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Evaluation of novel molecular markers for monitoring drug resistance in *Plasmodium falciparum* malaria

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Mestrado em Microbiologia Clínica
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Summary

The human malaria parasite *Plasmodium falciparum* is acquiring resistance to most drugs it has encountered, including the recently deployed Artemisinin Combination Therapy, on which much hope has been laid. Molecular markers for monitoring the evolution of resistance are, therefore, urgently required. Our group has recently made use of a rodent malaria model to identify a number of novel genetic markers of antimalarial drug resistance, namely a *clathrin mu adaptor gene* (*pfcmu*) involved in artemisinin resistance and an *amino acid transporter gene* (*pfaat1*) underlying chloroquine resistance.

The main aim of this thesis was to characterize and evaluate the contribution of the above genes to drug resistance in natural parasite populations of *P. falciparum* isolates from three endemic areas: Rwanda, Democratic Republic of Sao Tomé & Principe (DRSTP) and Brazil.

The global diversity of *pfcmu* and *pfaat1* was determined, resulting in the identification of several polymorphisms. The *pfaat1* gene appears to be highly conserved and no correlations were found between this gene and the *in vitro* resistance to 4-aminoquinolines. In contrast to *pfaat1*, the *pfcmu* gene was genetically diverse, with nine Single Nucleotide Polymorphisms and three different insertions identified across all isolates inspected. Samples could be grouped in to fourteen different *pfcmu* haplotypes, whose diversity was higher in both African sites than in Brazil (H_d = 0.964 ± 0.077, 0.750 ± 0.139 and 0.250 ± 0.180 in Rwanda, DRSTP and Brazil, respectively). Some of the identified polymorphisms showed geographical specificity. We found a significant association between a *pfcmu* G479A genotype in Rwanda samples and the *in vitro* sensitivity to dyhidroartemisinin (p = 0.0207). These constitute new findings to suggest that polymorphisms in *pfcmu* can be involved in *P. falciparum* defence mechanisms against artemisinin derivatives. Thus, further assessment of the gene in artemisinin responses is a top priority, in the context of effective surveillance of artemisinin resistance.

Resumo

A resistência do parasita *Plasmodium falciparum* aos fármacos é um dos principais obstáculos a uma contenção eficiente da malária. Os compostos utilizados para o combate a esta doença têm perdido sua eficácia ao longo dos anos, incluindo a artemisinina e os seus derivados, recentemente indicados como promissores no tratamento da malária. De facto, foram descritos recentemente os primeiros casos de resistência *in vivo* a estes compostos em parasitas de malária humana. Consequentemente, a identificação de marcadores moleculares de resistência a estes fármacos, previamente a um alastramento da resistência, apresenta-se como uma estratégia essencial.

Recentemente, fazendo uso de um modelo de malária de roedores (*Plasmodium chabaudi*), o nosso grupo identificou um número de determinantes genéticos de resistência a diferentes antimaláricos, nomeadamente, o gene *pfcmu*, que codifica uma subunidade *mu* do complexo adaptador de clatrina e se relaciona com resistência aos derivados da artemisinina e o gene *pfaat1*, que codifica uma proteína transportadora de aminoácidos e está envolvido na resistência à cloroquina.

O objectivo primordial deste trabalho centrou-se na caracterização dos genes acima descritos e na avaliação do seu possível papel em mecanismos de quimio-resistência em populações parasitárias provenientes de três regiões endémicas: Ruanda, República Democrática de São Tomé e Príncipe (RDSTP) e Brasil.

Identificaram-se e caracterizaram-se os polimorfismos existentes em ambos os genes. O gene *pfaat1* mostrou ser um gene altamente conservado e não revelou ter qualquer relação com a resistência às 4-aminoquinolinas. Pelo contrário, o gene *pfcmu* revelou possuir uma maior diversidade genética, tendo-se identificado nove mutações pontuais e três inserções, no conjunto de todos os isolados estudados. As amostras analisadas permitiram constituir catorze haplótipos, cuja diversidade demonstrou ser mais elevada nos países Africanos em comparação com o Brasil (H_d = 0,964 ± 0,077, 0,750 ± 0,139 e 0,250 ± 0,180 no Ruanda, RDSTP e Brasil, respectivamente). Alguns dos polimorfismos identificados revelaram

especificidade geográfica. Identificou-se uma associação significativa entre uma mutação no gene *pfcmu* (G479A) e a susceptibilidade *in vitro* à dihidroartemisinina, em isolados provenientes do Ruanda ($p = 0.0207$).

Estes resultados sugerem que polimorfismos no gene *pfcmu* podem estar envolvidos nos mecanismos de resistência do parasita *P. falciparum* aos derivados da artemisinina. Por conseguinte, estudos futuros envolvendo este gene e as respostas aos derivados da artemisinina, revestem-se de especial importância no contexto actual de uma vigilância eficaz da resistência a estes compostos.

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ACT	Artemisinin-based Combination Therapy
AP	Adaptor Proteins
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DRSTP	Democratic Republic of Sao Tomé & Principe
DV	Digestive vacuole
Hb	Haemoglobin
IC50	50% Inhibitory Concentration
Indel	Insertion/Deletion
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
kDa	kilodalton
LGS	Linkage Group Selection
PBS	Phosphate-Buffered Saline
pcaat1	*P. chabaudi amino acid transporter gene*
pccmu	*P. chabaudi clatrin mu adaptor gene*
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
pfaat1	*P. falciparum amino acid transporter gene*
Pfam	Protein families database
PfATPase6	Sarco/endoplasmic reticulum Ca^{2+} - ATPase orthologue of *P. falciparum*
pfATPase6	*Sarco/endoplasmic reticulum Ca^{2+} - ATPase orthologue of P. falciparum gene*
pfcmu	*P. falciparum clatrin mu adaptor gene*
PfCRT	*P. falciparum* chloroquine resistance transporter
pfdhfr	*P. falciparum dihydrofolate reductase gene*

pfdhps	*P. falciparum dihydropteroate synthase gene*
Pfmdr1	*P. falciparum multidrug resistance protein 1 gene*
PIRA-PCR	Primer-Introduced Restriction enzyme Analysis PCR
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-pyrimethamine
WHO	World Health Organization
WT	Wild-type

CHAPTER 1

INTRODUCTION

Chapter 1: Introduction

1.1. The burden of malaria

Despite considerable scientific advances and the development of modern drugs, malaria remains an important public health concern, affecting approximately 3 billion people, and resulting in 250 million clinical cases each year. Although it is disseminated throughout more than 100 countries, the bulk of this burden falls on sub-Saharan Africa with about 90% of all malaria cases and related deaths[1].

The effect of malaria extends far beyond this direct measure of mortality since morbidity in endemic countries leads to major socio-economic losses. Acute febrile illness, anaemia, chronic debilitation, complications in the course and outcome of pregnancy, and delays in cognitive and physical development contribute to a heavy public-health burden, which has a negative impact on the social and economic development of affected countries[2-6]. The disease contributes to approximately 1.3% annual reduction of economic growth in countries with high levels of transmission[7].

1.2. *Plasmodium*

Malaria is caused by protozoan parasites of the genus *Plasmodium*, which are inoculated into humans by female *Anopheles* mosquitoes. The genus contains more than 100 species of which four are infectious to humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*[8]. However, recent reports from Asia suggest the possibility that a different malaria

species, *Plasmodium knowlesi*, which usually infects primates, is on the verge of crossing the species barrier[9].

These species differ morphologically, immunogenically, in their geographical distribution, in their ability to relapse and in their response to drugs. By far, the most virulent specie is *P. falciparum,* which causes the majority of severe malaria morbidity and mortality[10].

1.3. Life cycle of *Plasmodium falciparum*

The *P. falciparum* life cycle comprises numerous transitions stages and occurs between two separate hosts (Figure 1.1). The cycle begins when haploid **sporozoites** are injected into the human host by an infected female mosquito of the genus *Anopheles* during a blood meal. These motile forms of the parasite rapidly access the blood stream and then migrate to the liver. There, each sporozoite traverses a few hepatocytes, before finally establishing in one of them[11], where it differentiates and undergoes asexual multiplication resulting in tens of thousands of **merozoites** which burst from the hepatoctye. Upon release from the liver, individual merozoites invade red blood cells (erythrocytes). Once inside the erythrocyte, *P. falciparum* develops into a **ring** stage/early trophozoite and then into a highly metabolically active late stage **trophozoite**. Finally, trophozoites develop into **schizonts** and asexual replication occurs. In the latter, new **merozoites** are formed within the schizont and are released to the bloodstream when the erythrocyte bursts. The clinical manifestations of malaria are associated with the synchronous rupture of infected erythrocytes. The free merozoites go on

to invade additional erythrocytes. Although the majority of invading merozoites will go on to develop into schizonts and continue the asexual cycle, a small proportion enter the sexual phase of the life cycle by differentiating into haploid male or female **gametocytes**, which are the only forms that are transmissible to the mosquito.

For transmission to occur, when an infectious host is bitten by a female anopheline mosquito, both male and female gametocytes must be taken up during the blood meal. Inside the mosquito midgut, gametocytes develop into gametes and sexual reproduction takes place, whereby 8 flagellated **microgametes** are released from a male gametocyte. These fertilize the female **macrogamete** to form a diploid zygote that converts into an **ookinete** which penetrates a cell wall in the midgut and subsequently develops into an **oocyst**. Each oocyst produces haploid sporozoites by asexual reproduction. The oocyst finally ruptures to release a large number of **sporozoites** into the haemolymph where from they eventually migrate to the mosquito salivary glands. The sporozoites remain here, ready to be inoculated into the next host and begin a new cycle of infection when the female mosquito takes a further blood meal.

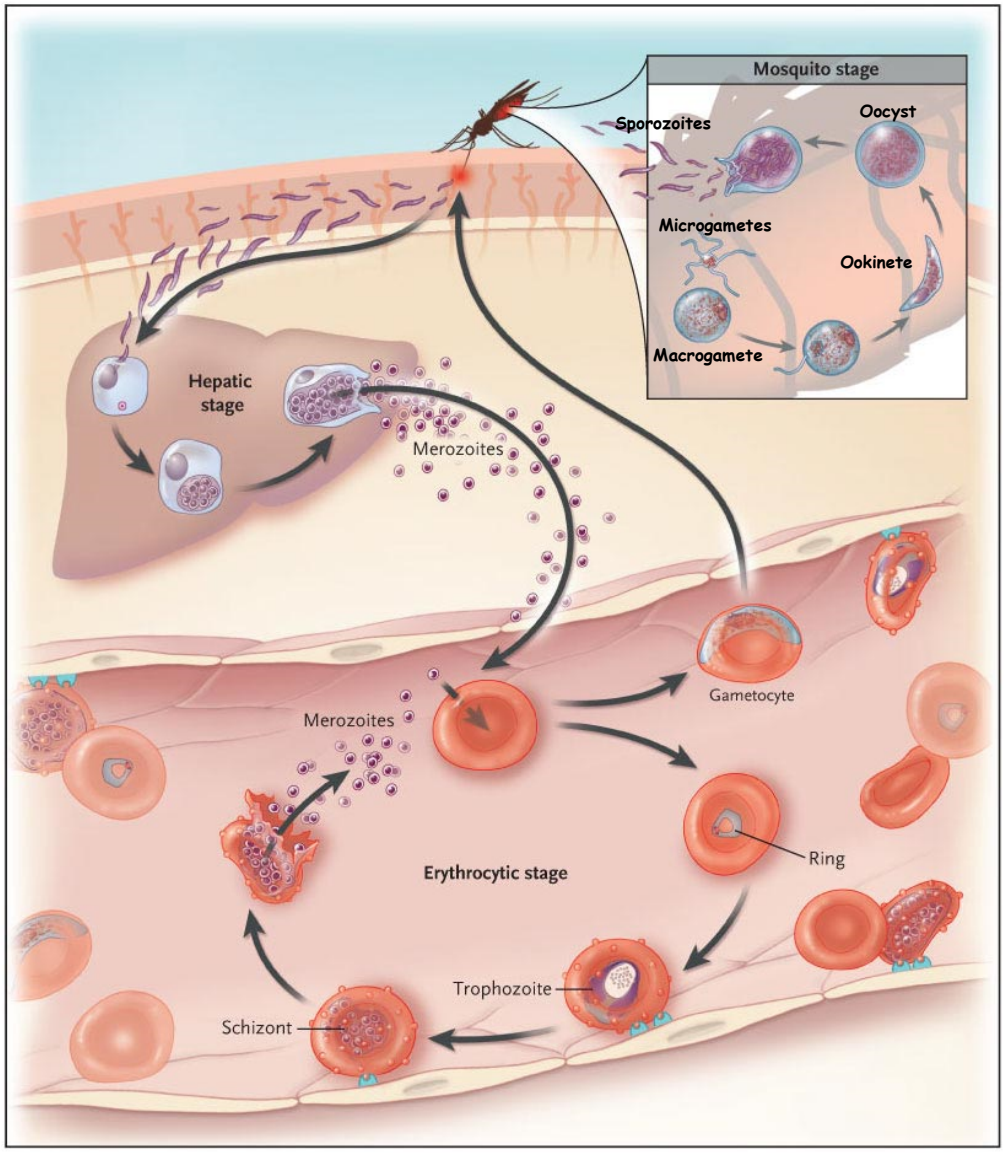

Figure 1.1. The life cycle of *P. falciparum* (adapted from Rosenthal[12])

1.4. Malaria control

Three living entities are involved in the malarial infection; parasite, host and vector. In order to reduce the burden of malaria, interventions can be designed to target several stages of the parasite lifecycle. For example, the parasite can be targeted in the host either directly with drugs, or indirectly with vaccines.

Similarly, strategies that reduce vector contact with the host or increase vector mortality can be employed to reduce the number of new infections[13].

Despite decades of intensive research, no effective vaccine capable of conferring an adequate level of immunity has been developed to date[10,14,15,16]. Thus, measures aimed against the parasite are based on the use of drugs either as treatment or as prophylaxis. Medication reduces morbidity and mortality by terminating a malaria infection in a patient and restricts malaria transmission by diminishing the parasite reservoir[7]. Antimalarial drugs are also used as a preventive measurement, both as chemoprophylaxis for travellers to malaria endemic areas and for intermittent preventive treatment (IPT) in high-risk groups such as pregnant women, infants and children[17,18].

There are two key approaches to malaria prevention by vector control: the use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS). These central interventions may be complemented by other methods such as larval control or environmental management[18,19].

1.5. Antimalarial drug resistance

Antimalarial drugs remain as one of the most powerful tools in the fight against malaria. So far, malaria control has relied largely on a comparatively small number of chemically related drugs belonging to 7 classes of compounds: 4-aminoquinolines, 8-aminoquinolines, arylaminoalcohols, antifolate compounds, artemisinin and derivatives, inhibitors of the respiratory chain and antibiotics[20,21].

Table 1.1. Main groups of antimalarial compounds according to their chemical class

Class	Compound
4-aminoquinolines	Chloroquine
	Amodiaquine
8-aminoquinolines	Primaquine
Arylaminoalcohols	Quinine
	Mefloquine
	Halofantrine
	Lumefantrine
Antifolates	Sulfadoxine
	Dapsone
	Pyrimethamine
	Proguanil
	Chlorproguanil
Artemisinin and derivatives	Artemisinin
	Artesunate
	Artemether
	Arteether
	Dihydroartemisinin
Inhibitors of the respiratory chain	Atovaquone
	Proguanil
Antibiotics	Doxycyclin
	Clindamycin

In recent decades, increases in malaria mortality rates in African children have been linked with the emergence and spread of parasites resistant to the antimalarial drugs in use[22-26]. Now, resistance to virtually all classes of antimalarial drugs is widespread in Africa and Southeast Asia.

Until recently, chloroquine was the most widely used drug to treat and prevent malaria infection. The success of chloroquine was based upon its rapid action, safety and low cost relative to other antimalarials[3]. With the spread of chloroquine

resistance, many countries adopted sulfadoxine-pyrimethamine (SP) combination as first line antimalarial treatment. Still, resistance to SP spread more rapidly than to chloroquine and is now widespread in Asia and South America and is spreading in Africa[27].

Drug combinations, rather than monotherapy, are now regarded as the best solution for treating malaria. The simultaneous use of two or more drugs is a chemotherapeutic strategy for improving treatment efficacy and retarding the development of resistance to the individual drugs in the combination[28]. The underlying principle for the impact of combination therapy on drug resistance is based on the assumption that drug resistance essentially depends on mutation. Provided that the constituent drugs administered in the combination have independent modes of action, the probability of a parasite developing resistance to both drugs simultaneously is significantly reduced compared to developing resistance to one drug. The probability that a mutant will arise that is at the same time resistant to two different antimalarial drugs is the product of the mutation rates per parasite for the individual drugs, multiplied by the number of parasites that are exposed to the drugs, in an infection[29].

In response to the increasing burden of malaria caused by *P. falciparum* resistance to the standard antimalarial medicines, World Health Organization (WHO) recommended the use of combination therapies, ideally those containing artemisinin derivatives in countries where *P. falciparum* malaria is resistant to the conventional antimalarial medicines chloroquine, SP, and amodiaquine[28]. Unfortunately, even artemisinin derivatives, the only drugs that had been fully

effective against *P. falciparum* until very recently, seem to be loosing their efficacy along the border between Cambodia and Thailand[30-32].

1.6. Artemisinin-based combination therapy

Artemisinins are currently the most important class of antimalarial drugs, presenting many advantages over other compounds. These advantages include several possible routes of administration, suitability for treating severe malaria replacing quinine and avoiding its side effects, rapid activity and activity against trophozoites, blood schizonts, and also against ring forms and gametocytes[33]. This latter property may help diminish transmission rates[34]. Altogether, artemisinin derivatives reduce the incidence of malaria, reduce drug use, and thus contribute to slowing the evolution of drug resistance[34,35].

Artemisinin-based combination therapy (ACT), the combination of an artemisinin derivative with another structurally unrelated antimalarial, is now being proposed as the best therapeutic alternative for treating drug-resistant malaria and delaying the development of resistance[17].

Artemisinins are highly effective, short-acting drugs derived from an herb, qinghao (*Artemisia annua*), a plant found in China. This class of compounds can reduce the number of parasites faster than any other class of antimalarial drug and this is also the reason for their ability to provide clinical relief fast[36,37].

Because of the short elimination half-lives of the artemisinin compounds, the administration of artemisinins with longer-acting agents is required[38]. In an ACT, the artemisinin derivative kills rapidly and drastically most of the parasites and those that remain are then eliminated by a high concentration of the longer-lasting

partner drug, after the short-lived artemisinin has dropped below therapeutic levels. In this way the artemisinin derivative should protect its partner drug. If the treatment is successful, the partner drug also protects the artemisinin derivative by removing all the remaining parasites that were initially exposed to the artemisinin. Consequently the probability that mutant parasites survive and emerge from these two drugs is low[37,39]. However, this question is under debate. Accordingly to Hastings and Watkins, combination therapies containing drugs with mismatched half-lives could jeopardize the efficacy of the artemisinins, in particular combination with partner drugs against which resistance is already spread[40]. Discrepancy in the half-lives of the partner drugs leaves windows of monotherapy during which parasites can acquire resistance, or those with pre-existing resistance to the partner drug, re-emerge and cause drug failure. Ideally, drugs with short half-lives should be preferred, in order to reduce the exposure of reinvading parasites to suboptimal drug levels which may induce the selection for tolerance and eventually the development of resistance.

1.7. Assessment and monitoring of resistance

Drug surveillance is necessary to ensure correct management of clinical cases and early detection of changing patterns of resistance to assure that national treatment policies remain effective[41]. Three approaches have been used to evaluate the efficacy of an antimalarial drug: clinical *in vivo* studies (also known as therapeutic efficacy testing), *in vitro* susceptibility testing, and more recently, molecular markers.

In discussing these different approaches, it is fundamental to differentiate intrinsic parasite resistance from decreased clinical efficacy. The term resistance means the ability of a drug to prevent parasite growth in culture, at defined drug concentrations, and in the absence of the host immune response. Alterations in efficacy are detected through clinical *in vivo* studies in which parasite intrinsic susceptibility is one of several factors that determine the outcome[42].

1.7.1. *In vivo* tests

The therapeutic efficacy test remains the "gold standard" method for detecting drug resistance[41]. These tests reveal the exact biological nature of drug treatment response. This response involves a complex interaction between the drugs, the parasites, and the host response (i.e. the therapeutic response of currently circulating parasites infecting the current population in which the drug will be used), while *in vitro* tests measure only the interaction between the parasites and the drugs[43].

In vivo tests involve the treatment of symptomatic *P. falciparum* infected patients with a standard dose of an antimalarial drug and subsequent follow-up of clinical and parasitological outcomes of treatment during a fixed period. Recent studies describe the benefits of 28-day, or even 42-day, follow-ups[44]. This proceeding allows the detection of any reappearance of symptoms or signs of clinical malaria and the detection of parasites in the blood as an indication of reduced sensitivity to a particular drug[41].

Due to the influence of factors such as the patient immunity, variations of drug absorption and metabolism, and possible misclassification of new infections as recrudescences, the results of therapeutic efficacy test do not necessarily reflect

the real level of antimalarial drug resistance. These studies include others drawbacks: the long duration of patient monitoring that may result in high patient loss to follow-up, the assessment of resistance to one drug regimen only and poor ability to compare different studies, because local adaptations and modifications of the standard protocol are usually made[42].

However, these tests provide decision-makers with a simple, readily comprehensible indicator of the efficacy of an antimalarial drug with reduced requirement for equipment and supplies[41].

1.7.2. *In vitro* tests

The *in vivo* method has allowed the thresholds of treatment failure that are crucial for adjusting antimalarial drug policies to be determined but it not sufficient on its own to confirm drug resistance[41].

To support the evidence of a failing antimalarial, an *in vitro* test can be used providing a more accurate measure of drug sensitivity under controlled experimental conditions, which removes variables such as patient immune status, re-infection and pharmacokinetics. *In vitro* tests allow a more objective approach to parasite resistance, since in these studies the parasite will be in direct contact with incremental drug concentrations. Several tests can be carried out with the same sample, and several drugs can be studied at the same time, including drugs that are still at the experimental stage[41].

Several *in vitro* tests exist, which differ with respect to the measure effect and the duration of exposure to the test compound. These include microscopic examination of blood films for the WHO mark III test (inhibition of maturation or

replication; Giemsa-stained), the radioisotopic test (incorporation of hypoxantine) and the enzyme-linked immunosorbent assay with antibodies directed against *Plasmodium* lactate dehydrogenase or histidine-rich protein II[45].

The importance of these tests has become evident with the increasing use of combination therapy, since they can be used to monitor susceptibility to each drug in a combination. It is often impossible to perform *in vivo* tests for each component, due to ethical problems, non-availability of the drug as monotherapy and the need to study a large number of patients[46].

Although this method is useful, its application is limited. *In vitro* methods require trained personnel with access to a laboratory capable to perform culture of malaria parasites. Even when provided with such facilities, it is often difficult to establish cultures and not all the primary parasites will adapt to *in vitro* culture conditions[47]. Moreover, in part because these tests remove the host factors, the correlation between results of *in vitro* and *in vivo* tests is not always reliable and is not well understood. *In vitro* drug sensitivity data may provide early evidence of increasing drug tolerance prior to parasitological/clinical resistance. Whereas, may give misleading indications if the alterations in sensitivity are so small that they do not result in parasitological/clinical resistance[48].

These limitations of *in vivo* and *in vitro* methods have led to the search for genetic markers of resistance.

1.7.3. Molecular markers

Molecular markers for drug resistant malaria provide promising public heath tools of great potential value[49]. Studies that can detect genetic markers of drug resistance are relatively fast, quantitative and less expensive compared with clinical studies involving more patient care and follow-up[50]. In addition, collection, storage and transport of specimens for subsequent molecular analysis are far easier than for *in vitro* tests[41].

Molecular markers for drug resistance malaria are based on genetic changes that confer parasite resistance to drugs. These genetic mechanisms of *P. falciparum* drug resistance have not been completely elucidated. However, five genes that appear to play a role in regulation of resistance to the principal chemical families of antimalarials in current use have been identified. Multiple mutations in the *P. falciparum* chloroquine resistance transporter (PfCRT) confer resistance to chloroquine. In particular a substitution at amino acid position 76 (K to T)[51,52] is crucial to the manifestation of *in vitro* resistance as well as therapeutic failure. Mutation in the P-glycoprotein homologue (Pgh1) encoded by *pfmdr1* (*P. falciparum multidrug resistance gene 1*) may further modulate the extent of chloroquine resistance[53]. Polymorphisms and/or amplification of *pfmdr1* have also been shown to affect the susceptibility to structurally unrelated antimalarial drugs, including mefloquine, artesunate, lumefantrine and quinine[34,52]. Resistance to sulphadoxine and pyrimethamine (SP) is conferred by mutations in the *dihydropteroate synthase* (*pfdhps*) and *dihydrofolate reductase* (*pfdhfr*) genes, respectively[55,56]. One mutation in the plasmodial *ATPase6* gene has been proposed as a marker for artemisinin resistance but this association has not yet been confirmed[57].

As it is the case with *in vitro* tests, the presence of particular molecular markers does not necessarily directly predict treatment outcome. Mutations in the above genes contribute to drug failure but the outcome is not certain: some patients with "resistant" alleles clear the infection, and some patients with "sensitive" alleles failed treatment. Therefore is better to think in terms of mutations encoding rising probabilities of drug failure that ultimately depends on factors such as host immune response, the drug dose taken, and variation in drug absorption and metabolism[58].

Much debate has occurred concerning the relative qualities of one test over another, with the suggestion always being that one type of test should be used preferentially. These methods can be considered as complementary sources of information about resistance, rather than competing, regarding the different types of information each approach gives.

1.8. Mechanisms of drug resistance

The development of resistance is a two steps process; de-novo emergence and subsequent spread. Resistance arise through point mutations or gene duplications, mainly during asexual reproduction, a single genetic event may be all that is required, or multiple events (epistasis). The rare de-novo emergence event is thought to be independent of drug selection pressure[59]. These mutants are then selected for survival, multiplication, and subsequent spread as a result of the drug pressure which provides a selective advantage to resistant parasites[2,59,60]. Resistant parasites are then transmitted to other individuals by mosquitos[61]. In addition,

sometimes mosquitoes bite two gametocyte-carrying humans offering the possibility of recombination with the formation or breakdown of multigenic resistance[2]. The parasites carrying the mutant alleles are selected if antimalarial drug concentrations are sufficient to inhibit the development of susceptible parasites but are insufficient to inhibit the mutants, a phenomenon known as "drug selection"[62].

Advances in the understanding of the mechanisms of drug action during the last two decades led to the identification of the putative molecular targets and the genetic basis responsible for parasite resistance to antimalarial drugs. The genetic events that confer antimalarial drug resistance include single point mutations in or changes of copy numbers of genes encoding drug targets, such as important enzymes or pumps that affect intraparasitic drug concentrations.

In the following sections the genes implicated in the resistance to the drugs investigated in this thesis, the 4-aminoquinolines and artemisinins derivatives, are described in detail.

1.8.1. Genes influencing 4-aminoquinolines susceptibility

Resistance to 4-aminoquilolines is multigenic and relates to changes in drug accumulation in the parasite's food vacuole. In 2000, the *P. falciparum chloroquine resistance transporter* (*pfcrt*) gene was identified as a primary candidate gene for chloroquine resistance by extensive mapping of genetic cross between chloroquine sensitive and resistant clones. A single nucleotide polymorphism (SNP) at position 76 (replacement of lysine by threonine), has been

reported to be completely associated with chloroquine resistance in 40 laboratory-adapted *P. falciparum* clones from wide geographic areas[51]. Other *pfcrt* mutations occur, but their patterns tend to vary in different geographical areas and their role remains undetermined[63]. The causal relationship between *pfcrt* mutations and chloroquine resistance was further confirmed by genetic transfection experiments[64]. Several *in vivo* studies showed total selection of the *pfcrt* K76T mutant allele in clinical failure[65]. However, the presence of parasites carrying this allele was not always predictive of the clinical outcome or predictive of *in vitro* resistance[66-68]. This mutation appears to be a prerequisite for resistance but may not be sufficient on its own[69,70].

In addition to mutations in *pfcrt*, polymorphisms in the *P. falciparum multidrug resistance gene 1* (*pfmdr1*) have been suggested to modulate the degree of resistance in parasites harbouring the *pfcrt* T76 mutation[71]. Transfection and allelic exchange tests have shown strong associations between *pfmdr1* polymorphisms and enhancement of the degree of *in vitr*o chloroquine resistance[72]. However, many clinical studies have failed to find associations between *pfcrt* and *pfmdr1* mutations and chloroquine resistance[67,73,74] and isolates carrying the same *pfcrt* and *pfmdr1* genotypes often reveal different IC_{50} values, indicating that several other genes may also be responsible for the increase level of chloroquine resistance[75].

This suggests involvement of additional genetic loci in modulating chloroquine resistance, more studies are necessary to elucidate the mechanisms of resistance to this class of drugs.

1.8.2. Genes influencing artemisinin susceptibility

Some scientists have questioned whether resistance to artemisinin would ever arise. However, the recent cases of resistance reported, characterized by slow parasite clearance *in vivo*, indicate that these assumptions where wrong[30-32]. The specific mechanism of action of artemisinin is not well understood, and there is ongoing research directed at elucidating it. Uhlemann and colleagues provided strong evidence that resistance to artemisinins may depend on a single nucleotide polymorphism in the drug's putative chemotherapeutic target, the SERCA-type ATPase protein of *P. falciparum* (PfATPase6)[76]. A subsequent study report that *P. falciparum* parasites from French Guiana harboring mutant forms of the *pfATPase6* gene (S769N) displayed significantly increased IC_{50s} to artemisinins, suggesting its role in artemisinin resistance[57]. However, mutations in this gene are not associated with resistance in field isolates from elsewhere. Moreover, our group could not identify any mutation or altered copy numbers of the *ATPase6* gene in artemisinin and artesunate-resistant clones of *P. chabaudi*. Causal unequivocal association between mutations in the *pfATPase6* gene and resistance to artemisinins has not yet been established though, whilst other genes have also been implicated in this phenotype.

Amplification of the *multi-drug resistance 1 gene* has been linked to the parasite's ability for modulating artemisinin responses in previous work[53,77,78]. In an attempt to clarify the role of *pfmdr1* amplification in this phenotype, Sidhu et al.[79] genetically abolished one of the two copies of the gene in the multi-drug resistant parasite *P. falciparum* FCB. *In vitro* assessment of the resulting knock-out mutant harboring a single gene copy revealed that its response to

artemisinin had approximately halved, providing the first direct evidence as to the ability of *pfmdr1* copy number to modulate this phenotype.

Surprisingly, no correlation was found between *pfATPase6* and *pfmdr1* genes and *in vivo* resistance to artemisinins in the first confirmed cases of resistance along the Thai/Cambodian border[31,80]. Thus we still lack validated genetic markers to monitor the evolution of artemisinin resistance in natural parasite populations. Significant efforts should now be directed at elucidating the mechanisms of parasite resistance to this class of compounds, in order to develop strategies that allow maximizing their useful longevity.

1.9. *Plasmodium chabaudi* model system

Field studies of human malaria parasites provide very limited amount of information about the mechanisms underlying drug resistance. Consequently, the genetic basis of drug resistance in malaria must be investigated by other ways. One of the most appropriate ways is to use genetically stable resistant mutants selected through drug pressure from cloned sensitive parasite lines. In this way, drug sensitive and drug resistant parasites are genetically identical (isogenic) except for the mutation involved in resistance; such mutations can then be identified using different approaches. *In vitro* studies with human malaria parasites present several limitations. Identification of causative mutations requires controlled *in vivo* laboratory experiments, which are simply not feasible with human malarias. Therefore rodent malaria parasites have been widely used to study the mechanisms of drug resistance *in vivo*[81-85]. *Plasmodium chabaudi*

chabaudi is a malaria parasite of murine rodents and it has been used as a model to study various aspects of parasite biology and disease which are difficult to investigate using human malaria parasites. There are many reasons why *P. chabaudi* is considered to be a good model for the human malaria parasite, especially in studies on drug resistance. This species shares a number of similarities in their basic biology with *P. falciparum*, including preferential invasion of mature erythrocytes, the synchronicity of their asexual blood form, sequestration of schizont-infected erythrocytes, and gametocyte development late in the infection[86].

Our group has determined the genetic basis of drug resistance in a congenic lineage of multi-drug resistant mutants of *P. chabaudi*, selected from sensitive progenitors, using advanced technology like Linkage Group Selection (LGS) and Solexa (second generation sequencing technology). LGS allows the identification of areas of the whole genome associated with drug resistant phenotypes, using the uncloned, recombinant progeny of genetic crosses[87] and Solexa permit the rapid and accurate sequencing of whole parasite genomes and thus the identification of any mutation occurring within drug resistant clones[88].

These studies have identified three genes, two that could potentially mediate artemisinin resistance a *de-ubiquitinating enzyme* (*ubp1*)[89] and an *adaptor protein subunit* (clathrin mu adaptor - *cmu*) (Cravo *et al*., unpublished); and one *amino acid transporter* (*aat1*) underlying chloroquine resistance (Cravo *et al*., unpublished). However, the importance of the above mentioned markers in modulating susceptibility to the different drugs in the human malaria parasite has not been assessed yet.

In the above context, the objective of this work was to evaluate the importance of two of these newly identified molecular markers of antimalarial drug resistante, the *pfcmu* and *pfaat1* genes, in natural parasite populations of *P. falciparum*. To achieve this, we used an extensive collection of previously samples isolates of *P. falciparum* originating from 3 endemic regions (Rwanda, Democratic Republic of Sao Tomé & Principe and Brazil) which were tested for *in vitro* sensitivity to several drugs[90-92]. In these samples we investigated the correlation between the presence of mutations in the *pfcmu* and *pfaat1* genes and the *in vitro* sensitivity to artemisinin derivatives and 4-aminoquinolines, respectively.

1.10. Aims of the thesis

The main aim of this thesis was to determine whether particular mutations in the *pfcmu* and *pfaat1* genes can be used as molecular markers for predicting parasite's *in vitro* responses to chloroquine and artemisinin derivatives.

The specific aims were as follows:

- To genotype sensitive and resistant parasites for single nucleotide polymorphisms in *pfcmu* and *pfaat1* genes identified in our group.
- To evaluate the potential role of these genes (mutations) as putative modulators of the resistance in *P. falciparum*.

CHAPTER 2

MATERIAL AND METHODS

Chapter 2: Material and Methods

2.1. *Plasmodium falciparum* isolates

This study included the analysis of a collection of previously sampled isolates of *P. falciparum* which were freshly collected and tested *in vitro* for their response to several antimalarial drugs, as described below.

Blood samples were collected in Rwanda during November and December 2003[92], in the Democratic Republic of Sao Tomé & Principe (DRSTP) during February 2004[90] and in Brazil during September 2005[91].

After confirming *P. falciparum* monoinfection each sample from DRSTP and Brazil was tested for its *in vitro* susceptibility to drugs, using a modified version of the standard WHO MarkIII micro-test[93]. The *in vitro* drug sensitivity of Rwanda isolates was assessed by use of a classical isotopic microtest technique[94].

Fifty six *P. falciparum* isolates from Brazil were characterized for the *in vitro* susceptibility to artesunate and artemether. A set of 74 isolates from Rwanda were characterized for their in vitro susceptibility to chloroquine, amodiaquine and dihydroartemisinin. The isolates from the DRSTP were characterize for their *in vitro* susceptibility to amodiaquine (53 isolates), artesunate (42 isolates) and artemether (51 isolates).

The *in vitro* values of half maximal inhibitory concentration (IC_{50}) were calculated and organized according to increasing concentrations.

2.2. DNA extraction

DNA was extracted according to the method described by Plowe and co-workers[95]. Briefly, dried filter paper (Whatman TM no. 4) onto which venous blood had been blotted and kept at room temperature was cut and placed into 1.5 ml tubes. One millilitre of 10 % saponin in phosphate-buffered saline (PBS) was added to each tube. Tubes were then incubated overnight at 4 ºC and subsequently washed in PBS. Afterwards, genomic DNA from each sample was obtained by boiling in Chelex-100 followed by ethanol precipitation. DNA was stored at 4 ºC until use. A similar method was used to extract genomic DNA from the reference strain 3D7.

2.3. Molecular analysis of drug-resistance associated genes

2.3.1. Primer design

The *P. chabaudi chabaudi* genes previously proposed as putative modulators of drug resistance were the following: *clathrin mu adaptor gene* (*cmu*) and *amino acid transporter gene* (*aat1*). The DNA sequences of these genes were available online at the PlasmoDB genome database (http://plasmodb.org/plasmo) with the following accession numbers: PCAS_143590 for *pccmu* and PCAS_112780 for *pcaat1*. In order to obtain the *P. falciparum* orthologues of the genes we used the genetic synteny maps provided by PlasmoDB (Figures 2.1 and 2.2). Individual gene sequences of *P. falciparum* orthologues were retrieved from the synteny maps and used as template for designing primers to amplify each open reading frame using an overlapping PCR fragment strategy.

Figure 2.1. Synteny map of the region surrounding PCAS_143590 gene [showing orthologous genes in *P. falciparum, P. vivax, P. knowlesi, P. yoelii* and *P.berghei*. The horizontal lines indicate the chromosomal backbone, with the chromosome number shown on the left. The bars indicate the genes. Genes with introns are shown has interrupted bars. The color of the bars indicate the direction of transcription; blue for forward and red for reverse transcription directions. Genes with sequence identity between species are connected by shaded gray lines. UTRs- Untranslated regions; M– mega base pairs; K– kilo base pairs (adapted from PlasmoDB)].

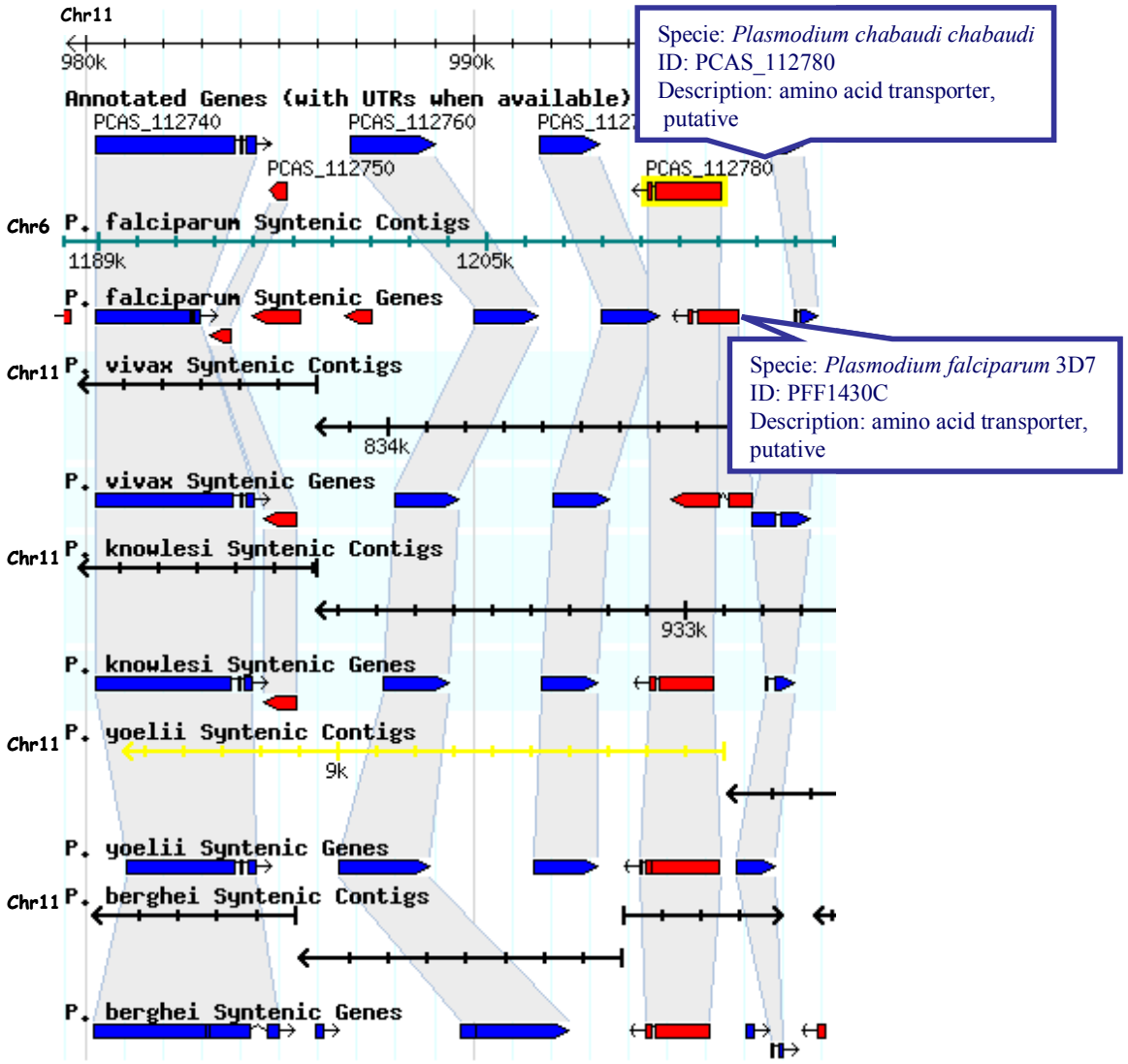

Figure 2.2. Synteny map of the region surrounding PCAS_112780 gene [showing orthologous genes in *P. falciparum, P. vivax, P. knowlesi, P. yoelii* and *P.berghei.* The horizontal lines indicate the chromosomal backbone, with the chromosome number shown on the left. The bars indicate the genes. Genes with introns are shown has interrupted bars. The color of the bars indicates the direction of transcription; blue for forward and red for reverse transcription directions. Genes with sequence identity between species are connected by shaded gray lines. UTRs- Untranslated regions; M– mega base pairs; K– kilo base pairs (adapted from PlasmoDB)].

Primers were manually designed for each of the genes, according to the following parameters: avoidance of the formation of self and hetero-dimers, hairpins and self complementarity, primer length and melting temperature. These properties were verified using a primer design software, entitled Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html).

Several primers were designed and used in order to amplify the whole genes and those producing positive results are shown in Table 2.1. These primers were then used in PCR amplifications, as described below.

Table 2.1. Primer sequences and PCR reactions for *pfcmu* and *pfaat1* genes.

Gene	Primers	Sequence (5' to 3')	Coordinates Length	PCR Program	
pfcmu	**PfClR-1F**	GTTAACACGATTAGCGTCATTTG	(-54 to 524) 578 bp	94 ºC, 3 min ; [94 °C, 30 s; 53 °C, 45 s; 72 °C, 60 s;] 72 °C, 10 min;	35 cycles
	PfClR-2R	GTCCTATTATGTATATGTGGATC			
	PfClR-3F	GATATCCACAAACATTAGAAGTG	(358 to 1199) 841 bp	94 ºC, 3 min ; [94 °C, 30 s; 52 °C, 45 s; 72 °C, 60 s;] 72 °C, 10 min;	35 cycles
	PfClR-4R	CCATCTGGTGGTGTGAAGG			
	PfClR-5F	GCATATTTCATCATTGTGTTACC	(1126 to 1879) 753 bp	94 ºC, 3 min ; [94 °C, 30 s; 53 °C, 45 s; 72 °C, 60 s;] 72 °C, 10 min;	35 cycles
	PfClR-6R	ACACCCGATTGAACTATTTATAC			
pfaat1	**Pfaat1-1F**	CATGAATATGTGAATCCTAAGT	(-144 to 676) 820 bp	94 ºC, 3 min ; [94°C, 30 s; 52 °C, 45 s; 72 °C, 60 s;] 72 °C, 10 min;	35 cycles
	Pfaat1-2R	CTAATGACGAGGTACATAATATA			
	Pfaat1-3F	CTGATGGGGATTATACTAATGAT	(418 to 1112) 694 bp	94 ºC, 3 min ; [94 °C, 30 s; 52 °C, 45 s; 72 °C, 60 s;] 72 °C, 10 min;	35 cycles
	Pfaat1-4R	CATGCATTTGGTTGTTGAGAG			
	Pfaat1-5F	CCATAACTGTATTAACTATAGGAC	(964 to 1661) 697 bp	94 ºC, 3 min ; 94 °C, 30 s; 52 °C, 45 s; 72 °C, 60 s; 72 °C, 10 min;	35 cycles
	Pfaat1-6R	GATGTGATACCTCCTCCTATAC			
	Pfaat1-9F	GATCCATACCAAGAAACACATG	(1479 to 2153) 674 bp	94 ºC, 3 min ; [94 °C, 30 s; 54 °C, 45 s; 72 °C, 60 s;] 72 °C, 10 min;	35 cycles
	Pfaat1-10R	GTATGTTTTATTAAGCATCCTTC			

Positions of the primers, expected sizes of amplified products, and amplified gene regions are shown in Table 2.1. The primer coordinates were taken considering the A of the ATG start codon as base pair zero.

2.3.2. PCR

A subset of *P. falciparum* isolates were initially selected according to their origin and response to each drug. These isolates were then inspected for changes in the sequence of each of the genes under investigation (Table 2.2).

For the *pfcmu* gene we picked 8 samples from Rwanda, 8 from DRSTP, and 8 from Brazil, in agreement with their response to artemisinin derivatives (4 samples with the lowest IC_{50} and the 4 with the highest values, from each country) (Table 2.2). For the *pfaat1* gene we selected 8 samples from Rwanda and 8 samples from DRSTP according to their response to 4-aminoquinolines (4 samples with the lowest IC_{50} values and the 4 with the highest, from each site) (Table 2.2).

PCR assays were performed with 1 µl of DNA into a 50 µl mixture containing 0.2 µM of each primer, 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates and 1.25 units of GoTaq® Flexi DNA Polymerase (Promega).

PCR products were analysed by ethidium bromide-stained agarose 2 % gel electrophoresis.

Table 2.2. *In vitro* IC_{50} responses of a subset of *P. falciparum* isolates from Brazil, Rwanda and DRSTP, selected for genetic studies.

Gene	Site of Sample Collection	Sample ID	Drug	IC50 (nM)
pfcmu	**Brazil**	BR58	Artesunate	0.1
		BR05		0.2
		BR15		0.2
		BR45		0.2
		BR38		3.4
		BR07		4.8
		BR67		4.8
		BR68		4.9
	Rwanda	RW10	Dihydroartemisinin	0.3
		RW32		0.3
		RW58		0.4
		RW30		0.5
		RW57		8.8
		RW106		9.6
		RW77		10.1
		RW40		14.3
	DRSTP	STP27	Artesunate	0.02
		STP29		0.03
		STP41		0.04
		STP46		0.05
		STP35		1.7
		STP34		2.0
		STP44		3.4
		STP58		9.0
pfaat1	**Rwanda**	RW16	Chloroquine	7.6
		RW10		8.9
		RW20		15.9
		RW12		17.3
		RW101		341.5
		RW103		348.9
		RW98		386.0
		RW105		443.7
	DRSTP	ST07	Amodiaquine	1.0
		ST19		1.0
		ST20		1.0
		ST28		1.0
		ST15		126.1
		ST13		129.3
		ST50		132.8
		ST58		156.0

2.3.3. DNA sequencing

After DNA purification using a commercial kit (QIAquick PCR purification kit - Quiagen™), amplified fragments were sequenced using the commercial services of Stab Vida (http://www.stabvida.com). DNA sequences were generated from both sense and antisense primers and aligned to check for genetic polymorphisms. Only unambiguous single nucleotide polymorphisms (SNPs) were considered. Potential sequence ambiguities were resolved through close inspection of the corresponding chromatogram, using ChromasPro software Version 1.5 (www.technelysium.com.au/ChromasPro.html). Sequences of insufficient quality were either resequenced or rejected. Sequence assembly was done manually or with the CAP3 Sequence Assembly Program available online [96].

2.3.4. PIRA-PCR and RFLP

One particular mutation in the *pfcmu* gene (G479A) was determined in all samples characterized for their *in vitro* response to artemisinin derivatives using a PCR-restriction fragment length polymorphism (RFLP) assay. Since this point mutation does not create a natural endonuclease restriction site, we investigated the possibility of using the primer-introduced restriction enzyme analysis PCR (PIRA-PCR) method.

This method is based on incorporation of a deliberate mismatch in one primer so that a new restriction enzyme recognition site is created in the PCR amplicon in the presence of a specific allele in the target DNA. The programme available

online[97] searches all mismatches on both sites of the mutation and on both strands and would create an artificial restriction site either for the wild-type (WT) or the mutant allele.

A PIRA primer PfCLR-G479A-F (forward) with sequence 5'-ATC AAA TGA ATT ATT AAA TGT AAc TA -3' was thus designed, where an A-to-C mismatch was incorporated at the 3rd base position from 3' end terminus (shown in small letter in primer sequence). The terminal cTAG-3' sequence of the PIRA primer creates a recognition site for the restriction enzyme *BcuI* (Figure 2.3).

For PIRA-PCR analysis, a PCR product was amplified using primers PfCLR-G479A-F and PfCLR-G479A-R (with sequence 5'- AAA ATG ATT TGC TGT CTT ATT A -3') with final concentration of 0.2 μM, of each primer, 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates and 1.25 units of GoTaq® Flexi DNA Polymerase (Promega). Conditions of PCR were: initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 51 °C for 45 s and 72 °C for 60 s, and a final extension step of 72 °C for 10 min. 10 μl the PCR reaction mixture was digest with 20 units of *Bcu*I restriction enzyme (Fermentas) with 1 ×Buffer Tango in a final volume of 20 μl and incubated at 37 ºC for 3 h. The restriction products were run on 3 % agarose gel containing ethidium bromide and visualized by UV transillumination.

Classification of each sample was made after inspection of its respective restriction pattern.

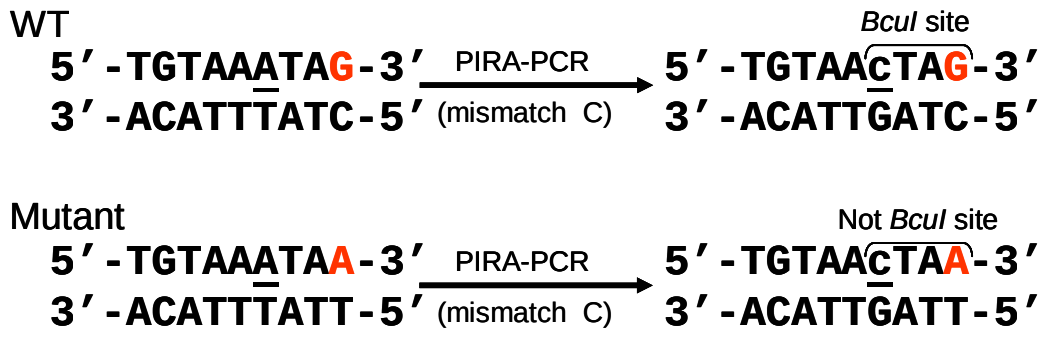

Figure 2.3. Diagram of PIRA-PCR. [Underling nucleotide is the local of the mismatch C introduction. The A to C mismatch incorporation is shown in small letter. The G479A single nucleotide polymorphism is coloured in red].

2.4. Population genetics analysis

Genetic diversity is generally measured as either "haplotype" or "nucleotide" diversity. Gene or haplotype diversity (i.e., the probability that two randomly selected haplotypes are present in the sample) looks only at the number and frequencies of haplotypes, whereas nucleotide diversity also includes a measure of haplotype sequence similarity. The nucleotide diversity (π), which is the average number of base differences per site between two homologous sequences randomly selected from a population, is an important parameter used to understand the structure and history of populations. Nucleotide variability was also measured using the population parameter Theta (θ), which considers the number of segregating sites (S) and the length of the sequences (i.e. the number of sites under study) and is one of the most important parameters in population genetics (http://www.ub.edu/dnasp/DnaSPHelp.pdf).

Levels of genetic diversity were quantified using DnaSP 4.10 program [98]. The following parameters were considered: N (sequences analysed), S (polymorphic sites), H (number of haplotypes), H_d (haplotype diversity) and its standard deviation (SD), π (nucleotide diversity) and SD and θ (Theta), the variability in a

population, calculated from *S*. Insertions and deletions were excluded from all estimates.

2.5. Data analysis

Peirce's criterion was used to test for and exclude statistical outliers. Peirce's criterion is a rigorous method, devised by Benjamin Peirce that may be used to eliminate suspect experimental data using probability theory[99]. This method was used to identify statistical outliers in the data used in genotype-phenotye association studies.

The unpaired *t*-test was applied to assess the association between *in vitro* artemisinin derivatives response and the G479A mutation in the *pfcmu* gene. When P value < 0.05, result was considered as significant. Outliers were excluded from statistical analysis.

CHAPTER 3

RESULTS

Chapter 3: Results

3.1. Identification and characterization of the *Plasmodium falciparum clathrin mu adaptor* and *amino acid transporter* genes and corresponding predicted proteins

We aimed to identify and characterize the above genes in natural parasite populations of *P. falciparum*, namely: i) to define circulating genotypes in different endemic areas, ii) to investigate whether particular genetic polymorphisms could relate to differential parasite drug response levels and iii) to establish measures of genetic diversity and relatedness between genotypes from the different geographical sampling sites.

The DNA sequences of the *P. chabaudi cmu* and *aat1* orthologues were available online at the PlasmoDB database (http://www.genedb.org). In *P. chabaudi*, these genes are located on chromosome 14 and chromosome 11, respectively. According to the genetic sinteny map, the location of these genes in the rodent malaria parasite should correspond to blocks on *P. falciparum* chromosomes 12 and 6 in case of the *cmu* and *aat1* genes, respectively. The *P. falciparum* orthologues of *pccmu* and *pcaat1* genes were also available at the PlasmoDB database with the following accession numbers: PFL0885w and PFF1430c, respectively. These sequences were retrieved and used to design primers to amplify both genes, as described below. The information provided in this database revealed that the *pfcmu* gene is located on chromosome 12 of *P. falciparum* genome, as expected by genetic synteny. The gene is encoded by a

1866 nucleotide open reading frame which predicts a translated protein of 621 amino acid residues with a deduced molecular mass of 72.9 kDa.

The *pfaat1* gene is located on chromosome 6 and the corresponding cDNA contains 2058 bp, with an expected structure comprising a single intron from base 1676 to 1913. The deduced protein consists of 606 amino acids and has a predicted molecular mass of 68.8 kDa.

3.1.1. Identification of conserved domains in the CMU and AAT1 predicted protein sequences

The *P. falciparum* CMU and AAT1 predicted protein sequences were used to identify conserved protein families, using the Pfam database (http://pfam.sanger.ac.uk/), a reliable collection of protein families that includes their annotations and multiple sequence alignments generated using hidden Markov models. This database allows finding all known motifs that occur in a sequence.

E-values (expectation values) were calculated and the resulting scores allowed the identification of a significant pfam-A match in each protein. The E-value is the number of hits that would be expected to have a score equal or better than this by chance alone (a good E-value is much less than 1). This significant match for the CMU predicted aminoacid sequence (E-value = 1.6e-64) corresponded to a protein domain family designated as *Adaptor complexes medium subunit family* (also known as *mu homology* domain) and located between residues 246-621 (Figure 3.1). In the case of AAT1 we identified a protein domain family corresponding to a *Transmembrane amino acid transporter protein* (E-value = 1.2e-44), located between the residues 167-606 (Figure 3.1).

These results provided additional confidence for the correct identification and retrieval of the genes under study.

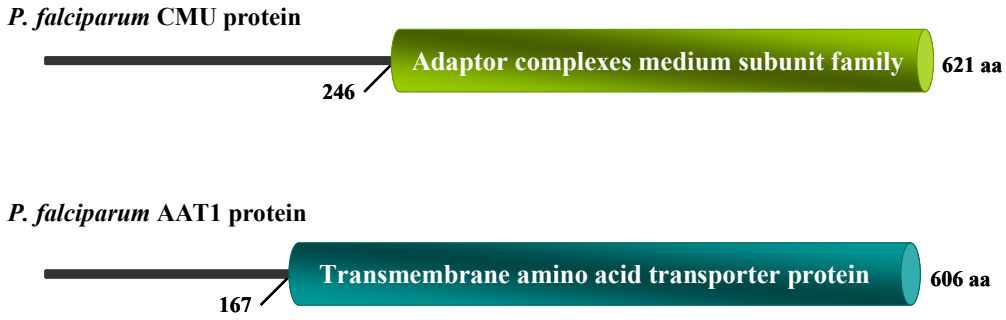

Figure 3.1. Graphical representation of potential Pfam domains of *P. falciparum* CMU and AAT1 proteins. [Domains were predicted by sequence-motif approach using Pfam database (http://pfam.sanger.ac.uk/)].

3.2. Design and optimization of PCR

In our study, we screened 40 field-collected isolates of *P. falciparum* from 3 endemic regions (Brazil, Rwanda and DRSTP) for the presence of mutations in *pfcmu* and *pfaat1* genes, using DNA sequencing. To do this, several primers were designed and used in novel PCR reactions in order to amplify and sequence the entire coding region of the two genes (see "Material and Methods" section for details).

After optimization of the PCR conditions for each primer set, the size of the amplification products was estimated by comparison with a 100 bp molecular marker DNA ladder (MassRuler^{TM} Low Range, Fermentas), in order to ensure that PCR products had the expected size. Typical results after assay optimization are shown in Figure 3.2 and in Figure 3.3 for *pfcmu* and *pfaat1* gene, respectively.

After several rounds of optimization all primer pairs produced the desired results except for *pfaat1* 7F/8R, which consistently failed to generate PCR

amplicons. Consequently a new set of, of primers targeting the same area of the gene, was designed (9F/10R).

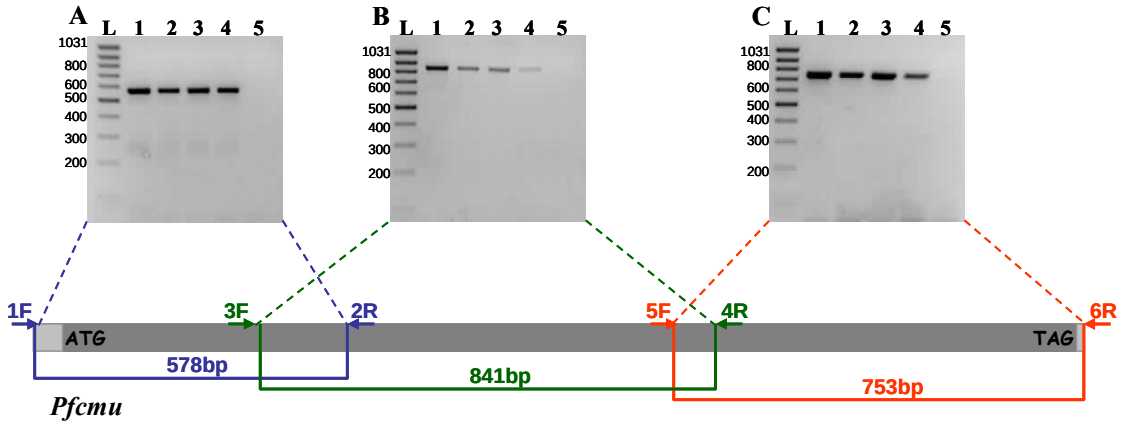

Figure 3.2. (Top) Ethidium bromide-stained 2 % agarose gel electrophoresis of *pfcmu* PCR products [obtained using the 1F/2R set of primers (A), the 3F/4R set of primers (B) or the 5F/4R set of primers (C) are represented. Lanes: L – 100 bp DNA ladder; 1 – Positive Control (3D7); 2, 3, 4 - *P. falciparum* isolates randomly selected; 5 – Negative Control.] **(Bottom)** Schematic representation of *pfcmu* gene structure [showing each primer position and fragments size; Arrows indicate the position of the primers used to amplify the different fragments; F and R indicate the forward and reverse primers, respectively].

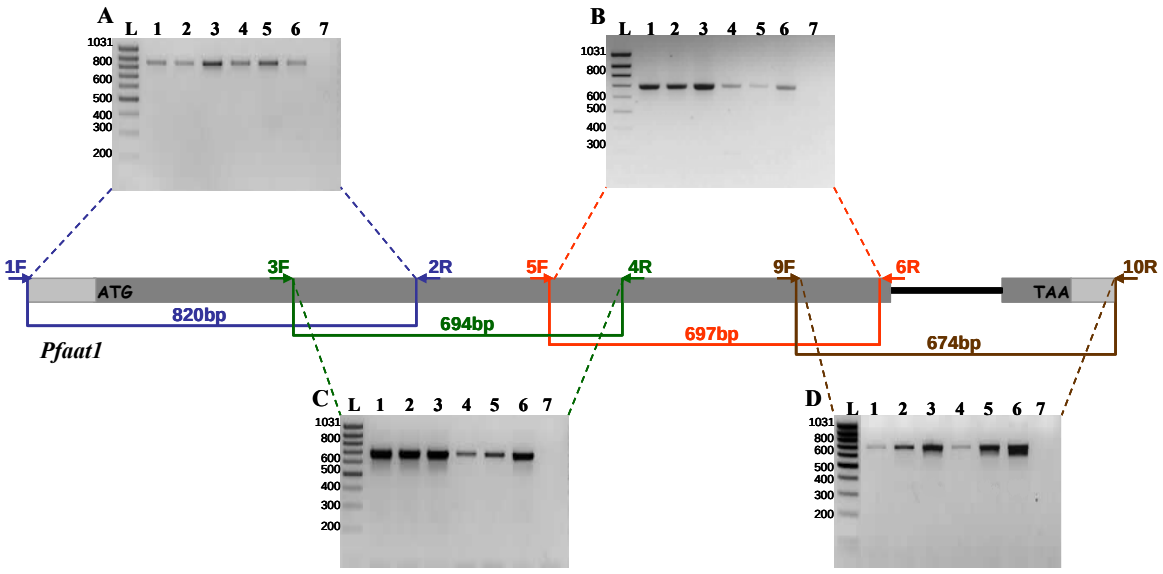

Figure 3.3. (Top and Bottom) Ethidium bromide-stained 2 % agarose gel electrophoresis of *pfaat1* PCR products [obtained using the 1F/2R set of primers (A), the 3F/4R set of primers (B), the 5F/4R set of primers (C) or the 9F/10R set of primers (D). Lanes: L – 100 bp DNA ladder; 1 - Positive Control (3D7); 2, 3, 4, 5, 6- *P. falciparum* isolates randomly selected; 7 – Negative Control]. **(Middle)** Schematic representation of *pfaat1* gene structure [showing each primer position and fragments size. Areas in black indicate the exon and arrows indicate the position of the primers used to amplify the different fragments. F and R indicate the forward and reverse primers, respectively].

3.3. Characterization of gene mutations in *Plasmodium falciparum* isolates

The PCR protocols above were applied to a subset of *P. falciparum* isolates previously selected according to their origin and response to each drug (see "Material and Methods" section for details). For the *pfaat1* gene we selected 16 samples, 8 from Rwanda and 8 from DRSTP according to their response to 4-aminoquinolines. For the *pfcmu* gene we picked 24 samples, 8 from each country (Brazil, Rwanda and DRSTP) according to their response to artemisinin derivatives.

In order to identify possible point mutations in these genes, the overlapping sequencing fragments were compiled to obtain a *pfcmu* and a *pfaat1* sequence for each sample. All sequences thus generated were compared with the reference strain *P. falciparum* 3D7 to search for potential SNPs and insertions/deletions (indels). Sequence alignments were carried out using online software denoted Multiple Sequence Alignment with hierarchical clustering[100].

3.3.1. *Plasmodium falciparum clathrin mu adaptor* gene

Sequence analysis of the *pfcmu* gene revealed a total of 12 polymorphic sites, consisting of 9 SNPs and 3 indels. Details on the genetic polymorphisms identified in the *pfcmu* gene and the corresponding encoded amino acids are shown in Table 3.1. Briefly, among the 12 identified polymorphisms, 7 SNPs and all the insertions were novel mutations compared with the information available in PlasmoDB database (this database incorporates the recently completed *P.*

falciparum genome sequence and annotation, as well as SNPs analysis for several *P. falciparum* strains and isolates).

Five (56 %) of these SNPs were non-synonymous mutations, causing changes in the amino acid sequence while 4 were synonymous mutations (Table 3.1).

Table 3.1. SNPs identified in the *pfcmu* gene with their consequent amino acid replacements.

Codon	Reference sequence (3D7)	Amino acid	Nucleotide substituition	Amino acid changes
100	ATA	Ile	ATC	none
127	GTG	Val	GTT*/GTC	none
146	**AGA**	**Arg**	**AAA**	**Lys**
160	**AGT**	**Ser**	**AAT**	**Asn**
162	ATT	Ile	ATC	none
163	GAA	Glu	GAG*	none
199	**AAA**	**Lys**	**ACA**	**Thr**
233	**AAT**	**Asn**	**AAG**	**Lys**
437	**TTC**	**Phe**	**TTA**	**Leu**

* - SNP present in PlasmoDB database. SNPs with amino acid changes shown in bold type.

All 3 indels observed lead to a 3-nucleotide insertion of the AAT codon that corresponds to an insertion of an asparagine residue in the polypeptide chain (Table 3.2). The insertions of the asparagine residues were found in 3 different stretches of asparagines.

Table 3.2. Indels identified in *pfcmu* gene, nucleotide insertion and consequent additional amino acid.

Codon	Insertion	Amino acid
234	AAT	Asn
325	AAT	Asn
330	AAT	Asn

Polymorphism distribution across functional domains

In order to investigate the possible meaning of the polymorphic sites found in PfCMU we analysed their distribution according to protein domains. Among the 12 mutations/insertions described only 3 lyed in the conserved *Adaptor complex medium subunit family* domain (Figure 3.4), namely, one non-synonymous mutation (F437L) and 2 insertions (325N and 330N).

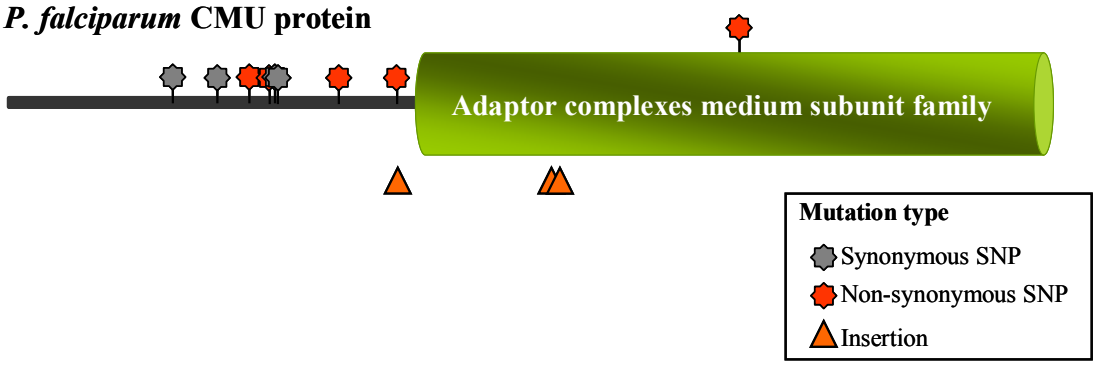

Figure 3.4. Schematic illustration of the described mutations/insertions in the context of functional domains of *P. falciparum* CMU protein. [The locations and the types of the 12 mutations/insertions are also shown].

Polymorphism distribution and prevalence

Analysis of *pfcmu* DNA sequences showed that mutant alleles of this gene were present in 21 of 24 samples (87.5 %). The most common allele of the *pfcmu* gene in the field isolates of *P. falciparum* was 381T (21.9 %). Six of the 13 (46 %) polymorphisms identified were infrequent, with the variant-type alleles occurring only in one isolate (singletons). Seven of the 8 (87.5 %) isolates from Brazil have the same sequence which differs from the 3D7 reference strain in only one point mutation (G381T) (Table 3.3). The remaining *P. falciparum* isolate from Brazil had an identical sequence to the 3D7 reference strain.

The Rwanda samples presented the higher degree of polymorphism, with 8 mutated sites affecting 8 codons and one insertion (Table 3.3). Six of the polymorphisms found were detected in only one *P. falciparum* isolate and variant alleles exist in frequencies ranging from 12.5% (1/8) to 37.5% (3/8).

In the DRSTP, 3 polymorphisms (G381C, G479A and A489G) and 3 insertions were observed (Table 3.3). The 701AAT insertion was predominant, accounting for 4 of 8 isolates (50%). The insertion 987AAT and the A489G SNP were present in 2 of the 8 samples, respectively, whereas the other two SNPs and the 973AAT insertion were detected in only one isolate.

Table 3.3. Prevalence of *pfcmu* mutations/indels identified in isolates of *P. falciparum* from Brazil, Rwanda and RDSTP.

Codon	Brazil (N=8)	Rwanda (N=8)	DRSTP (N=8)
300C	0	2 (25.0 %)	0
381T	7 (87.5 %)	0	0
381C	0	0	1 (12.5 %)
437A	0	1 (12.5 %)	0
479A	0	3 (37.5 %)	1 (12.5 %)
486C	0	1 (12.5 %)	0
489G	0	1 (12.5 %)	2 (25.0 %)
596C	0	1 (12.5 %)	0
699G	0	1 (12.5 %)	0
701AAT	0	0	4 (50.0 %)
973AAT	0	2 (25.0 %)	1 (12.5 %)
987AAT	0	0	2 (25.0 %)
1311A	0	1 (12.5 %)	0

Some of the polymorphisms described above appeared to have strong geographic clustering (Figure 3.5). Six variations identified were seen exclusively among Rwandan isolates (A300C, G437A, T486C, A596C, T699G and C1311A), 3 exclusively among DRSTP isolates (G381C, 701AAT and 987AAT) and 1 signature that was specific for Brazilian samples (G381T). Only 3 polymorphisms were common between Rwanda and DRSTP (G479A, A489G and 987AAT).

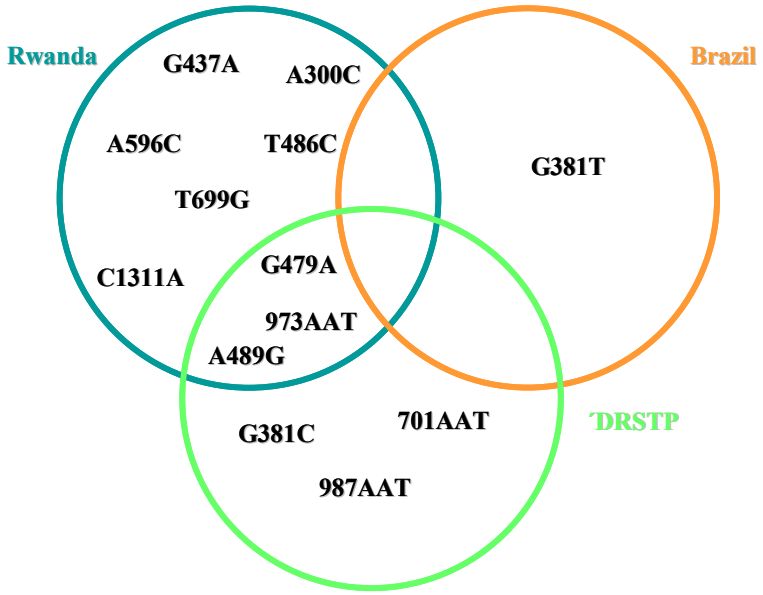

Figure 3.5. Representation of the geographic distribution of the polymorphisms described. [The polymorphic sites are shown by codon].

Haplotype distribution and prevalence

On the total 21 of the 24 isolates showed polymorphisms, a total of 14 different haplotypes were found and 10 of them leading to amino acid substitutions or insertions. The *pfcmu* allelic haplotypes present in each region are shown in Table 3.4.

Thirteen isolates carried single SNP/indel, 6 displayed double SNPs/indels and only 2 carried triple SNPs/indels (Table 3.4).

Unlike the Brazilian samples in which alleles had a single mutated position, 3 out of the 8 alleles from Rwanda were multiple mutants with 2 (haplotype RW1, RW2 and RW4) or 3 (haplotype RW6) mutated codons/insertions. In the DRSTP samples, we also observed multiple mutants with 2 (haplotype DRSTP1 and DRSTP3) or 3 (haplotype DRSTP2) mutated codons/insertions.

Table 3.4. Representation of the haplotypes observed in the different study areas.

		I100I A300C	**V127V** G381T	**V127V** G381C	**R146K** G437A	**S160N** G479A	**I162I** T486C	**E163E** A489G	**K199T** A596C	**N233K** T699G	**N** 701AAT	**N** 973AAT	**N** 989AAT	**F437L** C1311A
Brazil (n=8)	WT (n=1)													
	BR1 (n=7)		■											
Rwanda (n=8)	RW1 (n=1)					■			■					
	RW2 (n=1)						■			■				
	RW3 (n=1)							■						
	RW4 (n=1)	■										■		
	RW5 (n=1)					■								
	RW6 (n=1)				■	■						■		
	RW7 (n=1)													■
	RW8 (n=1)	■												
DRSTP (n=8)	WT (n=2)													
	DRSTP1 (n=2)							■			■			
	DRSTP2 (n=1)					■					■	■		
	DRSTP3 (n=1)			■							■			
	DRSTP4 (n=2)												■	

WT: wild type. The polymorphic sites observed in this study are shown by codon (numbers refer to position in the gene or in the protein sequence). Amino acids are indicated with single letter code in capital, the nucleotide changes are indicated below). Mutations are indicated in black rectangles.

3.3.2. *P. falciparum amino acid transporter* gene

We were not able to obtain the full sequence of the last amplicon of the *pfaat1* gene. Due to a repetitive AT-rich sequence in the terminal region of the gene a reliable sequence product could not be generated. Therefore it was not possible to

obtain the sequence of the coding region between nucleotide positions 1913 and 2058.

Sequence analysis of the *pfaat1* gene revealed only 2 SNPs, 1 non-synonymous mutation (C773T) leading to a serine to leucine substitution (Ser258Leu) and 1 silent mutation (C→A) at nucleotide position 1656. Both SNPs were present in all 16 samples sequenced and were previously described in the PlasmoDB data base.

The analysis of the distribution of the mutations across the protein domains revealed that the 2 mutations found lying in the transmembrane amino acid transporter protein domain family (Figure 3.6).

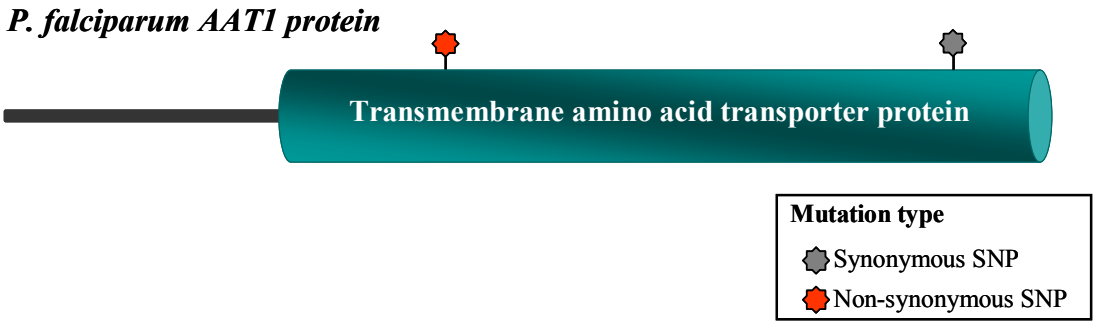

Figure 3.6 Schematic illustration of the described mutations/insertions in the context of functional domains of *P. falciparum* AAT1 protein. [The locations and the types of the 2 mutations are also shown].

3.4. Association between *pfcmu* point mutations and *in vitro* responses profiles to artemisinin derivatives

To investigate the association between the identified mutations and *in vitro* responses profiles to antimalarial drugs, we intended to screen particular mutations in the remaining samples using a strategy that would be more cost-effective than DNA sequencing and that could increase throughput.

The initial study of the full *pfcmu* gene through DNA sequencing allowed to uncover 5 non-synonymous mutations, and 3 asparagine insertions. Details on these genetic polymorphisms, and the corresponding range and mean of IC_{50} values associated with each of them are presented in Table 3.5.

Table 3.5. Details on polymorphisms in *pfcmu* gene in relation to *in vitro* susceptibility to artemisinin derivatives in *P. falciparum* isolates.

Mutant allele	Amino acid Change	Total no. of mutant type alleles (*n* = 24)	Artemisinin derivatives IC_{50} (nM) range (mean)
437A	Arg→Lys	1 (4 %)	9.6
479A	**Ser→Asn**	**4 (17 %)**	**0.3-9.6 (6.2)**
596C	Lys→Thr	1 (4 %)	0.3
699G	Asn→Lys	1 (4 %)	0.3
701AAT	+ Asn	4 (17 %)	0.02-2.0 (0.9)
973AAT	+ Asn	3 (13 %)	0.5-9.6 (3.9)
987AAT	**+ Asn**	**2 (8 %)**	**3.4-9.0 (6.2)**
1311A	Phe→Leu	1 (4 %)	10.1

Polymorphisms with high values of prevalence associated with higher IC_{50} values are in bold.

From the above list of genetic polymorphisms, the 479A SNP and the 987AAT insertion (Table 3.5; shown in bold type), were those associated with higher IC_{50} values (with a mean IC_{50} of 6.2 nM). Therefore, it was decided that these merited further analysis. However, due to time constraints and technical limitations we were unable to analyse the 987AAT insertion at this stage. Therefore, further work was carried out on screening of the G479A mutation solely.

3.4.1. Detection of G479A SNP by PIRA PCR and RFLP

In order to investigate the prevalence of G479A mutation in all isolates available, we developed a PCR-RFLP assay. This assay was then applied to all isolates that were tested for the *in vitro* susceptibility to artemisinin derivatives. From Brazil we analysed 56 isolates, tested for their *in vitro* susceptibility to artemether and to artesunate. From DRSTP we studied 51 and 42 *P. falciparum* isolates tested for their *in vitro* susceptibility to artemether and to artesunate,

respectively. From Rwanda analysed 73 isolates, tested for *in vitro* susceptibility to dihydroartemisinin.

PCR-RFLP is a simple method to identify a point mutation on the condition that the region of interest contains a restriction enzyme site. However, since the G479A point mutation does not create a natural endonuclease restriction site, we used the PIRA-PCR to amplify and restriction-digest the target region (see "Material and Methods" section for details).

The predicted size of the PIRA-PCR product resulting from amplification with primers PfCLR-G479A-F and PfCLR-G479A-R is 268 bp, which can then be cleaved into 245 and 23 bp fragments when digested with *Bcu*I, in the presence of WT allele (TAG). No cleavage is expected in the presence of mutant allele (TAA). The 268 and 245 bp fragments can be easily resolved on 3 % agarose gel, whereas the 23 bp fragment is normally difficult to visualize or discriminate from primer-dimers. Therefore, the 245 bp band was scored as WT and 268 bp as mutant. Figure 3.7 shows typical results of these PIRA-PCR assays.

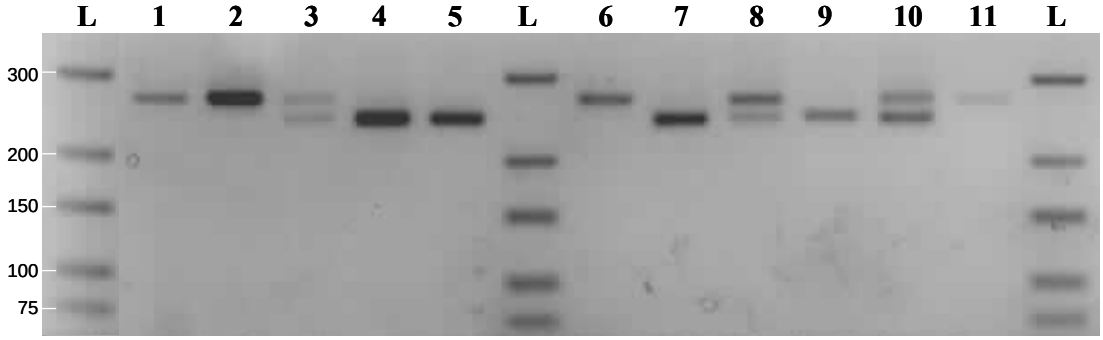

Figure 3.7 Ethidium bromide-stained 3 % agarose gel electrophoresis of *BcuI* digest of PIRA-PCR products [The products were amplified with the set of primers PfCLR-G479A-F and PfCLR-G479A-R. Lanes: L – DNA ladder (O'GeneRuler^{TM} Ultra Low Range); Lane 5 – Positive Control (3D7); Lanes 1 to 4 and 6 to 11 – corresponds to *Bcu*I digests of randomly selected *P. falciparum* isolates. Lanes 4, 7 and 9 corresponds to samples with the WT genotype (245 bp); Lanes 1, 2, 6 and 11 corresponds to samples with the mutant genotype (268 bp); Lanes 3, 8 and 10 corresponds to samples with a mixture of genotypes (WT and mutant)].

3.4.2. Analysis of G479A mutation frequency

To determine whether there was correlation between the G479A mutation and the *in vitro* susceptibility to artemisinin derivatives we analysed this polymorphism in all isolates available. In all 56 Brazil isolates tested for their *in vitro* susceptibility to artesunate and artemether we only found the WT allele. In Rwanda we found 83.6 % (61/73) of isolates with the WT allele, 12.3 % (9/73) with the mutant allele and 4.1 % (3/73) with a mixture of the two genotypes (mixed). In DRSTP isolates tested for *in vitro* susceptibility to artemether we found 86.2 % (44/51) of isolates with the WT allele, 11.8 % (6/51) with the mutant allele and only one isolate with a mixture of the two genotypes. In DRSTP isolates tested for *in vitro* susceptibility to artesunate we found 85.7 % (36/42) of isolates with the WT allele, 11.9 % (5/42) with the mutant allele and only one isolate with a mixture of the two genotypes.

3.4.3. Correlation of *in vitro* susceptibility to artemisinin derivatives and G479A polymorphism in *P. falciparu*m isolates

Results on the distribution of the G479A mutation in Rwanda and DRSTP isolates, according to *in vitro* drug responses, are shown in Figure 3.8.

The outliers were identified by Peirce's method[99] were excluded from further analysis.

The mean dihydroartemisinin IC_{50} in Rwandan isolates harbouring the G479A mutation was 4.78 nM was significantly higher than that in isolates with the wild type allele (mean = 3.01 nM ; p =0.0207). Three isolates showed a mixture of alleles (Wt and Mutant), with a mean IC_{50} of 4.17 nM. We found no significant

differences for the means of the IC_{50} between WT and mixed isolates. Isolates from DRSTP, carrying the G479A mutation had a mean artesunate IC_{50} of 0.63 nM compared to 0.35 nM among those with the wild type allele. Only one isolate showed a mixture of alleles (IC_{50} = 0.02). In case of *in vitro* susceptibility to artemether among DRSTP samples, mean IC_{50} values of 0.66 nM, 0.73nM and 0.02nM, were observed in the mutant, WT and mixed allele groups, respectively. For the DRSTP isolates we found no significant differences in the means of the IC_{50} values in WT and mutant isolates or in WT and mixed isolates. All the isolates from Brazil had the WT allele (artesunate had a mean IC50 of 1.26 and artemether had a mean IC50 of 4.87).

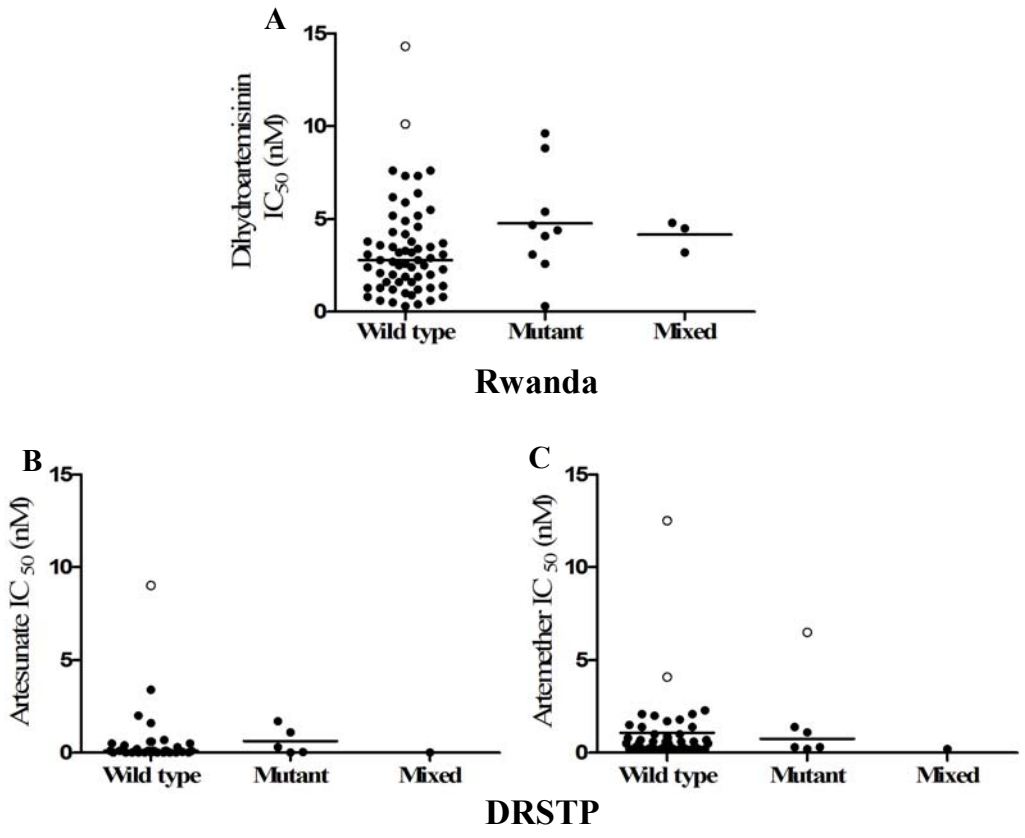

Figure 3.8 Distribution of artemisinin derivatives IC_{50} values and the G479A mutation. [(A): Rwanda isolates tested for *in vitro* susceptibility to dihydroartemisinin; (B) DRSTP isolates tested for *in vitro* susceptibility to artesunate; (C) DRSTP isolates tested for *in vitro* susceptibility to artemether. Wild type: G479; Mutant, A479; Mixed, G479 + A479. Individual values (circles) and mean values (bars) are shown. Outliers are marked as open circles].

3.5. Population genetic analysis of the *P. falciparum clathrin mu adaptor gene*

We analysed DNA polymorphisms of the nucleotide sequence data using the DnaSP software (http://www.ub.edu/dnasp/) in order to gain insights into the levels of genetic diversity. 10 haplotypes and 9 polymorphic sites were identified among the 24 *P. falciparum* individuals studied for which complete DNA sequence was available. Collectively, 8 segregating sites in Rwanda, 3 in DRSTP and 1 in Brazil were identified. These grouped into 7, 5 and 2 different haplotypes, respectively.

Genetic diversity indices for each parasite population are presented in Table 3.6. The theta value of all *P. falciparum* isolates was 0.00129 with the overall π diversity 0.00085 ± 0.00011. Haplotype diversity (Hd), nucleotide diversity per site (π) and theta value showed regional variations. Of the 3 locations, the Rwanda was found to be the most polymorphic population (π = 0.00132, SD = 0.00017). The mean value of nucleotide diversity of DRSTP isolates was 0.00050 ± 0.00011 with θ value of 0.00166. The Brazil population displayed the lowest values for all the indices of genetic diversity and the lowest theta estimates (Table 3.6).

Table 3.6: Estimates of genetic diversity per sample.

	N	***S***	***H***	H_d	**π**	***θ(S)***
Brazil	8	1	2	0.250 ± 0.180	0.00013 ± 0.00010	0.00021
Rwanda	8	8	7	0.964 ± 0.077	0.00132 ± 0.00017	0.00166
DRSTP	8	3	4	0.750 ± 0.139	0.00050 ± 0.00013	0.00062
All samples	24	9	10	0.870 ± 0.045	0.00085 ± 0.00011	0.00129

Legend: *N*: sample size; *S*: number of segregating sites; *H*: number of haplotypes; H_d: haplotype diversity (*i.e.* expected heterozygosity based on haplotype frequency) and its standard deviation, SD; π: nucleotide diversity (average number of nucleotide substitution per site between pairs of sequences in the data set) and SD; $\theta(S)$: θ from *S*.

CHAPTER 4

DISCUSSION

Chapter 4: Discussion

The human malaria parasite *P. falciparum* has acquired resistance to most drugs it has encountered, including the recently deployed Artemisinin Combination Therapy, on which much hope had been laid[31,32,80,101]. The spread of artemisinin resistance could significantly impair global malaria-control efforts. Therefore, the need for identification and monitoring of genes and mutations giving rise to resistance to this class of drugs is immediate. Dealing with this issue is now imperative, before the spread of resistance become uncontainable.

Resistance to artemisinin derivatives has been correlated with mutation-driven alterations in the drug's putative chemotherapeutic target, the SERCA-type ATPase protein of *P. falciparum* (PfATPase6)[57] and amplification of the *multi-drug resistance 1 gene*, *Pfmdr1*[53,77,78]. However, no correlation was found between these genes and *in vivo* resistance to artemisinins in the first confirmed cases of resistance along the Thai/Cambodian border[31,80]. Thus we still lack validated genetic markers to monitor the evolution of artemisinin resistance in natural parasite populations.

The *P. falciparum chloroquine resistance transporter gene* (*pfcrt*) and the *pfmdr1* gene have been linked to 4-aminoquiloline resistance[51,71,72]. Several *in vivo* studies showed absolute selection of the *pfcrt* K76T mutant allele in therapeutic failure. However, the presence of parasites having this allele was not always predictive of the clinical outcome or predictive of *in vitro* resistance [66-68]. Furthermore, the relationship between *pfmdr1* and chloroquine resistance remains unclear and is a matter of controversy[67,73-75]. This suggests involvement of

additional genetic loci modulating chloroquine resistance. For this reason, more studies are necessary to elucidate the mechanisms of resistance to this class of drugs.

Our group has used the *Plasmodium chabaudi* rodent model to investigate the genetic basis of antimalarial drug resistance. By using a novel paradigm based on genome-wide genetics and genomics approaches, we have recently identified a number of previously undescribed genetic markers of resistance to different drugs. Two of these genes are involved in mediating artemisinin resistance, namely a de-ubiquitinating enzyme (UBP1)[89] and a *clathrin mu adaptor gene* (*pfcmu*) (Cravo *et al.*, unpublished). Additionally, one further mutation in an *amino acid transporter gene* (*pfaat1*) was shown to underlie chloroquine resistance. Given the pressing requirement to contain the spread of artemisinin resistance in natural parasite populations and gain new insights into the dynamics of resistance to 4-aminoquiloline, the assessment and validation of new molecular markers for drug surveillance is now a top priority. However, the importance of the markers identified in the rodent model in modulating susceptibility to the different drugs in the human malaria parasite has not been assessed yet. In this context, the objective of this work was to characterize the *pfcmu* and *pfaat1* genes in natural parasite populations of *P. falciparum*. To achieve this, we used an extensive collection of previously sampled isolates of *P. falciparum* from three endemic regions (Rwanda, DRSP and Brazil) which were tested for *in vitro* sensitivity to several drugs. In these samples we investigated the correlation between the presence of mutations in the *pfcmu* and *pfaat1* genes and the *in vitro* sensitivity to artemisinin derivatives and to 4-aminoquinolines, respectively.

In previous studies, the same samples above had been inspected for mutations in a number of previously characterized candidate genes (*pfcrt*, *pfmdr1*, *pfATPase6*, *pfubp1*), but no statistically significant association between the response to drugs and the genes analysed was found[90-92,102,103], suggesting that other molecular determinants are likely to be involved in modulating the parasite's susceptibility to the compounds tested.

4.1. Characterization of the *P. falciparum amino acid transporter gene*

Analysis of the full length sequence of *pfaat1* in 16 isolates from Rwanda and DRSTP, indicated that the gene appears to be highly conserved, with only two SNPs present among the isolates examined, namely. i) a serine to leucine substitution at nucleotide 773; and one silent mutation (C1656A). Alternative explanations to justify this high degree of conservation are discussed below.

A biological argument is that the high level of conservation may be critical for the function of the protein. Alternatively, the *pfaat1* gene could be positioned in a genomic region of reduced variation. It has been demonstrated that genetic diversity suffers from natural degrees of variation across the genome among different chromosomes and/or particular loci[104]. The source of this variation is not fully understood in all cases, but it may be that the *pfaat1* gene could be positioned in a genomic region of intrinsically reduced variation. Well characterized sources of reduced genetic variation across the genome are areas that have undergone selective sweeps, such as those observed around the chloroquine locus, *pfcrt*[105]. In these regions, not only the target gene suffers from limited diversity, but also all the linked *loci* surrounding it. The *pfaat1* gene may lie in

one of these areas and may have, consequently, been exposed to the effects of selective pressures that ended up contributing to decrease its allelic diversity. Finally, the relatively small number of samples sequenced may be other explanation for the homogeneity observed. Thus, additional studies would be of great value to better understand the relative importance of biologic and/or epidemiologic factors responsible for the limited allelic diversity observed in this study.

The two mutations identified were found in all isolates sequenced, independent of the samples' response to 4-aminoquinolines. These data indicate that variations in susceptibility to 4-aminoquinolines in *P. falciparum* may not determinate by changes in the sequence of the *pfaat1* gene. Therefore it is possible that *P. falciparum* may reveal an alternative mechanism of resistance to those of *P. chabaudi*, as in the case of the *pfcrt* gene[84].

4.2. Characterization of the *P. falciparum clathrin mu adaptor gene*

DNA sequencing from 24 isolates distributed among the three regions, revealed nucleotide substitutions at 9 different positions, with five of these SNPs causing the following predicted amino acid shifts: R146K, S160N, K199T, N233K and F437L. A second source of genetic diversity detected in the *pfcmu* gene was sequence length variation due to the presence of indels (insertions/deletions). Three indels at different positions within the coding sequence were detected, all of them leading to a 3-nucleotide insertion of an AAT codon that corresponded to an insertion of an asparagine residue.

The F437L mutation, and the 325N and 330N insertions are predicted to lie in the conserved "*adaptador complexes medium subunit family*" domain (also known as "*mu homology*" domain) of the protein, possibly affecting its function. The other mutations/indels are expected to be positioned in a protein region with no known functional domains. One way to interpret this observation is that mutations can easily accumulate in the less conserved region, since they will not significantly affect the function of the protein. However, the biological significance of the above mutations/indels is difficult to forecast in the absence of functional assays.

Analysis of *pfcmu* DNA sequences showed that mutant alleles of this gene were present in 88% (21/24) of the isolates analysed. There was a clear geographic heterogeneity in the frequency and type of polymorphisms found and none of the mutations were found in *P. falciparum* isolates from all three study areas. In Brazil, the only mutation present was synonymous (G381T) and was highly predominant (88% - 7/8). Polymorphisms in this gene were more frequent in *P. falciparum* populations from Africa, where eight and six mutated sites were found in Rwanda and the DRSTP, respectively. Three of these mutations were detected in isolates from both study areas (G479A, A489G and 978AAT). The other polymorphisms appear to have strong geographic clustering, with six variants identified exclusively among Rwanda isolates (A300C, G437A, T486C, T699G and C1311A) and three among DRSTP isolates (G381C, 701AAT and 987AAT). The G381T mutation is a specific signature of the Brazilian samples.

All measures of genetic polymorphisms showed regional variation among the three *P. falciparum* populations. *Pfcmu* nucleotide and haplotype (gene) diversity

values in Brazil (π = 0.00013; H_d = 0.250) are much lower than those obtained in Rwanda (π = 0.00132; H_d = 0.964) and DRSTP (π = 0.00050; H_d = 0.750). These findings are congruent with other studies of genetic variation in the *P. falciparum* population[106,107,108]. At a global scale, dramatic differences in *P. falciparum* population structure in different locations have been reported. A possible explanation for these findings is the high transmission levels observed in Africa as compared with Asia and South America. High transmission levels are correlated with malaria prevalence and are a direct measure of the parasite reproduction. Thus, it is expected that higher transmission areas will maintain populations with higher effective population size were diversifying natural selection could be a important aspect for sustaining the parasite polymorphisms[107]. An alternative explanation is that *P. falciparum* most likely originated in Africa exhibits more genetic diversity in this continent[109]. The derived populations in Southeast Asia and the Americas may have less genetic diversity because of bottlenecks during the colonization process[110]. The geographic distribution of transmission levels correlate with history, thus both assumptions make the same predictions and are not mutually exclusive.

Understanding the genetic structure of *P. falciparum* is important for predicting how fast particular phenotypes, such as drug resistance, will arise and spread within and between populations.

4.2.1. Correlation of *in vitro* susceptibility to artemisinin derivatives and G479A polymorphism in *P. falciparum* isolates

Pfcmu genotyping and population genetics studies demonstrated that the gene's diversity is variable between different geographical regions. Considering that this variation can pose a confounder effect in the relation between *pfcmu* SNPs/indels and parasite drug susceptibility it may be necessary to further analyse phenotypic associations with particular mutations within a region. Consequently, we investigated the prevalence of the G479A mutation in all isolates tested for their *in vitro* susceptibility to artemisinin derivatives from Brazil, Rwanda and DRSTP. In Brazilian isolates only the WT allele was detected. This allele was found in more than 80 % of the isolates and the mutant allele in ≈ 12% of the isolates from Rwanda and DRSTP. We only found 3 and 1 isolate with a mixture of genotypes in Rwanda and DRSTP, respectively.

A significant association was observed between G479A mutation and the *in vitro* dihydroartemisinin responses in isolates from Rwanda. The mean dihydroartemisinin IC_{50} in Rwandan isolates harbouring the G479A mutation was significantly higher ($p < 0.05$) than that in isolates with the wild type allele. Whereas, the differences in IC_{50} values between the *pfcmu* WT and mutant in DRSTP isolates were not statistically significant. One possible explanation for this finding is that the direct correlation between point mutations and artemisinin susceptibility may be dependent of the geographic origin of the parasites. Alternatively, the isolates from the two countries could have been exposed to different antimalarial drug pressures, leading to different *in vitro* responses to artemisinins.

The G479A mutation leads to the replacement of a Serine by an Asparagine, substitution that could affect the protein function and ultimately affect the parasite susceptibility to artemisinin derivatives. Besides G479A mutation, other two polymorphisms (987AAT and 1311A) could be associated with high IC_{50} values for artemisinins (> 6 nM), but these were not explored in detail. These two mutations result in an insertion or amino acid change, respectively, at the conserved *mu homology* domain of the protein.

Collectively, all of the above polymorphisms are not indicators of resistance, but may reveal the initial stages of reduced susceptibility, with a subsequent process of stepwise mutation. The evolution of resistance to artemisinins can be a stepwise process during which the accumulation of mutations might modulate the response to artemisinins. The first step is the acquisition of some mutations that confer no "real resistance" on its own, followed by subsequent a mutation which augments resistance levels. Therefore, it is possible that some of these mutations are the necessary first step that allows the other changes to be selected.

These findings suggest that analysis of the *pfcmu* gene may be a potential tool for the surveillance of antimalarial drug resistance to artemisinins. However, more in-depth studies are necessary to determine the implications of these and other genetic polymorphisms on *in vitro* drug susceptibility to artemisinin derivatives.

It is known that natural parasite populations of *P. falciparum* vary significantly with respect to their susceptibility to artemisinin derivatives[91,111]. However, the clinical meaning of this phenotype variability is difficult to determine as there is no established *in vitro* cut-off value for resistance to artemisinins. The results of the *in vitro* drug tests to artemisinin derivatives performed in our samples

corroborate these findings, IC_{50} values ranged from 0.02 to 14.3 nM[90-92]. A possible explanation for these variations could be the result of cross-resistance to other antimalarial drugs, that as been proposed in several other studies[81,111,112] . Considering that our samples were collected before the implementation of ACTs, the diversity in *pfcmu* may represent the baseline variation of the gene without artemisinin selection in these regions. In alternative, the inappropriate use of artemisinin compounds in the studied areas may also explain this finding. The natural occurring variation in *pfcmu* may constitute a starting point for artemisinin driven selection of resistant variants. Therefore, it is important to identify the presence of SNPs in *pfcmu* for future monitoring of potential rise and spread of artemisinin resistance based on modifications in this gene.

4.3. Possible model of PfCMU mediated artemisinin resistance

During the blood stage of malaria infection, the parasite internalizes and degrades massive amounts of haemoglobin (Hb) in the digestive vacuole (DV). One of the processes recently described for the delivery of Hb into the DV was the mechanism mediated by membrane-bound vesicles (clathrin coated vesicles)[113]. These membrane-bound vesicles have specific coat proteins (such as clathrin) that are important for cargo selection and direction of transport between donor and acceptor compartments. Clathrin coats contain both clathrin (acts as a scaffold) and adaptor proteins (AP), that link clathrin to receptors in coated vesicles. AP complexes connect cargo proteins and lipids to clathrin at vesicle budding sites, as well as binding accessory proteins that regulate coat assembly and disassembly[114-116]. Interestingly, the *pfcmu* gene encodes a clathrin *mu* adaptor

protein that is a subunit of the AP2 adaptor, associated with the plasma membrane and responsible for endocytosis. Consequently, mutations in this protein may alter the adaptor binding specificity affecting the transport of Hb by clathrin coated vesicles to the DV.

The implications of these mutations to artemisinin resistance phenotypes are unclear in the absence of further studies. We suggest a highly speculative model (Figure 4.1) for the involvement of *pfcmu* mutation in resistance to artemisinins based on the biological process of Hb internalization/degradation in the parasite's DV and in the mechanism of action of artemisinins. Recent work has proposed that artemisinin trioxane derivatives rapidly accumulate in the DV and are activated by neutral lipid-associated haem[117]. The formation of the later depends on Hb-clathrin coated vesicles. Once inside the DV, the Hb-clathrin coated vesicles are digested, releasing amino acids, lipid components (phospholipids) and free haem. The phospholipids are afterwards degraded and its components are reconstituted to form neutral lipid-associated haem[117,118]. We propose that mutations in the *pfcmu* gene may impair Hb transport into DV via clathrin coated vesicles. By using alternative pathways of Hb uptake, the parasite could have access to Hb without the release of phospholipids (required for artemisinin activation), consequently modulating its response to artemisinin.

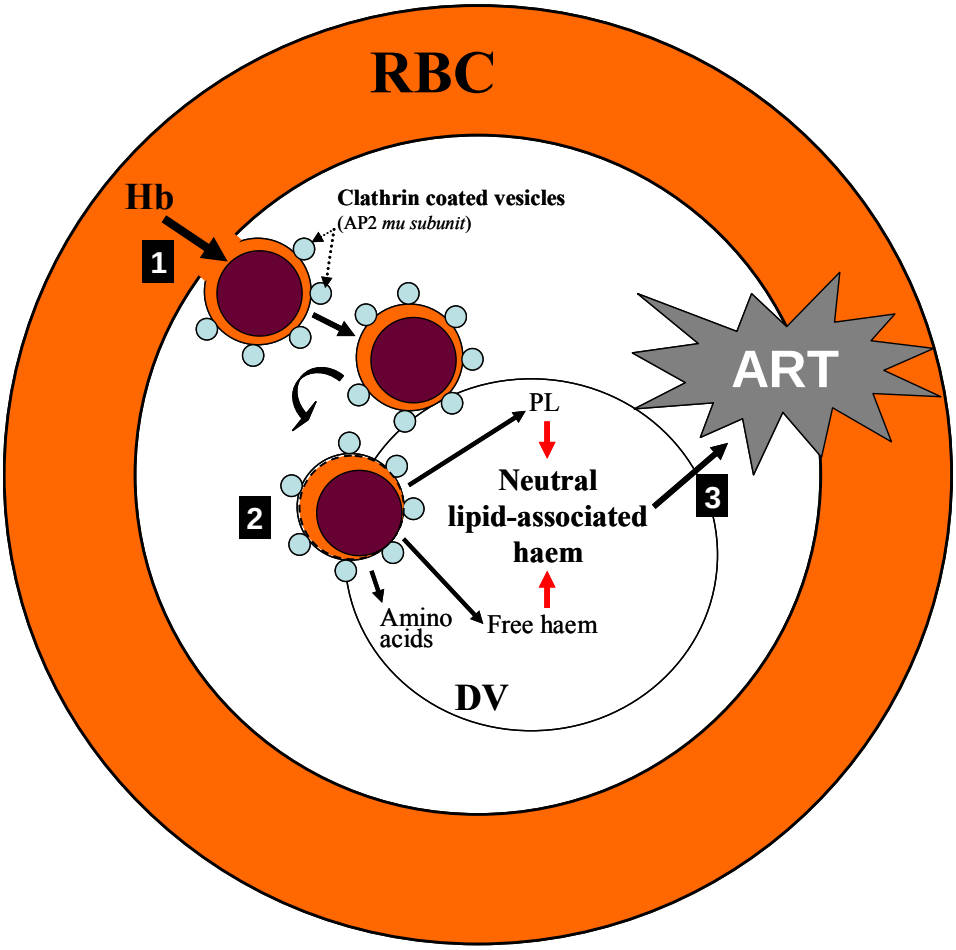

Figure 4.1. Putative mechanism of PfCMU mediated artemisinin resistance. Legend: RBC: Red Blood Cell; 1) Haemoglobin (Hb) transport into the digestive vacuole (DV) by a mechanism mediated by clathrin coated vesicles; 2) Digestion of Hb-clathrin coated vesicles with the release of amino acids, phospholipids (PL) and free haem. Phospholipids are subsequently degraded and reconstituted to form neutral lipid-associated haem. 3) Activation of artemisinin (ART) by neutral lipid-associated haem.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Chapter 5: Conclusions and future perspectives

We identify and characterize the *P. falciparum cmu* and *aat1* genes and investigate its possible involvement in the *in vitro* responses to artemisinin derivatives and 4-aminoquinolies, respectively.

The *Pfaat1* gene appears to be highly conserved and no correlations were found between this gene and the *in vitro* resistance to 4-aminoquinolines. *P. falciparum* may reveal an alternative mechanism of resistance to those of *P. chabaudi.*

In contrast to *Pfaat1*, the *Pfcmu* gene was genetically diverse, with some of the identified SNPs and indels showing geographical specificity. This diversity may provide useful baseline data for future studies on this candidate drug resistance locus.

We found a significant association between the *pfcmu* 479A genotype in Rwanda samples and the *in vitro* sensitivity to dyhidroartemisinin. These constitute new findings to suggest that polymorphisms in *pfcmu* can be involved in *P. falciparum* defence mechanisms against artemisinin derivatives. Thus, further assessment of the gene in artemisinin responses is a top priority, in the context of effective surveillance of artemisinin resistance.

In conclusion, malaria is a major public health problem which is further enhanced by *P. falciparum* drug resistance. The understanding of the mechanisms of resistance to antimalarial drugs is crucial. This thesis has shed light on the

complex issue of *P. falciparum* resistance to artemisinins and will foster new studies to understand its mechanisms.

CHAPTER 6

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Chapter 6: References

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