 2008 [118, 119].

1.6.1.3 Development and spread of pyrimethamine, sulphadoxine-pyrimethamine and chlorproguanil resistance

PYR resistance appeared in Asia and East and West Africa during or shortly after trials of mass eradication or prophylaxis during the 1950's and 1960's. [120-125]. Cross-resistance between PYR and CPGN was noted in Ghana, Nigeria and Liberia during the same period [125, 126].

SP resistant *P. falciparum* were described on the Thai-Cambodia border in 1967, the same year that SP was introduced. SP resistance subsequently spread to other regions in Southeast Asia [31]. In 1996, high-level resistance was found simultaneously in a large part of Southeast Asia, Southern China and Amazon area [127, 128]. Lower degrees and frequencies of resistance were observed on the Pacific coast of South America [31]. Sensitivity to SP in Africa started to decline in the late 1980s following increased use of PYR-dapsone prophylaxis [31].

In early reports from the 1950's *P. vivax* appeared to be resistant to SP. However, recently it was suggested that the early perception of poor efficacy may have been a product of confusion with the failure of SP to prevent relapse [129]. However, clinical failure following SP treatment has been reported from Papua New Guinea and Indonesia in 1992 [130, 131]. By 2005 resistance had also been reported from, Myanmar, Vietnam, Vanuatu and India [132, 133].

1.6.1.4 Artemisinin resistance

In 2005, there was a report of reduced *in vitro* susceptibility to arthemeter in isolates from French Guyana and Senegal [134]. *In vivo* artemisinin resistance or perhaps more correctly tolerance or reduced susceptibility is characterized by a slower rate of parasite clearance. This has been described from Western Cambodia [63, 64, 135, 136], Thailand [137], South-eastern Burma and South-eastern Vietnam [138]. A recent report from Western Cambodia describes how the proportion of *P. falciparum* with reduced artesunate susceptibility increased over time. The study showed that mean parasite clearance time decreased from 2.6 hrs in 2001 to 5.5 hrs measured between 2007 to 2010 [136].

1.6.2 Resistance associated genes

P. falciparum and *P. vivax* parasites contain approximately 5 500 genes on 14 chromosomes. By 2002 more than 95% of *P. falciparum's* genome was sequenced and sequencing of the *P. vivax* genome was completed in 2008 [139, 140]. Different genes from these parasites appear to be involved in resistance to different antimalarial drugs.

Resistance appears to be caused by a change in the structure, function or quantity of a protein. The change in the protein is in turn mediated by genetic changes such as single nucleotide polymorphisms (SNP), gene amplifications and gene expression. An alteration in the structure of a protein may prevent the drug from binding to its target as in SP resistance [141, 142]. An alternative mechanism is to enhance or block a transport protein so that a drug is removed from its site of action (e.g. CQ efflux from the DV) or prevented from entering into its site of action (e.g. import of CQ or QN in to DV) [143]. Changes in two genes may act in combination to produce a specific phenotype [144].

1.6.2.1 Quinolines

The two principle genes that appear to be involved in quinoline resistance are *P. falciparum* chloroquine resistance transporter (*pfcrt)* and *P. falciparum* multidrug resistance 1 (*pfmdr1*) [143]. Both are located in the membrane of *P. falciparum's* digestive vacuole [143]. Both are believed to be transporters and different SNPs hinder or enable the transport of different quinolines [143].

Additional genes that have been suggested to be involved in quinoline resistance include the multidrug resistant-associated protein 1 (*pfmrp1*) as well as the Na+/H+ exchanger (*pfnhe1*). Both are located in the plasma membrane and it has been suggested that they participate in the efflux of metabolites (e.g. oxidised glutathione, CQ, QN, folates) out of the parasite. In addition *pfmrp1* was suggested to efflux drugs out of the parasite [145-147].

The search for molecular markers of resistance in *P. vivax* has focused on the orthologs *P. vivax* multidrug resistance gene 1 (*pvmdr1*) and chloroquine resistance transporter gene (*pvcrt-o*) [41].

1.6.2.2 *P. falciparum* chloroquine resistance transporter – *pfcrt*

More than 40 years were necessary to go from clinical recognition of CQ resistance to the molecular basis of the phenomena which was unravelled in 2000 when *pfcrt* was identified [148]. *Pfcrt* is located in the membrane of the DV and different haplotypes that contain between 4 and 10 mutations have been associated with CQ resistance around the world [59, 143]. Specific haplotypes at positions 72-76 are linked to the regional evolution of CQ resistance [59] and the K76T SNP has been shown to be essential for CQ resistance *in vivo* and *in vitro*[149-152]. The replacement of Lysine (K) with Threonine (T) removes a positive charge enabling *pfcrt* to transport protonated CQ down its electrochemical gradient, out of the DV [153, 154]. The net result is lower nontoxic CQ concentrations in the DV and continued parasite growth. Recently the ordered evolution of the SNPs described K76T and N75E or N326D SNPs as the first step to drug resistance followed by additional SNPs and how these changes affected CQ transport [155].

Pfcrt has also been shown to influence susceptibility to other antimalarial drugs. Amodiaquine resistance is linked to *pfcrt* 76T just as CQ and allelic exchange experiments have linked *pfcrt* 76T to reduced susceptibility to mefloquine, artemisinin and QN [150, 156, 157]. Furthermore, K76 has been linked to reduced susceptibility to lumefantrine [158].

1.6.2.3 *P. falciparum* multidrug resistance 1 – *pfmdr1*

Before the discovery of *pfcrt* most attention was given to *pfmdr1* [159]. The first *pfmdr1* polymorphism that was correlated to drug resistance was gene copy number [160-162]. Subsequently, multiple *pfmdr1* copies were shown to be a molecular marker of *in vitro* and *in vivo* mefloquine resistance [62, 163]. Decreased *in vitro* susceptibility to lumefantrine, halofantrine, QN and artemisinin have also been linked to amplifications [62, 164, 165]. Furthermore, amplifications have been associated with an increased risk of treatment failure following use of arthemeter-lumefantrine [65, 166, 167].

Drug resistance associated SNPs in *pfmdr1* include N86Y, Y184F, S1034C, N1042D, F1226Y and D1246Y. Various combinations of these SNPs have been shown to modulate the level of drug resistance/tolerance to QN, CQ, amodiaquine, mefloquine halofantrine lumefantrine [61, 62, 75, 168-184] and artemisinin [179-184]. SNPs in *pfmdr1* do not confer CQ resistance [179, 180] however *pfmdr1* N86Y together with *pfcrt* K76T has been associated with high levels of CQ resistance [185, 186].

1.6.2.4 *P. falciparum* V-type H^+ pyrophosphatase – *pfvp2*

Pfvp2 is a class of H^+ pump found in plants and some protozoa [187-191]. *Pfvp2* is located in the DV membrane and increased transcription of *pfvp2* has been observed *in vitro* when *P. falciparum* are exposed to CQ [191] and lumefantrine [158]. Specifically a ten-fold up-regulation of *pfvp2* was observed when the CQ resistant (*pfcrt* 76I) *P. falciparum* 106/1 clone was exposed to CQ but no up-regulation was seen with the CQ sensitive 106/1 (*pfcrt* 76K) clone. A two-fold up-regulation of *pfvp2* was seen when the lumefantrine tolerant *P. falciparum* V1S (*pfcrt* 76K) clone was exposed to lumefantrine [158]. The up-regulation of *pfvp2* suggests that it could be involved in maintaining the H^+ balance in the parasite DV and to compensate for H^+ loss caused by removal of protonated CQ [191]. As described above, CQ resistance involves the removal of protonated CQ from the DV. This is likely to cause a loss of H^+ that needs to be replaced if the DV is to remain acidic.

1.6.2.5 *P. falciparum* Na+/H+ exchanger-1 – *pfnhe1*

The *pfnhe1* protein is believed to be located in the plasma membrane of the parasite [147, 192]. Though *pfnhe1* function has not been fully determined, it has been proposed that *pfnhe1* actively effluxes protons out of the parasite to maintain a pH of 7.4 within the malaria parasite [192]. Polymorphisms in *pfnhe1* have been associated with different susceptibilities to QN [54, 75]. Specifically, >1 DNNND repeat and one DDNHNDNHNND repeat in the coding microsatellite ms4760 have been associated with reduced *in vitro* and *in vivo* QN sensitivity [75, 79, 97]. These *pfnhe1* repeats have been associated with changes in the cytosolic pH affecting the parasite susceptibility to QN [147]. However, *pfnhe1* is not the major pH regulator in the parasite [193] and the cytosolic pH was not affected when *pfnhe1* expression was reduced by 50% [194]. *Pfnhe1* has also been suggested to play a role in CQ resistance [195, 196], but this was subsequently disputed [193, 197].

1.6.2.6 *P. falciparum* multidrug-resistant protein 1 – *pfmrp1*

Pfmrp1 is an active transporter located at the plasma membrane of the plasmodium parasite [198]. It is thought to be involved in the transport of CQ and QN, out of the cell. In line with this, *P. falciparum in vitro* susceptibility to CQ, QN, artemisinin, piperaquine and primaquine increased and CQ accumulated when *pfmrp1* function was disrupted [146]. Various *pfmrp1* SNPs and haplotypes have been associated to different responses to CQ, QN, mefloquine, pyronaridine, SP and ACTs in Africa, Southeast Asia and Oceania. However, in some countries associations were not found as described below. *Pfmrp1* H191Y and S437A SNPs were previously associated with increased susceptibility to CQ and QN in Southeast Asia

[168] but later no association were found in the same area [199]. *Pfmrp1* I876V SNP has been associated with reduced susceptibility to CQ (China-Myanmar border), artesunate-mefloquine (Thai-Myanmar border), and artemether-lumefantrine (Africa) [200-202]. However in north-east Myanmar no associations were found to these drugs [203]. *Pfmrp1* F1390I has been associated with reduced *in vitro* susceptibility to lumefantrine [204] and *in vivo* resistance to artesunate and mefloquine in Cambodia [205]. The R1466K SNP has also been associated with recrudescence after treatment with SP [206], however in recent studies from Benin and Myanmar no associations were found [184, 203].

1.6.2.7 *P. vivax* chloroquine resistance transporter – *pvcrt-o* and *P. vivax* multidrug resistance 1 - *pvmdr1*

So far no association between mutations in the *pvcrt-o* and *in vivo* or *in vitro* response to CQ have been found [119, 207-212]. The *pvmdr1* 976F allele has been associated with reduced susceptibility to CQ and amodiaquine [209, 213] and increased susceptibility to mefloquine and artesunate in Thailand, Indonesia and PNG [209, 213-215]. However, the correlation is not absolute as *P. vivax* CQ resistant can show Y976 and 976F parasites can be successfully treated [119, 208, 209, 216]. Amplifications of *pvmdr1* have been associated with reduced susceptibility to amodiaquine, mefloquine and artesunate in Thailand and Myanmar [209, 210, 213, 217]. However, in Indonesian *P. vivax*, resistant to the same drugs, no *pvmdr1* amplifications were found [213]. The role of *pvmdr1* in *P. vivax* drug resistance thus remains under discussion.

1.6.3 Antifolates

1.6.3.1 Dihydrofolate reductase – *dhfr* and dihydropteroate synthase – *dhps*

The *dhfr* coding region was cloned from *P. falciparum* in 1987 [218] and the *P. vivax* gene was identified in 1998 [219]. The *dhfr* genes from both species are ~66% identical and the active site regions are strongly conserved [220].

The gradual acquisition of resistance associated SNPs (N51I, C58R, S108N and I164L) in *pfdhfr* results in increasing levels of drug tolerance [221]. The triple dihydrofolate reductase (*pfdhfr*) haplotype N51I/C59R/S108N has been associated with SP treatment failure and when dihydropteroate synthase (*pfdhps*) SNPs A437G and K540E are added, highly SP resistant *P. falciparum* are generated [217, 222-225].

Twenty single nucleotide polymorphisms have been described in *P. vivax* dihydrofolate reductase (*pvdhfr*) including F57L, S58R, T61M and S117N/T that correspond to codons 50, 51, 59 and 108 in *pfdhfr*, respectively [132, 226-228]. *Pvdhfr* S58R and S117N result in decreased binding of PYR [220] and quadruple (F57L, S58R, S117N and I173L) SNPs have been associated with SP treatment failure [132, 226, 227].

2 AIMS OF THE THESIS

Overall Aim:

The overall aim was to determine temporal and geographic frequencies of known and putative genetic polymorphisms linked to *P. falciparum* and *P. vivax* drug resistance after exposure to various antimalarial drugs

Specific aims:

Paper I: Determine the proportion of chloroquine and sulphadoxine-pyrimethamine resistance associated genetic polymorphisms in *P. falciparum* and *P. vivax* collected in Honduras.

Paper II: Identify genetic changes in *pfvp2* and to describe their prevalence in 8 different countries and association with polymorphisms in *pfcrt* and *pfmdr1*.

Paper III: Study the early in vivo selection of genetic markers associated with antimalarial drug resistance after repetitive exposure to chloroquine, pyrimethamine or chlorproguanil compared with placebo in a previously virtually antimalarial drug naïve area of Liberia

Paper IV: Determine the temporal and seasonal frequency of genotypes associated with reduced susceptibility to chloroquine, artemether-lumefantrine and quinine between 2003 and 2012.

3 MATERIALS AND METHODS

3.1 Origin and sample collection

3.1.1 Honduras

In Honduras malaria transmission is seasonal and *A. albimanus* and *A. darling* are the two main vectors that transmit malaria during the rainy and dry seasons, respectively.[229]. Honduras contributes approximately 46% of malaria cases in Central America. More than 6,000 cases were reported in 2012 following an estimated incidence decrease of >75% since 2000 [9] The majority of malaria cases are caused by *P. vivax* and 12-15% are due to *P. falciparum* [9, 230].

CQ is recommended for treatment of uncomplicated *P. falciparum* and *P. vivax* infection. In addition, primaquine is used for treatment of *P. falciparum* gametocytes and *P. vivax* hypnozoites [231]. These drugs appear to remain effective despite being used for six decades and despite the spread of CQ resistant *P. falciparum* across most of the rest of the world [102, 232].

Filter paper samples for paper I were collected from adults that sought medical attention or were referred to the Hospital Escuela in Tegucigalpa between 2004 and 2006, and the regional hospitals in Trujillo, La Ceiba and Juticalpa, two primary health centres in Puerto Lempira and Iriona and one regional laboratory in Juticalpa during 2009. At the Hospital Escuela sample collection was considered to be part of routine malaria surveillance and did not involve additional sampling or collection of patient data. At the other health facilities patients who sought medical attention and were diagnosed with malaria were invited to participate in the study after written informed consent.

3.1.2 Colombia

Malaria transmission is endemic/epidemic and unstable throughout the country. Malaria is caused mainly by *P. vivax* (73%) and *P. falciparum* and occasionally by *P. malariae* [9, 233]. Samples for paper II were culture adapted isolates collected between 2001 and 2005 from endemic regions and were provided by Centro Internacional de Entrenamiento e Investigaciones Medicas (CIDEIM) in Cali, Colombia. When the samples were collected the national drug policy was amodiaquine + SP [233].

3.1.3 Liberia

Malaria was holoendemic when the samples were collected [234]. In 1976 (prior to deployment of monthly presumptive treatment) the overall parasite prevalence in the study area assessed by blood slide microscopy in 2-9 years old children was 82% for *P. falciparum*, 39% for *P. malariae* and 9% for *P. ovale* [235]. At the time of sample collection the official antimalarial drug policy was CQ [126] however, in 1976 the study area was virtually drug naïve.

Samples were collected in 1978 (paper III) and 1981 (paper II) as part of clinical studies in a rural area of northern Liberia 25 to 35 km from Yekepa. Four villages, about 5 km from each other, were included in study III: Bondi, Baytonwee, Bonah and Kinon. Access to healthcare and specifically antimalarial treatment was very limited when the study started in 1976. Children 2-9 years old were invited to participate in the study in 1976. Included children were given single doses of CQ, CPGN, PYR or placebo every four weeks for two years as follows. Bondi: 8-15 mg base/kg dose of CQ. Baytonwee: 1.0-2.0 mg base/kg of CPGN. Bonah: 1.3-2.5 mg base/kg of PYR. Kinon: 1 or 2 tablets of Vitamin B (placebo). Drug intake was supervised by the investigators [234]. After 2 years of monthly presumptive treatment, capillary whole blood samples were collected 4-6 weeks after last intake of study drug (i.e. from 28th of February to 17th of March, 1978) from all children (n=191) present and still included in the study. Samples selected for paper II were from children 2-9 years old from Bonah village in 1981.

3.1.4 Guinea-Bissau

Malaria is generally considered to be meso or hyperendemic in Guinea-Bissau. The main malaria species is *P. falciparum* (100%) [9]. Malaria is transmitted throughout the year with seasonal peaks before and towards the end of the rainy period between June and November. The mean annual incidence decreased from 16 per 1000 (1995–1997) to 3 per 1000 in 2007 and then increased to 26 per 1000 by 2011 and finally decreased to 10 per 1000 in 2012 [236]. Long lasting insecticide treated bed nets (LLIN) were distributed in 2011 and by 2012 there was 1 net per 2 people and 97% usage [236]. In June 2008 AL replaced an efficacious high dose CQ treatment regime (that was used instead of the officially recommended 25 mg/kg of CQ) for the treatment of uncomplicated malaria [237-239]. The high dose CQ treatment regime consisted of ~75mg/kg as 2-3 daily doses for 5 days [237]. QN remained the drug of choice for severe malaria throughout. CQ was also commonly used for home treatment of fever presumed to be malaria, at least until the introduction of AL. Paper II was

based on blood samples collected between 2003 and 2004 as part of a clinical trial and paper IV was based on all blood samples collected for genotyping from 2003 to 2008 and from 2010 to 2012 as part of five clinical trials. All trials were conducted within the Bandim Health and Demographic Surveillance Site (HDSS).

The drugs used as part of the studies were CQ or amodiaquine (2001-2004), CQ (2004-2006), CQ or AL (2006-2008), dihydroartemisinin-piperaquine or AL (November 2012-to date). As part of an effectiveness study (2010-2012) antimalarial drug use was monitored and AL was prescribed to 47%, QN to 44%, QN + AL to 9% and CQ to 1% of 799 children with uncomplicated malaria. Children with recurrent parasitaemia during study follow up were retreated with SP, CQ (50 mg/kg) or AL depending on the study.

3.1.5 Tanzania

Malaria is transmitted throughout the year with seasonal peaks during periods of increased rain in March-May and October-December. The main malaria species is *P. falciparum* (100%) [9]. Filter paper samples from paper II were collected from children ≤10 years as part of a community based cross sectional survey conducted at Fukayosi primary health care centre in 2008. The Fukayosi village is located in Bagamoyo district on mainland, Tanzania. Artemether + lumefantrine were used as first line treatment in Tanzania during the sample collection.

3.1.6 Iran

Malaria transmission occurs during the whole year and 90% of the cases are attributed to *P. vivax* and 10% to *P. falciparum* [9]. Whole blood samples were collected from adults at the Chabahar City Public Health Department in the Sistan-Baluchistan province of Iran. The study was conducted from April 2001 to March 2002 and the first line treatment was CQ according to national guidelines [240].

3.1.7 Thailand

Malaria in Thailand is endemic especially in the forest regions and the border areas. Malaria cases are attributed to *P. falciparum* (40%) and *P. vivax* (60%) [9]. Thailand's Western border with Burma/Myanmar and eastern border with Cambodia are epicentres of emerging antimalarial drug resistance. *P. falciparum* were collected from clinical cases (adults and children) from 2002 to 2008 in Mae Sot, in the Tak Province in west of Thailand and adapted for *in vitro* culture. The isolates were provided by the Shoklo Malaria Research Unit.

Artesunate + mefloquine were used as first line treatment for uncomplicated confirmed *P. falciparum* during sample collection [241].

3.1.8 Vanuatu

Vanuatu is a South Pacific archipelago made up of over 80 islands, each with varying levels of malaria. Malaria transmission is perennial but seasonal in intensity [242]. Malaria cases are attributed to *P. falciparum* (32%) and *P. vivax* (68%) [9]. Blood samples were taken from adults and children as part of community-based cross sectional survey in Ambae Island in 2002 and stored on filter paper. At the time CQ + SP were the first line treatment for uncomplicated *P. falciparum* malaria.

3.2 Sample selection

For papers I, III and IV all samples collected Honduras (2004-2006 and 2009), Liberia (1978) and Guinea-Bissau (2003-2008 and 2010-2012) were analysed. For paper II, previously extracted DNA from fifty randomly selected samples from Colombia (1999-2001), Liberia (1981), Guinea-Bissau (2001-2004), Tanzania (2008), Iran (2001-2002) and Vanuatu (2002) were selected and all available samples from Honduras (35) and Thailand (49) where were analysed.

3.3 Ethical considerations

All clinical studies had regional ethical approval as follows: Ethical Review Committee of Instituto Cardio Pulmonar in Tegucigalpa, Honduras, Liberian Institute of Biomedical Research. National Institute for Medical Research Tanzania (NIMR/HQ/ R.8A/Vol. IX/344) , Institute Pasteur in Iran (No. 502) , Ethical Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand , Ethical Review Committee of the Centro Internacional de Entrenamiento en Investigaciones Médicas (CIDEIM), Cali, Colombia. Guinea-Bissau studies were approved by Ethical review board in Bissau, Guinea-Bissau (Parecet NCP/N19/2006, 019/DHE/2004, 064/DGSP/2006) and Central Scientific Ethics Committee in Denmark (624-01-0042). Studies in Vanuatu were approved by the Ethical Committee in Tokyo Women's Medical University, Tokyo, Japan. Molecular analyses were approved by the Stockholm regional ethics board (2005/111-31/1, 2006/1151-31/1, 2011/832-32/2 and 2013/836-32).

3.4 Molecular Analysis

3.4.1 Sample storage, DNA extraction and amplification

Filter papers samples were collected in Honduras, Guinea-Bissau, Tanzania, Iran and Vanuatu. Capillary blood samples were collected in Liberia. Samples from Colombia and Thailand were culture adapted parasites. Filter papers were stored at room temperature while capillary blood was kept at -20°C.

DNA was extracted using the following methods: ABI Prism® 6100 Nucleic Acid Prep Station (Applied Biosystems, Fresno, CA) according to the manufacturer's instructions with minor modifications [243], QIAamp Blood Mini Kit (QIAgen Biosciences, Germantown, MD) and cheelex™ based method. Samples from study III (Liberia 1978) were extracted separately from other samples to minimise the risk of contamination Extracted DNA was stored at -20°C.

3.4.2 Restriction Fragment Length Polymorphism (RFLP)

Previously described multiplex PCR-RFLP methods were used to identify the following SNPs; *pfcrt* K76T, A220S, Q271E, N326D/S, I356L/T and R371I; *pfmdr1* N86Y and 184; *pfdhfr* N51I, C59R and N108T/S and *pfdhps* S436F/A, G437A and K540E [151, 244-246]. *Pfvp2* SNPs V405I and P711S were identified using nested PCR amplifications followed by restriction. A first set of primers were used to amplify nucleotide 1112 to 2260 of *pfvp2*. Two primer pairs were then used to amplify fragments that included codons 405 and 711. Primers were designed using Primer Express software (Applied Biosystems, Fresno, CA, USA) based on published sequence of *P. falciparum* (Gene Bank Accession No. AF283528). Primers and reactions conditions details are specified in paper II. All reactions included Taq polymerase reaction buffer, magnesium chloride, dNTPs, forward and reverse primer pair and GoTaq® DNA polymerase. Restriction enzymes (New England Biolabs) used to analyse SNP's 405 and 711 were AseI and DpnI.

PCR products were resolved on 2% agarose gels (Amresco, Solon, OH). All gels were stained with ethidium bromide or GelRed™ (Biotium Inc. Hayward, CA, USA) and visualized under UV transillumination (GelDoc®, Biorad, Hercules, Ca, USA).

3.4.3 Sequencing

Pfcrt 72-76, *pfdhfr* 16-185, *pfdhps* 436-632, *pfmdr1* 1034-1246, *pfnhe1* ms-4760 haplotypes were amplified using previously described PCR protocols [97, 151, 178, 247, 248]. New primers (nucleotides 1484-1929) were designed for a nested PCR amplification for identification of the *pfvp2* K582R allele. Primers and detailed reaction conditions are specified in paper II.

Previously described nested PCRs were used to amplify codons 917 to 1118 of *pvmdr1* and codons 1 to 238 of *pvdhfr* [133, 209]. PCR products were purified and sequenced commercially (Macrogen Inc. Seoul, Korea).

The Sequencher ™ software version 4.6 (Gene Codes Corporation, Ann Arbor, MI) was used for sequence analysis. The *P. falciparum* 3D7 clone sequence obtained from the NCBI database was used as references for *pfcrt* (Accession no. NC_004328), *pfmdr1* (Accession no. XM_001351751.1), *pfvp2* (Accession no. AF283528), *pfdhfr* (Accession no. XM_001351443.1) *pfdhps* (Accession no. XM_001349382.1) and *pfnhe1* (Accession no. XM_001349726). *Pfnhe1* ms4760 sequences were also compared with previously described isolates and clones [97, 249]. For *pvdhfr* the *P. vivax* ARI/Pakistan isolate sequence (Gen-Bank accession no. X98123) and for *pvmdr1* the *P. vivax* Sal-1 isolate sequence (Gen-Bank accession no. AY618622) were used as references.

3.4.4 Real-Time PCR

Pfmdr1 and *pvmdr1* copy numbers were determined using real time PCR (ABI Prism® 7000 Sequence Detection System) as previously described [62, 210]. All samples were run in triplicate. For *pfmdr1* 3D7, D10 and K1 clones were used as single copy calibrators and FCB and Dd2 were multiple copy controls. *Pvmdr1* single and double copy calibrators were created by the insertion of *pvmdr1* nucleotides 2751-3354 and *pvbtubulin* nucleotides 860-1056 in the pCR2.1 vector using the TOPO TA-cloning kit (Invitrogen, Carlsbad, CA) at 1:1 and 2:1 proportions, respectively. The sample copy numbers were calculated using a comparative threshold method ($\Delta\Delta C_t$). Copy number >1.6 and copy number >2.6 were defined as 2 and 3 copies of *pfmdr1*, respectively. Assays were repeated if the following results were obtained: copy number 1.3-1.6 and 2.3-2.6 or Ct value >35 or standard deviation value >0.5

3.5 Statistics

Data for papers I to III were entered, validated and analysed on Microsoft Excel 2003. Allele proportions were calculated by dividing the number of samples with a certain allele by the number of samples with an identifiable allele at that position. Thus mixed infections contributed to the proportion of both alleles. Associations were determined using Fishers Exact test using StataCorp 12.

Data for paper IV were entered on EpiData or Microsoft Excel and transferred to Stata Corp 12 for analyses. As above SNP frequencies were calculated as the number of samples with a certain allele. Haplotype frequencies were calculated after excluding mixed infections. Yearly and seasonal changes of allele and haplotype frequencies were estimated using logistic regression with bootstrapping (100 repeats) with year or month as continuous covariate and the earliest time point as baseline. For calculation of seasonal changes, February was the earliest time point as the high transmission season ends in January. Parasite densities and age were estimated and compared using quantile regression with bootstrapping (100 repeats).

For paper II and IV, linkage disequilibrium was calculated as a correlation coefficient between pairs of loci after excluding mixed infections. For paper II, absolute linkage was indicated by a value D=1 whereas D=0 indicated no linkage. For paper IV a correlation coefficient (r) of 1 indicated absolute correlation, 0 indicated no correlation and -1 total negative correlation.

4 RESULTS AND DISCUSSION

4.1 Genetic Polymorphisms in *P. falciparum*

4.1.1 *P. falciparum* chloroquine resistance transporter

4.1.1.1 *Pfcrt* 72-76 haplotypes

The *pfcrt* 72-76 haplotype was determined in samples from Honduras (30), Colombia (50), Liberia (50), Guinea-Bissau (50), Tanzania (50), Iran (50), Thailand (49) and Vanuatu (38) representing different origins of CQ resistance in papers I and II. In paper III, the *pfcrt* 72-76 haplotype was determined in 178 samples after repeated antimalarial exposure in a previously virtually antimalarial drug naïve area of Liberia. The haplotypes found and the frequencies in the various countries are shown in Figure 2. Three (of 5) different *pfcrt* 72-76 CQ resistant haplotypes were found, specifically, CVMNT from Colombia, SVMNT from Papua New Guinea and CVIET from Thailand (which subsequently spread to Africa) [59].

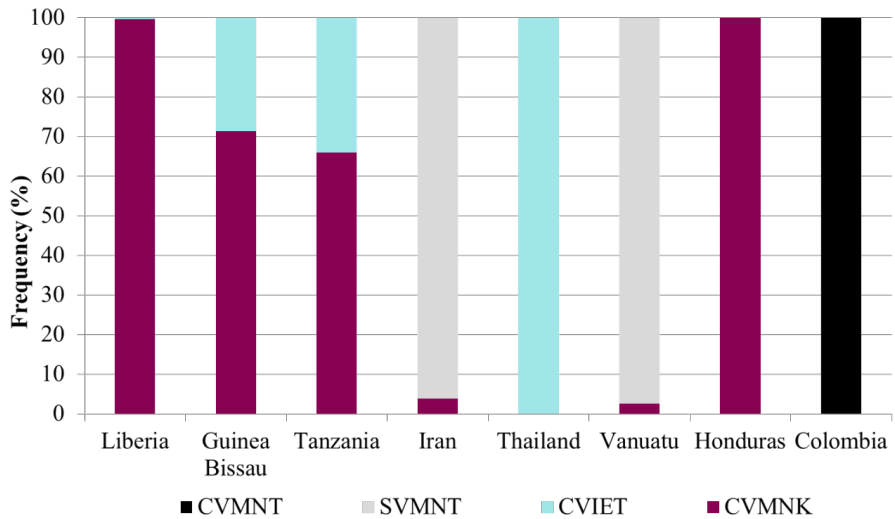

Figure 2.- *Pfcrt* 72-76 haplotypes and frequencies. Infections with mixed *pfcrt* 72-76 haplotypes were excluded. One *pfcrt* 72-76 CVIET haplotype was found in Liberia.

All 30 samples from Honduras carried the *pfcrt* CVMNK haplotype that is linked to CQ sensitivity (paper I and II). The results are in line with continued efficacy to CQ seen *in vitro* and *in vivo* [232, 250, 251]. This was, to our knowledge, the first report of these haplotypes from Mesoamerica. The *pfcrt* CVMNK haplotype was also found in 227/228 samples collected in Liberia in 1978 (paper III) and 1981 (paper II). Finding the CVMNK haplotype

in Liberia was in line with *in vivo* and *in vitro* CQ sensitivity results found at the time of sample collection [234, 252, 253]. The CVMNK haplotype found in Liberia, was the same as that found in 226/226 samples from Gambia collected in 1984 [254] and in CQ sensitive parasites in Guinea-Bissau [239] indicating that this was the wild type haplotype in West Africa before CQ resistance arrive to the area.

A single sample carrying *pfcrt* CVIET was detected in Liberia in 1978 after treatment with CQ (paper III). Though this *pfcrt* CVIET might have been imported from Southeast Asia it did not show the additional SNPs at codons 220, 271, 326, 356 and 371 usually present in Southeast Asian *pfcrt* [255]. It is thus probable that *pfcrt* 72-76 CVIET arose in the study area after intermittent treatment with CQ for only two years. A recent study described how resistance associated *pfcrt* SNPs accumulated in an ordered fashion controlled by a balance between improved CQ transport versus loss of fitness / function induced by mutations [155]. Having CVIET alone with no other SNPs in *pfcrt* only resulted in a low increase of the ability to transport CQ and hence to confer resistance. Thus CVIET might have developed in Liberia but it probably did not provide a selective advantage in this holoendemic area accounting for the haplotype not becoming widespread as indicated by all samples from Liberia collected in 1981 only having the CVMNK haplotype. In line with this, CQ resistant *P. falciparum* took approximately 12 years to develop in Southeast Asia and South America [31] from where it was eventually imported to Africa. Nevertheless, the possibility that this haplotype seems to have arisen de novo is intriguing and warrants further study to confirm this.

4.1.1.2 *Pfcrt* K76T

High *pfcrt* K76 proportions were found in Honduras (30/30 [100%]) and Liberia (227/ 228 [99.5%]) as shown in papers I to III, in Guinea-Bissau (614/766 [80%]) from 2003 to 2007 and in Tanzania (33/50 [66%]) as shown in papers II and IV. *Pfcrt* 76T proportions were high in Iran (49/50 [98%]), Vanuatu (38/38 [100%]), Colombia (50/50 [100%]) and Thailand (49/49 [100%]) as shown in paper II.

The ~100% *pfcrt* K76 frequency in Honduras and Liberia was in line with continued CQ efficacy in both countries [234, 256]. Surprisingly and for principally unknown reasons *P. falciparum* in Honduras therefore remain susceptible to CQ despite six decades of use, the worldwide spread of CQ resistance and the sporadic occurrence of CQ resistant *P. falciparum* in Nicaragua [257]. The high *pfcrt* K76 proportion in Guinea-Bissau concurrently with continued CQ use was also surprising but probably due to the routine use of high doses of CQ (~75mg/kg as 2-3 daily doses for 5 days) as discussed previously [258]. Briefly, higher

concentrations of CQ can effectively treat *P. falciparum* with *pfcrt* 76T. Furthermore *pfcrt* 76T causes a loss of fitness as discussed above. Thus *pfcrt* 76T did not provide a survival advantage when high CQ doses were routinely used and therefore did not accumulate. In line with this *pfcrt* K76 (1800 *P. falciparum*/μl) was associated with a significantly higher (p=0.005) day 0 parasite density compared to *pfcrt* 76T (15120 *P. falciparum*/μl) in Guinea-Bissau. Further evidence of the cost to fitness caused by *pfcrt* 76T is the rapid decrease of *pfcrt* 76T and increase of *pfcrt* K76 when CQ pressure decrease as seen in The Gambia and Malawi [259, 260]. Most probably the high *pfcrt* 76K in Tanzania was similarly an effect of a change of treatment policy from CQ to AL 7 years prior to sampling. Considering this loss of fitness and the use of artesunate + mefloquine it is interesting to note that the *pfcrt* 76T prevalence was 100% in Thailand where CQ resistance developed de novo unlike Africa where CQ resistance was imported. The high *pfcrt* 76T frequencies in Iran, Vanuatu and Colombia were on the other hand as expected given that CQ or AQ were used as first line treatment when samples were collected.

Pfcrt K76T frequencies were determined in 1806 children aged less than 15 years with uncomplicated *P. falciparum* malaria between 2003 and 2012 in Guinea-Bissau (Figure 3) as part of study IV. The *pfcrt* K76 frequency decreased from ~80% (2003-2007) to 67% (2008-2012) (p<0.001). Concurrently, the *pfcrt* 76T frequency increased significantly (p<0.001) between 2007 and 2012. In more detail, the *pfcrt* 76T frequency gradually increased from 25% (10/40) in 2007 to 57% (195/345) in 2011 and then appeared to decrease to 34% (41/121) in 2012. The annual median age increased from 5 years and 8 months to 10 years between 2003 and 2012 (p<0.001). The increased *pfcrt* 76T and decreased *pfcrt* K76 frequencies following the introduction of AL in 2008 contradicted expectations as *pfcrt* K76 has been associated with reduced susceptibility to lumefantrine and has been selected for after treatment with AL [183, 238, 261]. Furthermore, *pfcrt* 76T has, as discussed above, been associated with a fitness cost.

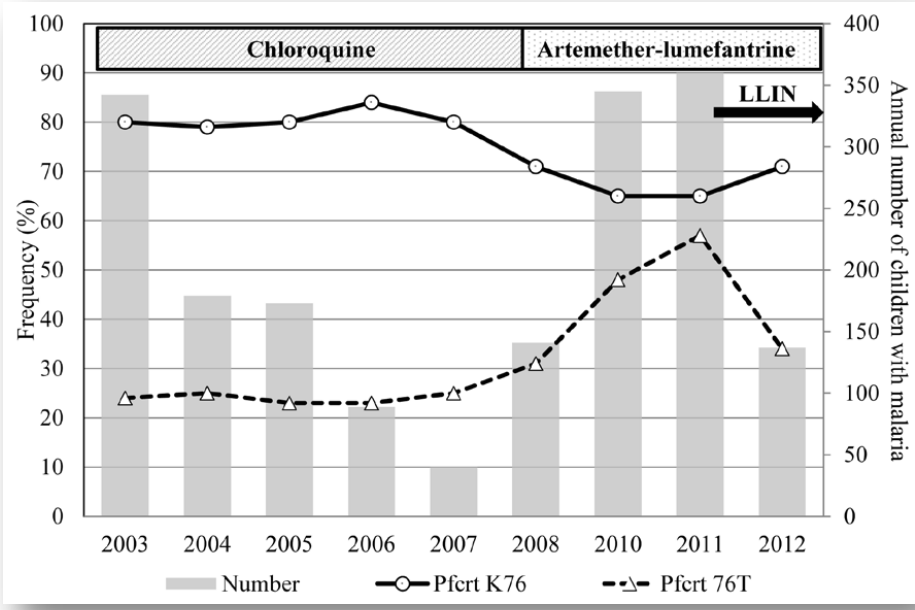

Figure 3.- *Pfcrt* K76T allele frequencies in children aged less than 15 years attending the Bandim health centre, Guinea-Bissau between 2003 and 2012. *Pfcrt* 76T frequency increased to 57% between 2007 and 2011 ($p<0.001$) and then decreased to 34% in 2012 ($p<0.001$).

Between 2010 and 2012 QN was prescribed to 44% of children diagnosed with *P. falciparum* malaria [236]. Several studies have linked *pfcrt* 76T to decreased QN susceptibility [168, 262-264]. In line with this, the *pfcrt* 76T frequency was higher amongst recrudescing *P. falciparum* compared to day 0 after treatment with QN in Guinea-Bissau [236]. Furthermore, *pfcrt* 76T increased from an average 37% (50/136) during the low transmission period (February to July), to 55% (69/126) at the end of the high transmission period (December, $p=0.03$) between 2008 and 2012 as shown in Figure 4. The accumulation of *pfcrt* 76T indicates that *pfcrt* 76T conferred a selective advantage as drug pressure increased.

Thus is seems highly probably that the increased 76T frequency was due to QN use. Interestingly this was not seen when high dose CQ was used indicating that 76T provided a greater selective advantage when QN was used compared to when high dose CQ was used. A possible explanation is that sub-therapeutic doses of QN were probably used. QN has traditionally been prescribed as a short 3 day treatment in Guinea-Bissau [265] and this appeared to be common between 2010 and 2012 as well.

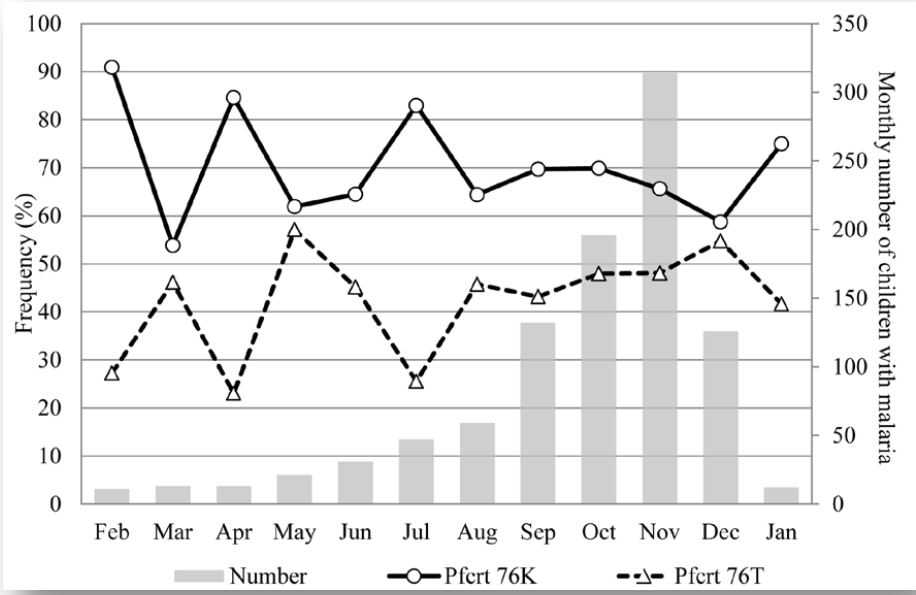

Figure 4.- Monthly *pfcrt* K76T allele, frequencies between 2008 and 2012 in Guinea Bissau. *Pfcrt* 76K possibly decreased from an average 74% (100/136) during the low transmission period (February to July) to 59% (74/126) in December (p=0.05).

Alternative explanations for the accumulation of *pfcrt* 76T include selective pressure by AL. However, a study completed in 2008 indicated that AL selected *pfcrt* K76 in Bissau as elsewhere suggesting that this is unlikely [238, 261]. Continued CQ use might also select for *pfcrt* 76T. However, based on prescription data collected 2010-2012 and informal questioning, CQ is hardly used anymore [266]. Furthermore, if CQ was the driving force, a novel lower and less efficacious CQ dose compared to that routinely used in the past would have had to have been used. Increased *pfcrt* 76T frequency could also be the result of selection after the 2007 bottleneck event (when malaria drastically decreased) due to better inherent fitness or a selective advantage in older children. For example older children may be more likely to self-medicate with CQ at home prior to seeking care at Bandim Health Centre. However, SNP frequencies did not vary with age and proxies for fitness such as lower parasite density and lower *pfcrt* 76T frequency during the low transmission period suggest that *pfcrt* 76T was associated with a fitness cost - not benefit.

4.1.2 *P. falciparum* multidrug resistance 1

4.1.2.1 *Pfmdr1* N86Y

All samples (30/30) from Honduras (paper I) and all Liberian samples (161/161) collected in 1978 (paper III) carried *pfmdr1* N86. Amongst the 47 samples from Liberia collected in 1981 one had *pfmdr1* 86Y and one had mixed *pfmdr1* N86 + 86Y (Paper II). Detailed *pfmdr1* N86Y frequencies from papers I to IV are shown in Table 1.

Country	***pfmdr1***	
	N86	**86Y**
Liberia 1978	191/191 (100%)	0/191
Liberia 1981	46/47 (98%)	2/47 (4%)
Guinea-Bissau 2003-2007[a]	564/770 (73%)	332/770 (43%)
Guinea Bissau 2007-2010	682/880 (77%)	274/880 (31%)
Tanzania	34/50 (68%)	16/50 (32%)
Iran	13/50 (26%)	37/50 (74%)
Thailand	49/49 (100%)	0/49
Vanuatu	0/32	32/32 (100%)
Honduras	30/30 (100%)	0/30
Colombia	50/50 (100%)	0/50

Table 1 *Pfmdr1* N86Y frequencies in data pooled from studies I-IV
[a]Fifty samples randomly selected collected between 2003-2004 were used for paper II

Pfmdr1 N86 was determined in 1650 samples from Guinea-Bissau collected between 2003 and 2012 in paper IV. *Pfmdr1* N86Y allele frequencies did not change significantly before 2007. *Pfmdr1* N86 increased significantly (63% to 83%, $p<0.001$) and *pfmdr1* 86Y decreased significantly (48% to 34%, $p<0.001$) between 2007 and 2012, as shown in figure 5.

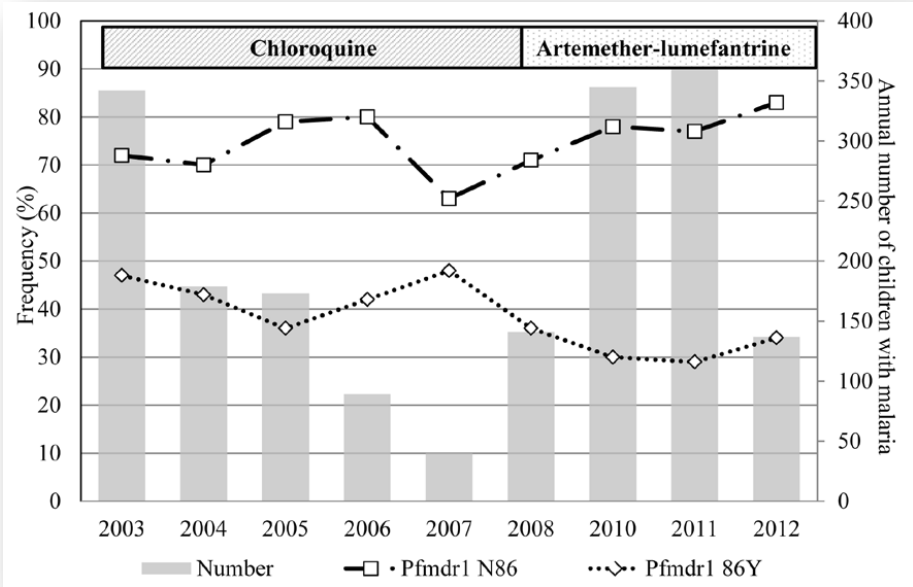

Figure 5.- *Pfmdr1* N86Y allele frequencies in children aged less than 15 years attending the Bandim health centre, Guinea-Bissau between 2003 and 2012.

Pfmdr1 86Y has previously been associated with CQ and amodiaquine resistance *in vitro* and *in vivo* [172, 182, 245, 267-271]. However, some studies have contradicted this association [272, 273]. Nevertheless there is a consensus that *pfmdr1* enhances CQ resistance [151, 185, 274] and or compensates for loss of fitness in *P. falciparum* with *pfcrt* 76T [275, 276]. The high proportion of *pfmdr1* N86 found together with *pfcrt* K76 in Liberia (paper II and III) indicates that KN was the wild type haplotype. Two samples collected in 1981 in Liberia were found to have *pfmdr1* 86Y. Similar low frequencies of *pfmdr1* 86Y (5/132) were found in 1984 in The Gambia [254]. This is to our knowledge the earliest report of *pfmdr1* 86Y in West Africa and together with the Gambian data it shows that *pfmdr1* 86Y occurred in West Africa prior to the spread of CQ resistance. Both Liberian samples were from the village in which monthly CQ was previously administrated and where a single sample with *pfcrt* CVIET was found. Though 86Y may have occurred naturally it is also possible that 86Y was an early response to CQ exposure.

In line with previous reports linking *pfmdr1* 86Y to enhanced CQ resistance [185, 274], high proportions were found in Vanuatu and Iran were *pfcrt* 76T frequencies were ~100% and CQ was first line treatment for uncomplicated *P. falciparum* malaria at the time of sample

collection. High *pfmdr1* 86Y frequencies in Iran and Vanuatu were also in line with reports of CQ failure in these countries [277, 278]. However, without data from the time before CQ resistance it is not possible to draw any conclusions. *Pfmdr1* 86Y did not accumulate between 2003 and 2007 in Guinea-Bissau suggesting that 86Y just as 76T did not provide a significant selective advantage when high dose CQ was used in Guinea-Bissau.

4.1.2.2 *Pfmdr1* N86Y and *pfcrt* K76T haplotypes in Guinea-Bissau.

Further analysis showed that *pfcrt* 76 + *pfmdr1* 86 KN ($p=0.002$) and KY ($p<0.001$) haplotypes decreased significantly whereas TY remained stable at ~10% between 2003 and 2012 in Guinea-Bissau. The TN frequency was ~10% between 2003 and 2007 but then increased to 27% by 2012 ($p=0.001$). Frequencies of *pfcrt* K76Y + *pfmdr1* N86Y haplotypes are presented in table 2. Median parasite densities were significantly higher with *pfcrt* 76 + *pfmdr1* 86 KN (18000 *P. falciparum*/μl, $p=0.008$) and KY (18400 *P. falciparum*/μl, $p=0.004$) compared to TY (14640 *P. falciparum*/μl) but there was no significant difference between KN or KY compared to TN (15840 *P. falciparum*/μl).

As discussed above QN may have selected *pfcrt* 76T. In line with this, the increase of *pfcrt* 76T + *pfmdr1* 86N between 2007 and 2012 in Guinea-Bissau could be due to direct selection by QN. However, decreased QN susceptibility has more often been linked with the TY haplotype [168, 264]. The discrepancy may be due to the probably multigenic basis of QN resistance [75, 168]. Alternatively, the TN haplotype is inherently fitter compared to TY, at least in Guinea-Bissau. The significantly lower parasite density found with TY but not with TN compared to KN or KY might support this, assuming that day 0 parasite density is a proxy for inherent parasite fitness. The improved fitness of TN compared to TY may also contribute to the fixed *pfcrt* 76T + *pfmdr1* N86 haplotype seen in Colombia and Thailand.

Year	*Pfcrt* K76T + *pfmdr1* N86Y haplotypes				
	Total Number	KN[a]	KY[a]	TN[b]	TY
2003	254	56% (142)	23% (59)	9% (24)	11% (29)
2004	129	56% (72)	21% (27)	9% (11)	15% (19)
2005	139	65% (91)	14% (20)	10% (14)	10% (14)
2006	65	63% (41)	15% (10)	11% (7)	11% (7)
2007	36	47% (17)	31% (11)	11% (4)	11% (4)
2008	121	46% (56)	23% (28)	22% (27)	8% (10)
2010	263	50% (132)	10% (26)	26% (68)	14% (37)
2011	263	46% (122)	12% (31)	29% (75)	13% (35)
2012	89	54% (48)	10% (9)	27% (24)	9% (8)

Table 2. *Pfcrt* K76T and *pfmdr1* N86Y haplotype frequencies between 2003 and 2012 amongst children diagnosed with *P. falciparum* malaria at the Bandim health centre.
[a] KN (p=0.002) and KY (p<0.001) frequencies decreased over time. [b] TN increased between 2007 and 2012 (p=0.001).

4.1.2.3 *Pfcrt* K76T and *pfmdr1* N86Y / F184Y / D1246Y haplotypes in Guinea-Bissau

Pfmdr1 86 + 184 NF frequency increased significantly from 39% (87/223) in 2003 to 66% (33/50) in 2012 (p=0.004) whilst YF decreased significantly (p<0.001) from 37% (82/223) to 10% (5/50) in the same period as shown in table 3. The *pfmdr1* 1034, 1042 and 1246 haplotype was SND in 137/138 samples between 2003 and 2004 and 315/316 in 2011.

Year	*Pfmdr1* N86Y + *pfmdr1* F184Y haplotypes				
	Total number	NF[a]	NY	YF[b]	YY
2003	223	39% (87)	22% (49)	37% (82)	2% (5)
2010	221	52% (116)	25% (55)	19% (41)	4% (9)
2011	246	46% (114)	27% (66)	22% (54)	5% (12)
2012	50	66% (33)	24% (12)	10% (5)	0% (0)

Table 3.- *Pfmdr1* N86Y and *pfmdr1* F184Y haplotype frequencies between 2003 and 2012 amongst children diagnosed with *P. falciparum* malaria at the Bandim health centre. [a] NF frequency increased (p=0.004). [b]YF frequency decreased over time (p<0.001).

Pfcrt 76 + *pfmdr1* 86 + *pfmdr1* 184 haplotypes were determined in 219, 200, 191 and 42 samples in 2003, 2010, 2011 and 2012, respectively. The KNF frequencies did not change significantly (p=0.8), KYF decreased from 24% to 7%, 11% and 7%, respectively (p<0.001), TNF increased from 7% to 17%, 17% and 21%, respectively (p=0.002) and TNY increased from 3% to 10%, 13% and 7% (p=0.001).

Reduced susceptibility to lumefantrine and recurring parasites after treatment with AL have been linked to *pfmdr1* N86, the *pfmdr1* 86+184+1246 NFD haplotype and *pfmdr1* amplifications in addition to *pfcrt* K76 [61, 183, 238, 261, 279-282]. Increased *pfmdr1* 86N and *pfmdr1* 86+184 NF haplotype frequencies following the introduction of AL are in line with these previous observations. As *pfmdr1* D1246 was fixed at ~100% frequency in our study area the selection of *pfmdr1* 86+184 NF actually represented a selection of the *pfmdr1* NFD haplotype that has been linked to a 15 fold higher *in vivo* AL tolerance [279]. As discussed above QN probably selected the *pfcrt* 76 + *pfmdr1* TN haplotype. As both the *pfcrt 76* + *pfmdr1* 86 + 184 TNF and TNY haplotypes increased it seems likely that neither *pfmdr1* 184 allele conferred a selective advantage against QN. The increased NF frequency was therefore more likely driven by AL than QN.

4.1.2.4 *Pfmdr1* copy number (CN) variation

Pfmdr1 CN variation was determined in 320 samples from 8 different countries in paper II. One *pfmdr1* copy was found in Liberia (30/30), Tanzania (46/46), Iran (36/36), Thailand (26/49), Vanuatu (9/9), Honduras (28/28) and Colombia (44/44). Two (14/49) and three (5/49) copies of the *pfmdr1* gene were present in samples from Thailand. In Guinea-Bissau (paper II and IV) one gene copy was found in 1635 samples, 1.5 copies were found in two samples and one sample had two copies.

Increased *pfmdr1* CN has been linked to reduced susceptibility to artemisinin derivatives, lumefantrine, piperaquine and mefloquine as well as to artesunate + mefloquine treatment failure [166, 283-285]. Not finding *pfmdr1* CN variation in Honduras, Colombia, Liberia, Iran, Vanuatu and Guinea-Bissau before 2008 was therefore consistent with expectations as they were not using antimalarial drugs that select for increased *pfmdr1* CN when the samples were collected. The high frequency of >1 CN in Thailand is in line with previous reports and has been discussed elsewhere [65, 204]. It was encouraging that only 1 *pfmdr1* copy was found in Tanzanian samples despite AL being used there since 2001. Finding 3 samples with increased CN in Guinea-Bissau could be due to introduction of AL there. However, the first sample with two copies was detected in 2008 and the frequency did not subsequently increase suggesting that increased CN did not provide *P. falciparum* with a great selective advantage in Guinea-Bissau. Alternatively, finding so few samples with increased copy number might well be within the margin of error when doing PCRs suggesting that none exist in Bissau. Either way *P. falciparum* with multiple *pfmdr1* copies have not become widespread in Bissau

or Tanzania contrary to the situation in Ghana where relatively high frequencies were recently reported [281].

4.1.3 *P. falciparum* V-type H^+ pyrophosphatase

Frequencies of *pfvp2* V405I, K582R and P711S were determined in 8 different countries as shown in table 4. The most striking result was the lack of variation of the *pfvp2* alleles studied. Only 26 SNPs in 20 samples were found among 344 samples collected in 8 countries with varying origins and proportions of CQ resistant *P. falciparum* at the time of blood sampling. The results thus suggest that the parts of the *pfvp2* gene that were analysed are conserved.

Country	*Pfvp2*		
	405I	**582R**	**711S**
Liberia	3/48	1/48	4/49
Guinea Bissau	3/50	1/50	3/50
Tanzania	0/49	1/50	2/48
Iran	1/50	1/50	1/50
Thailand	1/49	0/49	1/49
Vanuatu	0/31	0/32	0/38
Honduras	2/30	0/30	1/30
Colombia	0/46	0/46	0/50

Table 4 – Frequencies and geographical distribution of polymorphisms in *pfvp2*.

Despite the lack of variation the *pfvp2* 405+582+711 VKP haplotype was found to be associated with *pfcrt* 76T (p=0.007) as shown in table 5. Furthermore, linkage disequilibrium analysis also showed that *pfvp2* VKP haplotype was linked with *pfcrt* 76T (D=0.5). As *pfcrt* 76T is essential for CQ resistance these results suggest that the *pfvp2* 405V, 582K and 711P haplotype is associated with CQ resistance. *Pfvp2* up-regulation has been shown to occur in *P. falciparum* with the CQ resistant allele *pfcrt* 76I but not with the CQ sensitive allele 76K under CQ pressure [191]. This was proposed to be due to a need for increased H^+ transport into the parasite DV to compensate for H^+ loss when protonated CQ was transported out [153, 154]. Assuming that *pfvp2* functions as suggested our results indicate that in *P. falciparum* with the *pfcrt* 76T genotype the *pfvp2* 405V, 582K and 711P haplotype provides the most efficient H^+ pump.

pfvp2	***Pfcrt***		***pfmdr1***	
	K76	76T	N86	86Y
V405	95% (138/145)	99% (198/201)[a]	97% (235/242)	98% (99/101)
K582	98% (143/146)	99% (199/200)[b]	99% (240/243)	99% (101/102)
P711	95% (138/146)	99% (204/207)[c]	96% (236/246)	99% (103/104)
VKP haplotype	90% (131/145)	97% (191/196)[d]	93% (224/240)	97% (95/98)
405I	5% (7/145)	1% (3/201)	3% (7/242)	2% (2/101)
582R	2% (3/146)	1% (1/200)	1% (3/243)	1% (1/102)
711S	5% (8/146)	1% (3/207)	4% (10/246)	1% (1/104)
I and/or R and/or S*	10% (14/145)	97% (5/196)	7% (16/240)	3% (3/98)

Table 5. - The frequency of *pfvp2* alleles in *P. falciparum* with varying *pfcrt* K76T and *pfmdr1* N86Y alleles. [a] V405 occurred non significantly more often with 76T P=0.1; [b] K582 occurred non significantly more often with 76T P=0.3; [c] P582 occurred non significantly more often with 76T P=0.06; [d] The *pfvp2* V405 + K582 + P711 haplotype was significantly more common with *pfcrt* 76T (P=0.007); * i.e. not the VKP haplotype. Patients with both *pfcrt* K76 and 76T and patients with both *pfmdr1* N86 and 86Y were excluded.

Compared to all other countries the proportion of patient samples with any of *pfvp2* 405I, 582R and/or 711S was significantly more common in the following countries (table 4); Liberia (P=0.01), African countries (Liberia + Guinea-Bissau + Tanzania, P=0.004) and countries where CQ resistance had not been described at the time of blood sampling (Liberia + Honduras, P=0.001) compared to the other countries studied. In line with these findings there was also an association between samples with any of *pfvp2* 405I, 582R and/or 711S with *pfcrt* 76K (P=0.007) and the *pfcrt* 76K + *pfmdr1* 86N haplotype (P=0.002). Furthermore, *pfvp2* 405I and/or 582R and/or 711S (i.e not the VKP haplotype) were linked with *pfcrt* 76K (D=0.5). Finding *pfvp2* 405I, 582R and/or 711S alleles in CQ sensitive settings in both Africa and the Americas suggests that there was a larger variation in the *pfvp2* genome prior to the spread of CQ resistance supporting the association between *pfvp2* and CQ resistance.

Fourteen of 20 patient samples with *pfvp2* 405I, 582R and/or 711S came from African countries of which, 11/20 came from West Africa. This might suggest that the association between *pfvp2* and *pfcrt* is incidental possibly due to geographical variation. However the *pfvp2* SNPs were also linked to *pfcrt* 76K in Tanzania. An alternative explanation for the relatively common occurrence in Africa is that CQ resistance had not reached Liberia at the time of sampling and the proportion of CQ resistant *P. falciparum* had remained relatively low ~25% in Guinea-Bissau [258]. There had thus been less selective pressure on *pfvp2* in these two countries and *P. falciparum* had not passed through the parasite population bottle neck of spreading CQ resistance. Despite the link between *pfvp2* V405 + K582 +P711 and

pfcrt 76T very little variation was found in the *pfvp2* gene, therefore the results should be interpreted with caution.

4.1.4 *P. falciparum* Na+/H+ exchanger-1

Eight of the 114 previously described *pfnhe1* variants [97, 249] and 47 novel ms4760 variants were found in 156 samples collected in Liberia in 1978 (paper III). Similar high diversity has previously been reported from different African countries but not in samples from Asia [249, 286]. Finding higher diversity in Africa might be linked to greater malaria endemicity of *P. falciparum*. Distribution of DNNND and DDNHNDNHNND repeats in Blocks II and V were similar in all villages. Having more than one DNNND repeat and one DDNHNDNHNND repeat in the coding ms4760 of *pfnhe1* and specifically the ms4760-1 haplotype that has 2 x DNNND and 1 x DDNHNDNHNND have been associated with QN resistance *in vitro* and *in vivo* in some studies but not in others [75, 97, 249, 286]. Though QN was not used in the Yekepa area at the time of this study the frequency of *pfnhe1* ms4760-1 (26%) was similar to the frequency found at day 0 in a recent study in Mali where ms4760-1 was associated with QN resistance [97]. Furthermore, the number and frequencies of DDNHNDNHNND repeats in this study were similar to data found in 393 clinical isolates recently reported from Senegal [286]. Despite the geographic separation, the similarity to that found in Liberia in 1978 , prior to a drug selective pressure, suggests that ms4760-1 did not play a significant role in the emergence of resistance in West Africa over subsequent years.

4.1.5 Polymorphisms associated with antifolate resistance: *P. falciparum* dihydrofolate reductase and *P. falciparum* dihydropteroate synthase.

Pfdhps and *pfdhfr* SNPs were analysed in samples from Honduras collected between 2004 and 2006 and in 2009 (paper I) and in samples from Liberia collected in 1978 (paper III). The PYR resistance associated *pfdhfr* 108N and sulphadoxine resistant associated 613S were found in 38% (65/169) and 10% (14/135) of Liberian samples. No other SP resistance associated polymorphisms were found. Both *pfdhfr* 108N and *pfdhps* 613S were significantly lower in the village using PYR (1/46 and 0/38 respectively) compared to the other villages as presented in table 6. All Honduran samples (30/30) had haplotypes *pfdhfr* N51 + C59 + S108 and *pfdhps* A437 + K540 linked to sensitivity to SP.

The high frequency of *pfdhfr* 108N in the placebo village indicates that 108N was a naturally occurring allele prior to the introduction of antifolates for the treatment of malaria

in Liberia in 1978. Similarly, *pfdhfr* 51I, 59R, 108N SNPs were found in 9/66 samples collected in The Gambia in 1984 prior to SP use [254]. Contrary to our expectations *pfdhfr* 108N was not selected in the PYR village despite development of *in vivo* resistance to PYR within a year of initiating monthly intermittent presumptive therapy [234]. This indicates that there was an alternate mechanism of PYR resistance as has previously been suggested [287, 288]. Furthermore, the 108N did not provide a selective advantage in the presence of this alternate mechanism but possibly the opposite. Amplifications of the *pfdhfr* or GTP-cyclohydrolase (*gch1*) genes have been linked to PYR resistance in the past [289, 290]. Gene amplifications could have occurred in Liberia and might thus constitute an early method of drug resistance in line with the correlation between *pfmdr1* amplifications and drug resistance to mefloquine and artemisinin derivatives [283]. An alternative mechanism of PYR resistance might be a greater influx of folate into the parasite as has been suggested to be mediated by *pfmrp1* 1466K [206]. However, in Liberia only *pfmrp1* R1466 was found. PYR is not likely to directly exert a selective effect on *pfdhps* yet the frequency of *pfdhps* 613S (that is related to sulphadoxine resistance) was lower in the PYR treated village (0/38). Perhaps this represents an indirect effect of selection of other genes.

Village	***Pfdhfr***			***Pfdhps***	
	S108	108**N**	108**S+N**	A613	**613S**
Chlorproguanil	21/39 (54%)	17/39 (45%)	1/39 (3%)	29/32 (91%)	3 (9%)
Pyrimethamine	45/46 (98%)	**1/46 (2%)[b]**	0	38/38	**0**[c]
Chloroquine	21/39 (54%)	18/39 (46%)	0	19/26 (73%)	7(27%)
Placebo [a]	15/45 (33%)	29/45 (65%)	1/45 (2%)	35/39 (90%)	4 (10%)

Table 6. Allele frequencies at resistance associated *pfdhfr* codon 108 and *pfdhps* codon 613 from samples collected in Liberia in 1978. Resistance associated alleles are shown in bold. [a] Vitamin B. [b] The frequency of samples with the resistance associated *pfdhfr* 108N was lower in the pyrimethamine treated village when compared with separate and pooled frequencies from the other villages (P<0.001). [c] The frequency of samples with the resistance associated *pfdhps* 613S was lower in the pyrimethamine treated village compared to pooled frequencies from the other villages (P<0.01) and the village using CQ (P=0.001), but the differences were not significant compared to the villages using chlorproguanil (P=0.09) or placebo (P=0.12).

Finding no SNPs associated with SP resistance in Honduras indicates that *P. falciparum* remained sensitive to SP. In line with this antifolates were not previously used as CQ remains effective for the treatment of uncomplicated malaria in Honduras. However, SP was recently recommended as the second line treatment for uncomplicated malaria in Honduras and given the rapid development of SP resistance in the past continued monitoring would be worthwhile. Furthermore, the lack of resistance associated SNPs in Honduras indicates that artemisinin based treatment combinations containing SP could be used if Honduras wishes to change to an ACT. However, the results from Liberia presented above caution against only relying on genotyping to monitor for drug resistance.

4.1.6 CQ and SP resistance in imported malaria in Honduras.

In Paper I two patients with imported malaria that most probably contracted *P. falciparum* on a Pacific Island and West Africa were included. The patient from the Pacific Island had *P. falciparum* with *pfcrt* 72-76 SVMNT, *pfmdr1* 86Y, *pfdhfr* 51I + 59R+ 108N and *pfdhps* A437 + K540 haplotypes. The patient from West Africa had *P. falciparum* with *pfcrt* K76, *pfmdr1* 86Y, *pfdhfr* 51I + 59R+ 108N and *pfdhps* A437/437G + K540 haplotypes. These two patients highlight the risk of importing drug resistance to Honduras. La Moskitia, from where 90% of *P. falciparum* cases of the country are reported is known to be used for drug trafficking from South America [291]. Commonly smugglers come by boat or aeroplane presumably from Colombia. It is not difficult to envisage resistant *P. falciparum* also being imported this way. The risk of resistant genes crossing over into local *P. falciparum* is thus probably highest here. Yet this has as yet not happened according to the latest survey conducted in 2011 [256]. Furthermore, neighbouring Nicaragua recently reported the presence of *P. falciparum* with the CQ resistant *pfcrt* CVIET haplotype and *pfdhfr* 51I + 59R and/or 108N and *pfdhps* 437G SP resistant SNPs in 3/49 samples during a clinical trial conducted in 2005 [257]. In a follow up survey in Nicaragua in 2011 no SP resistant SNPs were present. Considering this, it is worth noting that malaria is often diagnosed clinically in Honduras and that it is then treated presumptively with primaquine (0.25mg/kg) for five days in addition to CQ. Primaquine reduces gametocyte carriage time thus reducing transmission and it is certainly possible that this has contributed to stop resistant *P. falciparum* from becoming established [292, 293].

4.2 Polymorphism in *P. vivax*

4.2.1 *P. vivax* multidrug resistance gene 1.

Proportions of *pvmdr1* Y976F and F1076L are presented in table 7. In paper I we found *pvmdr1* 976F in 7/41 (17%) samples with *P. vivax*. These results might indicate a degree of CQ tolerance but probably not resistance in Honduras [209, 217, 294]. In line with this, an *in vivo* evaluation conducted in Honduras (1998-2000) found that 73/73 *P. vivax* infections were successfully treated with CQ and primaquine [251]. We also reported 2 *pvmdr1* gene copies in one sample (with 976Y). These findings should be interpreted with caution as neither mefloquine nor artesunate are commonly used in Honduras [213]. It may however suggest the natural occurrence of this genetic change in Honduras.

Haplotypes	Y976F	F1076L	Number (Proportion %)
1	Y	F	29 (71)
2	Y	L	1 (2)
3	**F**	F	3 (7)
4	**F**	L	4 (10)
5	Y	ND	4 (10)

Table 7 - *Pvmdr1* Y976F and F1076L haplotype proportions.
Resistance associated alleles are shown in bold. [ND] Not Determined

4.2.2 *P. vivax* dihydrofolate reductase and *P. vivax* dihydropteroate synthase.

Double (57L+117N), triple (57L+58R+117N) and quadruple (57L+58R+61M+117T) *pvdhfr* mutations have been associated with SP resistant *P. vivax* [226, 228, 295]. In paper I we report double mutation 57L + 58R in 2/57 (3%) samples. Allele Proportions are presented in table 8. Similar proportions have been reported in Asia but not in South America [133, 226, 228, 296-299]. Our results may suggest a degree of tolerance but probably not resistance to SP that should be efficacious for treatment of *P. vivax* in Honduras [129]. However, for *P. vivax* only CQ is used in Mesoamerica and the efficacy of SP has not been assessed in the area [58]. Finding the double mutation despite a probably low consumption of SP suggests that resistance might develop rapidly if SP usage increases. A possible explanation for the occurrence of 57L and 58R despite the low use of SP for malaria might be that trimethoprim/sulfamethoxazole is the first line drug for treatment of acute respiratory tract infections in Honduras [300].

Alleles	F57L	S58R	T61M	S117N/T	Number (Proportion %)
1	F	S	T	S	57 (97)
2	**L**	**R**	T	S	2 (3)

Table 8 – *Pvdhfr* F57L, S58R, T61M and S117N/T haplotype proportions.
Resistance associated alleles are shown in bold

5 CONCLUSIONS

Overall Conclusion

Samples from a previously treatment naïve area, areas of no and limited drug resistance despite considerable drug exposure and areas of established high level drug resistance collected between 1978 and 2012 were analysed in this thesis and provide novel insights into the selective effects of antimalarials on known and putative genetic markers of antimalarial drug resistance.

Paper I

In *P. falciparum* infections originating in Honduras only SNPs linked to chloroquine or sulphadoxine-pyrimethamine sensitivity were found indicating that chloroquine and sulphadoxine-pyrimethamine should remain efficacious.

Chloroquine and sulphadoxine-pyrimethamine resistance associated SNPs were found in patients that contracted *P. falciparum* overseas highlighting the risk of drug resistance being imported to and spreading in Honduras.

In *P. vivax* infections contracted in Honduras genetic polymorphisms associated with chloroquine and sulphadoxine-pyrimethamine tolerance were found in eight (13%) and two (3%) samples, respectively, suggesting that a degree of tolerance may exist in the country.

Paper II

The *pfvp2* V405, K582 and P711 alleles were predominant throughout the eight countries studied.

An association between the *pfvp2* 405V, 582K and 711P haplotype and *pfcrt* 76T was detected. These observations are in line with previous data indicating that PfVP2 may have a role in chloroquine resistance. However, *pfvp2* SNPs were only found in 20/385 patient samples. The correlations found should therefore be interpreted with caution.

Paper III

In line with the continued efficacy of chloroquine, resistance associated SNPs in *pfcrt* were not widespread and were not found in *pfmdr1* in Liberia despite 2 years of monthly intermittent presumptive therapy.

The *pfcrt* 72-76 haplotype CVIET possibly developed de-novo but without the downstream *pfcrt* SNPs commonly found throughout CQR genotypes in Africa today.

The PYR resistance associated SNP *pfdhfr* 108N probably existed prior to the introduction of PYR or SP in Liberia.

Reduced frequency of *pfdhfr* 108N coincident with evidence of *in vivo* PYR resistance suggests that decreased susceptibility to PYR developed through mechanisms other than previously described SNPs in *pfdhfr*.

Paper IV

Pfcrt 76T accumulated during the high transmission season indicating that it provided a selective advantage after 2007. This was probably largely driven by quinine that was as commonly used as AL.

P. falciparum parasite densities were lower with *pfcrt* 76T compared to *pfcrt* K76 suggesting a loss of fitness.

The pfmdr1 86 + 184 NF and probably 1246D haplotype frequency increased between 2003 and 2012 possibly driven by AL.

6 FUTURE

Our finding of the *pfcrt* 72-76 CVIET haplotype in a Liberian from 1978 requires further investigation as no other downstream SNPs occurred in the same sample. Microsatelites analysis will help to determine the origin of this sample.

The intriguing finding of decreased *pfdhfr* 108N frequency concurrently with *in vivo* pyrimethamine resistance warrants further study. The first step will be to determine the presence of amplifications of the *pfdhfr* or GTP-cyclohydrolase (*gch1*) and SNPs in the *pfmrp2* genes.

It would also be very interesting to further assess the occurrence of polymorphisms associated with drug resistance in the collection of Liberian samples from 1977 to 1989.

We have started an *in vitro* study to determine if *P. falciparum* are able to "shut down" when stressed. When both CQ sensitive and resistant *P. falciparum* are exposed to high CQ concentrations for periods of up to one week, very few small dots generally considered to be dead parasites remain. When these dots are followed for a long enough period of time they often revive. A hypothesis to explain this finding is that *P. falciparum* have an innate ability to shut down all non-essential systems and thereby survive. This could in turn explain why *P. falciparum* survive treatment even when adequate concentrations of a drug to which they are not resistant are achieved. Similar mechanisms have been described in bacteria and are believed to be a survival response to for example lack of essential nutrients.

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