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EARLY DETECTION OF THE BIOLOGICAL AND GENETIC DETERMINANTS OF RESISTANCE TO ARTEMISININ-BASED COMBINATION THERAPY IN MALARIA PARASITES

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

µl – microliter

µM – micromolar

aat1 – aminoacid transporter gene

ACT – Artemisinin Combination Therapy

ARMD – accelerated resistance to multiple drugs

ART – artemisinin

ATN – artesunate

ATP – adenosine triphosphate

BLAST – basic local alignment search tool

bp – base pair

cDNA – complementary deoxyribonucleic acid

Chr – chromosome

CI – comparative index

CQ – chloroquine

dhfr – dihydrofolate reductase gene

dhps – dihydropteroate synthase gene

DMSO – dimetilsulphoxyde

DNA – deoxyribonucleic acid

DNAse I – deoxyribonuclease I

dNTP – deoxyribonucleotide triphosphate

EDTA – ethylenediaminetetraacetic acid

EPO – erythropoietin

IC50 – 50% inhibitory concentration

IC90 – 90% inhibitory concentration

indel – insertion/deletion

Kb – kilo base pairs

LGS – linkage group selection

Mb – mega base pairs

mdr1 – multidrug resistance 1 gene

MF – mefloquine

mg/kg – milligrams of drug per kilogram of body weight

min – minutes

ml – mililiter

mM – milimolar

nM – nanomolar

PABA – para-aminobenzoic acid

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PYR – pyrimethamine

RBCp – parasitized red blood cells

RNA – ribonucleic acid

RNAses – ribonuclease

rpm – revolutions per minute

SDS – sodium dodecyl sulfate

SDX – sulphadoxine

SE – standard error

SERCA – sarco-endoplasmic calcium ATPase

SNP – single nucleotide polymorphism

TBE – tris-borate-EDTA buffer

TCTP – translationally controlled tumour protein

TE – tris-EDTA buffer

ubp1 – deubiquitinating 1 gene

WHO – World Health Organisation

ABSTRACT

Artemisinin Combination Therapy (ACT) is the last standing force against multi-drug resistant malaria parasites. ACTs consist of the simultaneous administration of an artemisinin derivative and a chemically non-related antimalarial drug. Many studies have debated the use of ACTs as having many advantages, relying on the high efficacy and fast action of ART derivatives and on long-half life drugs used as partners in order to circumvent ART derivatives' short half life. However, sub-standard drug administration, amongst other factors, has contributed to the appearance of resistance to each component of combination therapy. Pre-existing resistance to one partner drug of the combination may be an important factor contributing to the appearance of resistance to ACTs in the field. In addition, parasites which are resistant to each component may undergo genetic recombination in the mosquito and originate new strains showing resistance to both partner drugs simultaneously.

In order to test these hypotheses and in an attempt to model ACT resistance as it might occur in the field, the rodent model *Plasmodium chabaudi* was used in this study. Although rodent laboratory models may not mimic *Plasmodium falciparum* natural parasite populations in their every characteristic, they provide important insights on the parasite's biology, such as mechanisms used to escape drug action, and may provide tools for estimation of drug's useful life and the help in the design of drug policies for endemic countries.

Experimental evolution of the artesunate + mefloquine (ATN + MF) version of ACT was accomplished by selection through drug pressure using two different approaches: i) a MF-resistant clone, AS-15MF, was subjected to consecutive sub-inoculations in mice treated with stepwise increasing doses of ATN alone; and ii) an ATN-resistant clone, AS-ATN, was subjected to consecutive sub-inoculations in mice treated with stepwise increasing doses of the ATN + MF combination. In both cases, the AS-MFATN-5 and AS-ATNMF-1 clones generated

here showed higher levels of resistance to ATN + MF than their respective progenitors AS-15MF and AS-ATN.

Genetic analyses revealed that both AS-MFATN-5 and AS-ATNMF-1 gained an extra copy of the *mdr1* gene during the evolution of resistance, and this extra copy was met with an increase in RNA and protein expression in AS-ATNMF-1. In addition, Solexa whole genome re-sequencing showed that AS-ATNMF-1 carried other two further novel non-synonymous mutations in the PCAS_132020 and PCAS_143160 genes, the function of which are unknown.

AS-ATNMF-1 was also compared with its progenitor AS-ATN in within-host competition assays is order to estimate if ATN + MF resistance would impose a biological cost to these parasites. In addition, AS-ATNMF-1 was compared with AS-ATN passaged through mice twenty seven times (AS-ATN27P) with the purpose of checking if consecutive sub-inoculations in mice would be associated with increased parasite fitness. Whilst AS-ATNMF-1 outgrew its progenitor AS-ATN, the same trend was not observed when in competition with AS-ATN27P.

These results suggest that *mdr1* amplification and overexpression may play a central role in ATN + MF resistance in our model. However, the newly acquired mutations may also influence parasite's phenotype. In addition, acquisition of these mutations is not associated with increased fitness cost to the resistant clone when compared to its ATN + MF-sensitive progenitor.

In conclusion, the efficacy of ACTs may be hampered by the development of a generic mechanism of drug-evasion that allows parasites to prevent the action of both drugs of the combination simultaneously. In addition, resistant parasites may show little or no reduction in fitness which could contribute to their rapid spread. The results of this work may provide valuable insights for improved deployment of drug combinations and for molecular surveillance of ACT efficacy in the field.

RESUMO

A utilização de Terapia Combinada com Artemisinina (ACT), administração simultânea de derivativos de Artemisinina (ART) com outros antimaláricos quimicamente distintos, representa atualmente o último recurso eficaz contra parasitas de malária humana múltiplos fármacos. As vantagens da utilização de ACTs para o tratamento da malária foram debatidas em diversos estudos, baseando-se fundamentalmente na elevada eficácia e rápida ação dos derivados de ART associadas à utilização de fármacos de semi-vida longa, com o objetivo de complementar a curta semi-vida dos derivativos de ART. No entanto, administração destes fármacos em níveis sub-terapêuticos, entre outros factores, contribuiu para o aparecimento de parasitas resistentes a cada componente da terapia combinada. O aparecimento de resistência a um dos componentes da combinação pode contribuir para o aparecimento de resistência aos ACTs em populações parasitárias. Adicionalmente, parasitas resistentes a cada um dos componentes dos ACTs podem sofrer recombinação genética em mosquitos vectores, o que poderia originar novas estirpes resistentes simultaneamente aos dois fármacos da combinação.

Com o objectivo de testar estas hipóteses e visando criar um modelo capaz de representar a possível evolução de resistência aos ACT em parasitas de humanos, o parasita de malária murina *Plasmodium chabaudi* foi utilizado neste estudo. Apesar de poderem não reflectir os mecanismos ocorrentes em *Plasmodium falciparum* com exatidão, os modelos murinos representam um exelente recurso para obter indícios relativos à biologia do parasita, nomeadamente, aos mecanismos de evasão à acção de fármacos. Além disso, podem ainda fornecer ferramentas necessárias para que a vida útil de um determinado fármaco seja avaliada e contribuir para o desenvolvimento de políticas apropriadas para cada país onde a doença é endemica.

No decorrer deste trabalho, a evolução experimental de resistência à versão de ACTs que consiste na combinação de artesunato e mefloquina (ATN + MF) foi obtida através de seleção

por pressão de fármaco utilizando duas abordagens: i) o clone resistente à MF, AS-15MF, foi submetido a sub-inoculações consecutivas em murganhos tratados com doses crescentes de ATN; e ii) o clone resistente a ATN, AS-ATN, foi submetido a sub-inoculações consecutivas em murganhos tratados com doses crescentes da combinação ATN + MF. Em ambos os casos, os clones obtidos, AS-MFATN-5 e AS-ATNMF-1, apresentaram níveis acrescidos de resistência a ATN + MF quando comparados aos seus progenitores AS-15MF e AS-ATN, respectivamente.

A análise genética dos clones AS-MFATN-5 e AS-ATNMF-1 revelou que os mesmos haviam adquirido uma cópia extra do gene *mdr1*, e que a aquisição desta cópia extra foi acompanhada pelo aumento nos níveis de expressão de RNA e proteína no clone AS-ATNMF-1. Adicionalmente, a re-sequenciação do genoma completo do clone AS-ATNMF-1 através do método Solexa revelou a presença adicional de duas novas mutações não sinónimas nos genes PCAS_132020 e PCAS_143160, ambos de função desconhecida.

O clone AS-ATNMF-1 foi ainda comparado ao seu progenitor AS-ATN em ensaios de competição intra-hospedeiro, com o propósito de avaliar um potencial custo biológico da resistência à combinação ATN + MF. Além disso, o parasita AS-ATNMF-1 foi comparado com o clone AS-ATN sub-inoculado vinte e sete vezes em murganhos na ausência de tratamento com fármaco (AS-ATN27P), com o objectivo de verificar se sub-inoculações consecutivas estariam associadas a um aumento no *fitness* destes parasitas. Nestes ensaios, enquanto o clone AS-ATNMF-1 foi capaz de superar o seu progenitor AS-ATN em termos de multiplicação parasitária, esta mesma tendência não foi observada quando em competição com AS-ATN27P.

Colectivamente, os resultados descritos neste trabalho sugerem que a amplificação do gene *mdr1* desempenha um papel importante na resistência a ATN + MF gerada neste modelo experimental. No entanto, mutações adicionais podem também ter algum papel nessse fenótipo.

Adicionalmente, a presença destas mutações não está associada a uma redução no "fitness" do clone resistente AS-ATNMF-1 quando comparado ao progenitor sensível a ATN + MF.

Em conclusão, a eficácia do tratamento baseado em ACTs pode ser comprometida pelo aparecimento de parasitas portadores de um mecanismo genérico de evasão à acção farmacológica, que previna a actuação dos dois componentes da combinação em simultâneo. Além disso, parasitas resistentes podem não apresentar redução no "fitness", o que poderia contribuir para o seu rápido alastramento.

Os resultados apresentados neste trabalho e a suas implicações inerentes poderão servir de base para o aperfeiçoamento das terapias utilizando combinações de fármacos e das ferramentas moleculares para a monitorização da eficácia dos ACTs em populações parasitárias selvagens.

PREFACE

This thesis describes the generation of resistance to Artemisinin Combination Therapy in the rodent malaria parasite *Plasmodium chabaudi*, the genetic mechanisms associated with it and the potential implications of resistance in parasite fitness.

This dissertation is divided into three main chapters:

I. General Introduction;

II. Evolution of resistance to the artesunate + mefloquine combination and identification of genetic markers of resistance;

III. Fitness cost of resistance to artesunate + mefloquine.

Chapter I contains a brief review of the general aspects pertaining to the malaria parasite, such as pathogenesis, life cycle, genetics and control of the disease. Chapters II and III contain a brief introduction, in which a review of the literature will be presented, highlighting the most important points in the context of the work developed. The results obtained will be presented and discussed.

A list of six Appendices is provided. The Appendices contain results of unsuccessful experiments as well as sequence alignments and lengthy tables.

The Material and Methods section is presented last, where detailed description of procedures carried out throughout this work is provided.

Finally, the literature used as support for this work will be compiled and displayed in alphabetical order in the References section.

CHAPTER I

GENERAL INTRODUCTION

I.1. EPIDEMIOLOGY OF MALARIA

Malaria is one of the most concerning health problems of the present time. The disease is present in more than 108 countries, the most affected being in sub-Saharan Africa, where the majority of cases and deaths are reported. Approximately 3,000 million people are at risk of being infected. In fact, more than 247 million cases occur each year, causing almost one million deaths (WHO – Fact sheet 94, April 2010). The most susceptible individuals are children under 5 years of age and pregnant women. Also, travellers from non-endemic countries visiting endemic countries are highly susceptible due to their non-immune status (WHO – Fact sheet 94, April 2010).

Besides high mortality rates, malaria is also responsible for high levels of morbidity among the affected populations, and is intimately associated with, and also responsible for hindering the economical development of countries where it is endemic (Sachs and Malaney, 2002).

Malaria is caused by a protozoan parasite of the genus Plasmodium. The four important species infecting humans are: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*. The latter is the most virulent form, causing the most serious cases of the disease. In spite of *P. falciparum*'s high prevalence in endemic regions of Africa and South East Asia, other species, such as *P. vivax,* are spreading and the number of cases is increasing (Price *et al,* 2007).

The symptoms of the disease include high fever, headache, chills, and vomiting, and if not treated in time, *P. falciparum* malaria can cause severe anaemia and death (WHO, 2006). In the most susceptible individuals, *P. falciparum* infections can also evolve to a deadly condition called cerebral malaria, characterised by seizures followed by coma and death (Idro *et al,* 2005). Although malaria is a curable disease, relapses may occur, if patients infected with *P.*

vivax or *P. ovale* do not receive specific treatment against the "dormant" forms of the parasites called hypnozoites (see Section I.2).

I.2. LIFE CYCLE OF THE MALARIA PARASITE

Malaria is transmitted to humans by the bite of a female mosquito of genus Anopheles and Plasmodium parasites' life cycle is composed of three stages: i) hepatic schizogony; ii) erythrocytic schizogony; and iii) sporogony. Throughout the three stages of development the parasite inhabits two different hosts: the two first stages take place in the mammalian host, whilst the last one occurs in the female Anopheline mosquito.

Plasmodium's life cycle (Figure 1) begins when the female Anopheles mosquito bites the mammalian host, introducing motile parasites forms known as sporozoites. The sporozoites reach the blood stream, travelling through it until they reach the liver. The parasites then invade and cross a few hepatic cells and finally establish in one of them. There they undergo several rounds of mitotic replication during 5 to 7 days, generating tens of thousands of merozoites. This eventually causes the rupture of the hepatocyte, releasing the merozoites into the host's blood stream. Alternatively, *P. vivax* and *P. ovale* sporozoites can mature and evolve into a "dormant" form, known as hypnozoite that can re-awaken months or even years later, causing the relapses characteristic of the infection by these species.

Upon reaching the blood stream, the merozoites invade the host's erythrocytes where they feed on cell resources, grow, and develop into schizonts. The schizonts undergo many rounds of mitotic divisions, forming approximately 8-to-20 new daughter merozoites. The erythrocyte's membrane is then disrupted, releasing the merozoites in the blood stream, where they will invade new erythrocytes, and again, grow into schizonts. This synchronous cycle happens at regular intervals, and its duration depends on the infecting species, e.g. 48 or 72 hours in *P. falciparum* or *P. malariae* infections, respectively. In addition, the synchronous release of the

merozoites and the disruption of the erythrocytes are responsible for the release of the intracellular material, causing the fever episodes characteristic of the disease.

Alternatively, instead of undergoing mitosis and forming new merozoites, schizonts can differentiate into different types of cells, forming the precursors of micro or macrogametes (male and female, respectively), called gametocytes. When the female Anopheles mosquito bites an infected host, it must ingest both male and female gametocytes for the cycle to continue. Inside the mosquito's midgut, the male and female gametocytes differentiate into gametes, which then fuse and become a diploid motile cell known as ookinete (zygote). It is in the ookinete that recombination of the genetic material from each parent takes place (Section I.3.2). The ookinete crosses the midgut wall, where it settles and undergoes one round of meiotic division. Finally, it matures into an oocyst which undergoes many mitotic multiplication rounds, generating about a thousand sporozoites. 5-to-7 days later, the sporozoites are released into the mosquito's haemolymph and then migrate into the mosquito's salivary gland. They will remain there rendering this mosquito infective throughout its whole life.

Upon biting a new host, the infective mosquito injects the sporozoites into the mammalian host, restarting the cycle.

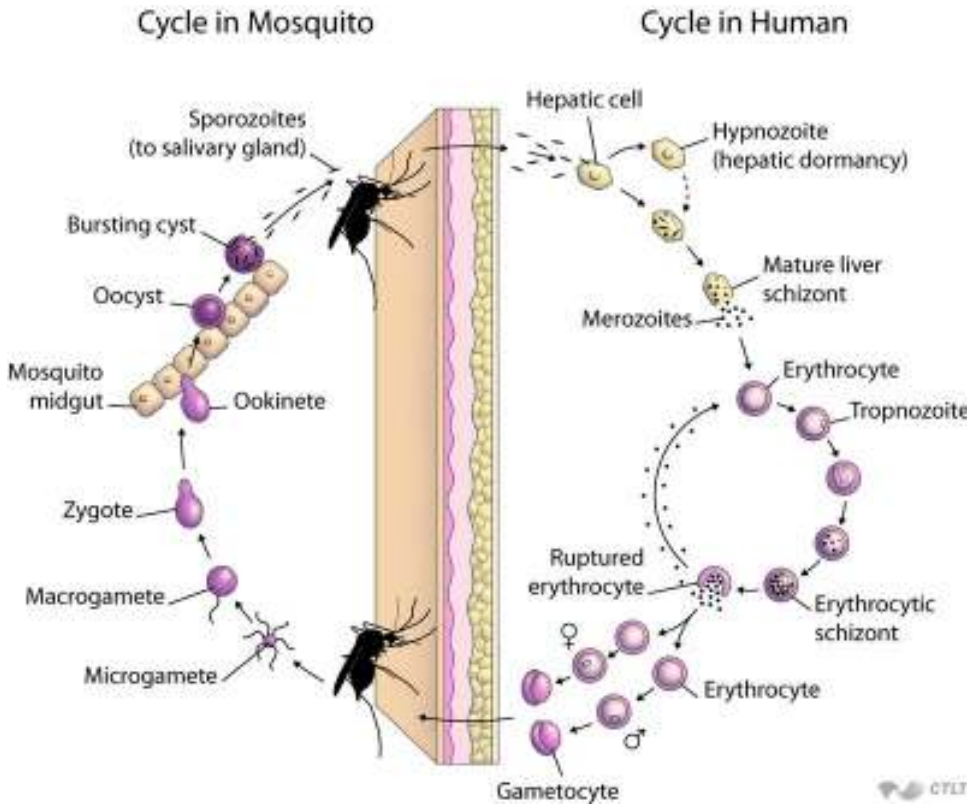


Figure 1 - *Plasmodium* spp life cycle. Graphic representation from "Life cycle of the malaria parasite" from Epidemiology of Infectious Diseases. Available at: http://ocw.jhsph.edu. Copyright © Johns Hopkins Bloomberg School of Public Health. Creative Commons BY-NC-SA.

I.3. GENETICS OF THE MALARIA PARASITE

I.3.1. GENOME ORGANISATION AND STRUCTURE

As described above, Plasmodium parasites are haploid during most parts of the life cycle, undergoing replication mostly by mitotic division. The only diploid phase takes place in the ookinete, the zygote resulting from the fusion of the two haploid gametes inside the mosquito midgut after the ingestion of infected blood (Sinden *et al,* 1985).

During the haploid phase, the parasite's genetic material is organised into three genomes: i) the mitochondrial genome, which is composed of 6 Kb in size and codes for a few specific mitochondrial genes, such as cytochrome b; ii) the plastid 35 Kb circular genome, coding mostly for tRNAs and rRNAs (Wilson and Williamson, 1997); and iii) the nuclear genome.

The nuclear genome of the malaria parasite is composed of 14 chromosomes of different sizes (~500 Kb – ~3 Mb) and totals approximately 20 Mb. The genome has a very high A + T content ranging from 55% to 80% depending on the *Plasmodium* species (Carlton *et al,* 2002; Gardner *et al,* 2002; Hall *et al,* 2005; Carlton *et al,* 2008). The A + T content is not constant along the chromosomes, with telomeric and sub-telomeric regions having higher A + T content than central chromosomal areas. In addition, non-coding regions are more A + T rich than coding regions (Carlton *et al,* 2005).

The whole genome of *Plasmodium falciparum* codes for approximately 5,300 genes, and at the time of the publication of the last *P. falciparum* genome assembly, 60% of all genes had no known function (Gardner *et al,* 2002). However, the genome is constantly being updated, as new molecular data on gene functions (Tuikue *et al,* 2008; Moyano *et al,* 2009) and new bioinformatics tools for comparative genomic analysis become available (Brehelin *et al,* 2008).

In general, the organisation (order and orientation) of genes in internal areas of the chromosomes is highly conserved between *Plasmodium* spp.; referred to as gene synteny (Hall *et al,* 2005; Kooij *et al,* 2005). On the other hand, sub-telomeric regions of chromosomes are far less conserved and contain different families of species-specific variable genes which are believed to be involved in antigenic variation and evasion from the host's immune system (van Lin *et al,* 2000).

When analysing genome sequence variations comparing parasites belonging to the same species, sub-telomeric genes show the most variable sequences mainly due to frequent recombination events (Mu *et al,* 2005). However, sequence variation can also occur in core regions of chromosomes. In addition to recombination, genome sequence variations can happen due to single nucleotide polymorphisms (SNPs) (Wootton *et al,* 2002) , insertions/deletions (indels) (Anderson *et al,* 2005), large scale deletions (Biggs *et al,* 1989), amplifications

(Cowman *et al,* 1994), inversions (Pologe *et al,* 1990), and translocations (Hinterberg *et al,* 1994).

Although no variation is found in chromosome number, variation in size of one chromosome when comparing different strains of the same species may be observed. This is believed to take place due to recombination events between homologous regions of different chromosomes, and also due to gene amplification events and/or indels. In the case of *in vitro* cultured strains, this variation may happen due to chromosome breakage and the consequent healing of blunt ends by the addition of telomeric repeats (Janse, 1993; Lanzer *et al,* 1995; Carlton *et al,* 2005).

The completion of *Plasmodium falciparum* genome in 2002 (Gardner *et al,* 2002) has opened the so-called "post-genomic" era in the investigation of the parasite's biology. The knowledge of the complete sequence of the *P. falciparum* genome, added to studies on conservation of gene order between different species (gene synteny)) (Hall *et al,* 2005; Kooij *et al,* 2005) provided new ways of identifying genetic markers involved in the expression of different phenotypes, such as drug resistance. Prior to this, advances in understanding the genetics of the malaria parasite were made relying on labour intensive performances of genetic-crosses, based on knowledge of a limited number of markers distinguishing different parasite strains.

The analysis of polymorphisms in target genes also led to some advances in understanding the mechanisms underlying different phenotypes. For instance, using a candidate gene approach, Sidhu and colleagues identified a mutation responsible for resistance to azithromycin in *P. falciparum* (Sidhu *et al,* 2007).

An important innovation in the search for genetic variations has been the recent developments in genome sequencing techniques. The advent of second generation sequencing (such as the Illumina Solexa and Roche 454 platforms) has greatly facilitated the rapid

discovery of genome-wide variants without prior knowledge of candidate genes (Morozova and Marra, 2008). The Illumina Solexa platform (Bentley, 2006) in particular has been adopted in the study of genetic variants in *P. chabaudi*, allowing the identification of candidate genes underlying various drug resistance phenotypes (Hunt *et al*, 2010, Martinelli unpublished results).

Another very useful genome-wide analysis tool is a technique known as Linkage Group Selection (LGS). This method was developed in *P. chabaudi*, and may potentially also be applied to other species for comparing two different strains displaying any selectable feature (Culleton *et al,* 2005; Martinelli *et al,* 2005; Carter *et al,* 2007). A map of genetic markers covering the whole genome of the parasites must be known, in order to allow the distinction between the two strains in a parasite mixture. LGS consists of performing a genetic cross (Section I.3.2) between the two strains of interest and then applying a selective pressure on the uncloned progeny obtained from the genetic cross. The selective pressure is meant to eliminate the most susceptible parasites from the cross progeny, promoting the survival of parasites showing the most favourable traits. Thus, the markers in closer linkage with the genes determining the phenotype of interest will be maintained, whilst the most distant will be eliminated. By comparing the percentage of each marker across the whole genome of the selected progeny and the unselected progeny of the same cross, it is possible to observe a selection valley around the locus/loci in linkage with the phenotype of interest. In theory, the gene that determines the phenotype under investigation should be located at the bottom of the selection valley (Culleton *et al,* 2005; Martinelli *et al,* 2005). In combination with second-generation sequencing, LGS provides an even more powerful and rapid method for the identification of mutations underlying selectable phenotypes (Hunt *et al*, 2010).

I.3.2. SEXUAL REPRODUCTION AND GENETIC RECOMBINATION

It is inside the mosquitoes' midgut that male and female gametes of the parasite will fuse, originating a diploid zygote, the ookinete. In high transmission areas infections usually consist of 3 to 7 genetically distinct parasite clones simultaneously circulating in human host blood stream (Arnot, 1998). When the mosquito ingests gametocytes deriving from different parasites clones, two outcomes are possible: i) self-fertilisation, when two gametes from the same clone fuse, and in this case, the resulting offspring is genetically identical to the parental clone; or ii) cross-fertilisation, when two gametes from different parasite clones form the zygote, and therefore, in this situation, recombination of the genetic material belonging to each parental takes place. The cross-progeny then will harbour a mixture of different alleles inherited from both parents (Figure 2).

The existence of genetic recombination was first shown in the rodent model *P. chabaudi*, in 1975 (Walliker *et al,* 1975). Since then, many advances were made in this field. It is now known that the rates of recombination events in Plasmodium are usually higher in sub-telomeric regions and non-coding repetitive sequences. Genes in internal regions of the chromosomes seem to be less prone to undergo rearrangement, unless they are under strong selective pressure (Carlton *et al,* 2005).

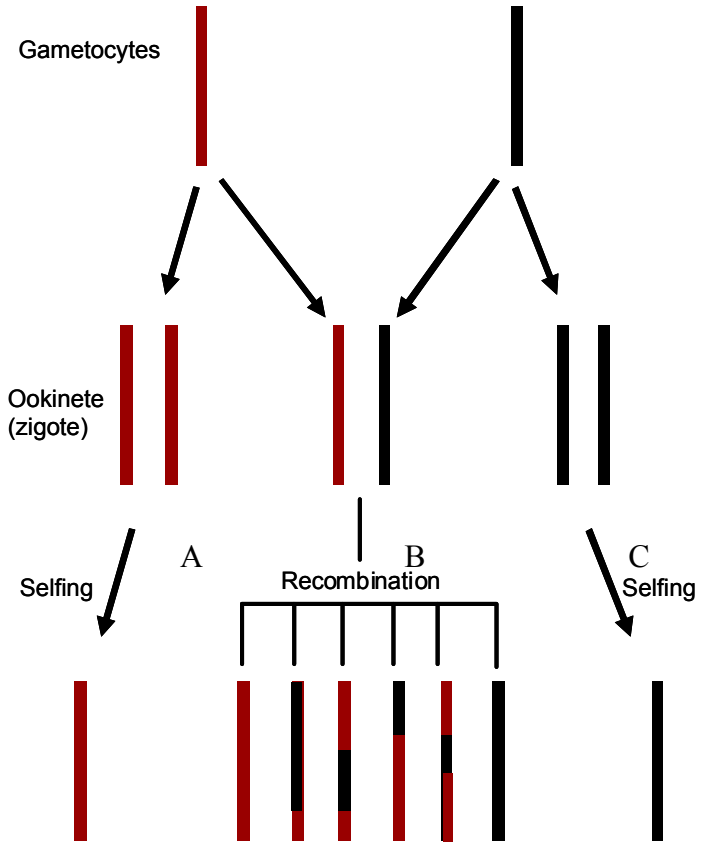


Figure 2 – Schematic representation of the possible outcomes when gametocytes are ingested in the bloodmeal of a female Anopheles mosquito. Two gametocytes are represented (red and black bars), indicating parasites carrying different genetic material. When two gametocytes carrying the same genetic material fuse forming an ookinete (A and C) the resulting progeny will have exactly the same alleles as the parental (Selfing). When two gametocytes carrying different alleles fuse (B), genetic recombination will occur, generating parasites that can either be like one of the parentals or carry a mixture of alleles belonging to each parental.

I.4. STRATEGIES FOR MALARIA CONTROL

The causative agent of malaria was discovered in 1880 by Alphonse Laveran. However, reports of the disease date from many centuries before (Cox, 2010). Nonetheless, malaria is still responsible for high morbidity and mortality to the present day. In the 1950's, many efforts were focused on the worldwide eradication of the disease. This program was successful in many regions, such as Europe, North America and the Caribbeans, however meeting with

limited success in Sub-Saharan African countries. In 1969, this ambitious goal was abandoned, and by 1992 efforts were focused on the control of the disease. This new strategy resulted in the reduction of the number of cases (Tanner and Savigny, 2008). However, due to the appearance and dissemination of resistance to the most widely used antimalarial drugs, and to mosquito resistance to insecticides, the number of cases increased once more. Very recently the aim of eradication has been revisited (Delacollette and Rietveld, 2006; WHO, 2007).

Strategies used for malaria control, as well as their pitfalls will be described below. Special focus will be placed on antimalarial treatment of infections, mechanisms of action and mechanisms underpinning resistance to the most important drugs currently available.

I.4.1. VACCINATION

Ideally, the prevention of new cases of malaria should be made by vaccination of people at risk of infection. This would provide the basis of control and possible eradication of the disease. However, in spite of the discovery of promising candidates, until the present date, no fully effective vaccines were produced (WHO, Parasitic Diseases - http://www.who.int/vaccine_research/diseases/soa_parasitic/en/print.html).

I.4.2. VECTOR CONTROL

A few species of Anopheles mosquitoes are highly efficient in transmitting the disease. In endemic areas, one strategy adopted for reducing transmission is the prevention of contact between mosquitoes and human hosts. This is done in two distinct ways: i) use of indoor residual spraying (IRS) of households in affected areas; and ii) the distribution of insecticide treated bednets (ITN) (WHO, 2006b).

WHO efforts for vector control between the 1940's and 1960's caused a drastic reduction in mosquito population, and consequently a decrease in the number of malaria cases. However,

logistical difficulties in reaching the breeding areas, in conjunction with the spread of resistance to insecticides amongst the mosquito population pose a threat to the effective control of the disease (Mabaso *et al,* 2004).

I.4.3. ANTIMALARIAL TREATMENT AND RESISTANCE

Since an effective vaccine is still to be achieved and control of the vector population has shown to be very difficult to sustain for long periods of time, malaria control relies mostly on treatment of patients with drugs targeted to the erythrocyte stages of the parasite's life cycle. Effective drugs, however, have their action limited by the gradual emergence of resistant parasites. The WHO defines "drug resistance" as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject", and "the form of the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action" (WHO, 1986).

At the present time, resistance to most drugs used in therapy of malaria has already emerged and spread worldwide. The only exceptions are the artemisinin (ART) derivatives, which are currently administered in combination with other drugs, constituting the so-called Artemisinin Combination Therapy (WHO, 2006c). However, recent reports indicate that the efficacy of ART compounds also seems to be declining (Carrara *et al,* 2009; Dondorp *et al,* 2009; Noedl *et al,* 2009; Lim *et al,* 2010). Taking into account the long time required for the development and implementation of newly synthesised drugs, if high levels of resistance to ART derivatives should become established in *Plasmodium* populations, malaria cases could become almost incurable.

In order to contain the advance of drug resistance it is important to understand the mechanisms behind the mode of action as well as the mechanisms underlying resistance to

antimalarial drugs currently in use. The history and evolution of resistance to the most important drugs used in malaria therapy will be briefly presented in Chapter II.

CHAPTER II

EVOLUTION OF RESISTANCE TO THE ARTESUNATE + MEFLOQUINE COMBINATION AND IDENTIFICATION OF GENETIC MARKERS OF RESISTANCE

This Chapter will deal with the selection and genetic analysis of artesunate + mefloquine (ATN + MF)-resistant clones and will be subdivided in three sections: i) Introduction - initially, a brief description of the mechanisms of action and the strategies evolved by the malaria parasites to escape the effect of the most important anti-malarials in use in endemic areas will be presented, mainly focusing on the use of Artemisinin Combination Therapies. Subsequently, the rodent malaria parasite *Plasmodium chabaudi*, used in this work as model for the selection of resistance to ATN + MF, will be described; ii) Results – firstly, the selection of ATN + MF resistance using different strategies will be described; and secondly, the presence of genetic mutations following the evolution of ATN + MF resistance will be presented; iii) Discussion – the results obtained in the previous section will be discussed.

II.1. INTRODUCTION

II.1.1. ANTIMALARIAL DRUGS – MECHANISMS OF ACTION AND DRUG RESISTANCE

II.1.1.1. CHLOROQUINE

In 1940's, a newly synthesised quinoline named chloroquine (CQ) was deployed as a treatment for malaria. CQ showed high efficacy, had little side effects and a very low production cost. For these reasons CQ was adopted as the first-line treatment against the disease in all endemic countries (WHO, 1984).

Many studies describe CQ as targeting the food vacuole (FV) of the *Plasmodium* parasite during the intra-erythrocitic stage. However, CQ's precise mode of action is still controversial (Ursos and Roepe, 2002).

The most accepted theory is that CQ acts by blocking the detoxification of haem radicals resulting from the digestion of haemoglobin by the parasite (Fitch, 1970; Chou *et al,* 1980). By uptaking and digesting the erythrocyte's haemoglobin in its FV, the parasite obtains most of the aminoacids necessary for the *de-novo* synthesis of proteins. The by-product resulting from haemoglobin digestion is the toxic ferrous radical haem which in abundance can cause severe damage to the parasite (Fitch, 2004). For that reason, the parasite inactivates haem toxicity by polymerising free haem radicals into an inert compound known as haemozoin (Pagola *et al,* 2000). CQ is believed to bind free haem inside of the FV, blocking haemozoin formation (Fitch, 1970; Chou *et al,* 1980). Free haeme or haeme-CQ adducts (Ginsburg *et al,* 1998) cause extreme oxidative stress, leading to the parasite's death (Fitch, 2004).

In spite of its initially high therapeutic efficacy, the long term selective pressure exerted by CQ administration associated with its indiscriminate prescription as treatment for all cases of fever in endemic areas (Trape, 2001), has led to the emergence of resistant *P. falciparum* parasites, less than 20 years after CQ's deployment. The first foci were reported simultaneously in South America (YOUNG and MOORE, 1961) and South-East Asia (Harinasuta *et al*, 1962),

and from there CQ resistance spread to other areas. At the present time, apart from few exceptions, CQ-resistant parasites are highly prevalent and use of CQ monotherapy is discouraged as a first-line treatment for malaria (WHO, 2008).

CQ resistance seems to be intimately related with the presence of mutations in the *pfcrt* gene (Bray *et al,* 2005). This gene codes for a transmembrane pump known as Chloroquine Resistance Transporter (CRT) expressed in the FV of the parasite (Fidock *et al,* 2000).

The physiological function of this protein or the mechanism by which mutated CRT would mediate CQ transport out of the FV (as a channel or a carrier) has been the focus of extensive investigation (Sanchez *et al,* 2010). Similarly, many efforts have been made for elucidating the role of mutations found in the *pfcrt* gene. Many mutations have been identified, but the substitution of a lysine to a threonine in aminoacid 76 (K76T) is the most frequently found in resistant *P. falciparum* parasites, and is described as having a central role in CQ resistance (Fidock *et al,* 2000; Sidhu *et al,* 2002; Wootton *et al,* 2002; Johnson *et al,* 2004; Lakshmanan *et al,* 2005). Nonetheless, other studies demonstrate that *pfcrt* K76T may not be the sole determinant of CQ resistance. Genetic transfection experiments carried out in *in vitro* cultured parasite lines indicate that parasites with distinct genetic backgrounds may differ in the level of resistance conferred by mutations in *pfcrt*, suggesting a role for other *loci* in CQ resistance resistance (Fidock *et al,* 2008; Valderramos *et al,* 2010).

For example, one gene also implicated in CQ resistance in *P. falciparum* is *pfmdr1*. It codes for a transmembrane efflux pump MDR1 (homologue of human P-gp1) belonging to the ATP-binding cassette family (Foote *et al,* 1989). Similarly to CRT, MDR1 is also expressed in the parasite's FV (Cowman, 1991; Sanchez *et al,* 2008), where it is believed to mediate the uptake of CQ into the FV (Sanchez *et al,* 2010).

Resistance to CQ seems to be associated with many polymorphisms in different residues of MDR1. The substitution of an asparagine by a tyrosine residue at aminoacid 86 (N86Y)

seems to be the most frequent in parasites displaying high levels of CQ resistance (Duraisingh and Cowman, 2005). Mutations in *pfmdr1* are believed to cause increased tolerance to CQ, in parasites that harbour the K76T mutation in *pfcrt* (Foote *et al,* 1990; Reed *et al,* 2000; Babiker *et al,* 2001).

Alternatively, rodent malaria models have provided additional candidate genes as mediators of CQ resistance, as is the case of the rodent malaria parasite *Plasmodium chabaudi*, which will be presented in Section II.1.2.1.

II.1.1.2. SULPHADOXINE + PYRIMETHAMINE

After the emergence and spread of chloroquine resistance, its use was replaced in some areas by the combination of two antifolate drugs: sulphadoxine + pyrimethamine (SP). As a consequence, a drastic reduction in cases of treatment failure was observed in the first year of SP application. However, resistant parasites quickly appeared in South East Asia, and rapidly spread to Africa (Roper *et al,* 2004).

SP's mode of action and its mechanisms of resistance are relatively simple: pyrimethamine (PYR) inhibits the parasite's dihydrofolate reductase (encoded by the *pfdhfr* gene) whilst sulphadoxine (SDX) acts on dihydropteroate synthase (encoded by the *pfdhps* gene) (Bruce-Chwatt *et al*, 1986). Consequently, SP acts by synergistically blocking the parasite's folate synthesis, which halts the parasite's pyrimidine synthesis and DNA synthesis/replication (Hyde, 2005).

Mutations in both the *pfdhfr* and *pfdhps* genes are described as causing resistance to SP in *P. falciparum*, and are frequently associated with treatment outcome (Wang *et al,* 1997). The first mutation described in natural *P. falciparum* populations was the substitution of a serine for an asparagine in residue 108 (S108N) of DHFR, which increases resistance to PYR by reducing the affinity between the drug and the enzyme (Walter, 1986). Further mutations were soon

identified in *pfdhfr*, such as the substitution of an asparagine to an isoleucine in position 51 (N51I), of a cysteine to an arginine in aminoacid 59 (C59R), and of an isoleucine to a leucine in aminoacid 164 (I164L). These mutations are believed to have appeared successively in that order, and acquisition of each one confers an extra increase in the level of resistance to PYR (Sirawaraporn *et al,* 1997).

Mutations in *pfdhps* have also been described to cause alterations in residues 436, 437, 540, 581, 623 (Duraisingh *et al,* 1998), being responsible for increased resistance to SDX (Triglia *et al,* 1997).

II.1.1.3. MEFLOQUINE

In 1985, Thailand was the first country to introduce the bis-quinoline-methanol mefloquine (MF) as first line treatment for *P. falciparum* malaria and its use was soon adopted in other endemic areas. Initially MF treatment was administered in combination with sulphadoxine + pyrimethamine, but in 1990 treatment that was changed, as MF doses were increased and the drug was administered as a monotherapy. By 1992, cases of therapeutic failure were about 40% in the Thai-Cambodian border (ter Kuile *et al,* 1992), indicating the emergence of MF-resistant parasites in that population (Nosten *et al,* 1991; Wongsrichanalai *et al,* 2001). In the face of widespread MF resistance, malaria treatment was consequently switched to MF in combination with artesunate (described in detail below).

Similarly to chloroquine (CQ), the most accepted mechanism of MF's anti-parasitic activity seems to be by inhibiting haemozoin formation (Sullivan, Jr. *et al,* 1998). There is also evidence that MF can inhibit the parasite's endocytic pathway, blocking the ingestion of nutrients (Hoppe *et al,* 2004).

MF has a very long elimination half-life of about 2-to-3 weeks (White, 1992). For that reason, it is postulated that a broad window of selection is created, i.e. sub-therapeutic levels of

the drug are present in the patient's bloodstream for a long period of time, allowing for the selection of parasites showing increased levels of tolerance to the drug (White, 1999).

As has been reported in CQ-resistant parasites, the N86Y polymorphism – amongst other point mutations – in the *pfmdr1* gene was described as conferring increased levels of resistance to MF (Duraisingh *et al,* 2000; Lopes *et al,* 2002; Price *et al,* 2004). Conversely, other studies have indicated that mutations in alternative codons of *pfmdr1* seem to increase sensitivity to MF (Sidhu *et al,* 2005).

However, the most accepted determinant of increased resistance to MF seems to involve the amplification of *pfmdr1* (Price *et al,* 1997; Price *et al,* 2004; Alker *et al,* 2007), associated with its increased transcription and expression (Wilson *et al,* 1989). In addition to field evidence, the induction of resistant laboratory strains was shown to select increased copy numbers, transcription levels and protein amounts of *pfmdr1* (Cowman *et al,* 1994; Peel *et al,* 1994), while the gene's deamplification has been reported as re-establishing sensitivity to MF and also other antimalarial drugs (Sidhu *et al,* 2006).

In the rodent malaria parasite *P. chabaudi*, the amplification of *pcmdr1* (the orthologue of *pfmdr1*), also seems to play an important role in MF resistance (Cravo *et al,* 2003; Borges, 2009). However, there are indications that other *loci* might also be implicated in MF resistance. For instance, a genetic cross performed between a MF-sensitive and a MF-resistant clone carrying two copies of *pcmdr1* produced sixteen clones, one of which showed MF resistance without a duplicated copy of the gene (Cravo *et al,* 2003). On the other hand, in this same experiment, none of the clones carrying a duplicated copy of *pcmdr1* gene showed a MF-sensitive phenotype.

Both field and *in vitro* experiments indicate that there seems to be an inverse relation between MF and CQ-sensitivity. Thus, the selection for high levels of MF resistance and the presence of increased *pfmdr1* copy number are associated with increased sensitivity to CQ

(Price *et al,* 1999; Price *et al,* 2004; Duraisingh and Cowman, 2005; Nelson *et al,* 2005). The opposite has also been observed, i.e. selection of high levels of CQ resistance leads to *pfmdr1* de-amplification and MF-susceptibility (Barnes *et al,* 1992).

II.1.1.4. ARTEMISININ DERIVATIVES AND COMBINATION THERAPY

Therapeutic application

Artemisinin (ART) or qinghaosu is extracted from the wormwood plant *Artemisia annua*. It has been used for about 2000 years by the Chinese traditional medicine for the treatment of fevers. ART has been "re-discovered" by Western medicine in the 1970's as a highly effective treatment for malaria. It is a sesquiterpene trioxane lactone, with an endoperoxide bridge. Its use became disseminated by the end of the 1990's, and at the present time it is recommended by WHO – in combination with other drugs – as first-line treatment for *P. falciparum* malaria worldwide (WHO, 2005).

ART acts extremely fast against all blood stages of the parasite (ter Kuile *et al,* 1993; Meshnick *et al,* 1996), including the sexual forms (Kumar and Zheng, 1990), which contributes to reducing transmission rates. After administration, ART reaches the blood stream within minutes, where it is quickly converted into its active form dihydroartemisinin (Balint, 2001). ART is also quickly metabolised, with a half-life of about 1h, which favours the reappearance of parasites after treatment is completed (Meshnick *et al,* 1996), a situation known as recrudescence. In addition, ART shows very low solubility in water and consequently a very poor bio-availability.

In order to improve ART's chemical properties, synthetic analogues were produced, the so-called ART derivatives. The most important ART derivatives are artesunate, artemether, artemisone, artelinic acid, and dihydroartemisinin (the active metabolite of all ART derivatives) (Meshnick *et al,* 1996). Although more soluble in water, these compounds still have a very

short half life, and in order to successfully eliminate the parasites from the bloodstream, monotherapy with ART derivatives must be carried out during 7 days (Luxemburger *et al,* 1995). Long treatment courses (mefloquine and chloroquine are usually administered in a 3-day regimen) usually lead to very low compliance by patients, increasing the chance of treatment failure (White, 1997). In order to circumvent these disadvantages, and also to "protect" ART derivatives against the eventual emergence of resistant parasites, the WHO recommends their administration in combination with long-lasting antimalarials (Olliaro and Taylor, 2004; Menard *et al,* 2005; WHO, 2005) a practice known as Artemisinin Combination Therapy (ACT).

Artemisinin Combination Therapy

As mentioned above, WHO's directive for the first-line treatment of uncomplicated *P. falciparum* malaria cases is to use an arteminsinin (ART) derivative in combination with a long-lasting partner drug. The partner drug must also be chemically unrelated to ART and have distinct intracellular targets or modes of action (WHO, 2001).

Many combinations are currently available in endemic areas and vary according to geographic region of administration. The most common versions of ACTs are: artesunate (ATN) + mefloquine (MF), widely used in South-east Asia and some countries of South-America; ATN + amodiaquine, widely used in West-African countries; ATN + sulphadoxine + pyrimethamine (SP), used in Eastern Mediterranean countries; artemether + lumefantrine (Coartem®), used in Central African countries; dihydroartemisinin + piperaquine, used in China, Indonesia, Viet Nam and Myanmar (Olliaro and Taylor, 2004; Menard *et al,* 2005; WHO, 2005).

In fact, the concepts behind Artemisinin Combination Therapies (ACTs) are not new. Other drug combinations have been used for the treatment of malaria. For instance, SP has been

used in combination with chloroquine or with MF (Salako *et al,* 1992; Menard *et al,* 2005). Indeed, SP is already a combination of two drugs. However, as an example of combination therapy, SP fails to meet the pre-requisites determined by the WHO, since sulphadoxine and pyrimethamine act in the same metabolic pathway (Sibley *et al,* 2001; WHO, 2001).

In the case of ACTs, on the other hand, ART derivatives are used in combination with chemically unrelated drugs, and ACT use should have many advantages. Firstly, it combines the rapid and efficacious action of ART derivatives with the long-term protection provided by the partner drug, such that any parasites surviving after the complete elimination of the ART derivative component would be killed by the partner drug (White, 1997; White, 1999). Secondly, if a parasite arises that is resistant to one of the drugs, the other drug should eliminate it, preventing the spread of newly arising resistant strains (White, 1999). However, it is now accepted that this mismatch in elimination half-lives may not be as advantageous as previously assumed (Hastings and Watkins, 2006; Martinelli *et al,* 2008). This is because after the removal of the ART derivative from the organism, the long-lasting drug remains in the blood stream in sub-therapeutic levels, blocking the growth of sensitive strains, but favouring the survival of the most tolerant ones (Hastings and Watkins, 2006; Martinelli *et al,* 2008).

Another theoretical advantage of the use of ART derivatives with chemically unrelated partner drugs would reside on the parasite's intrinsic mutation rate. Considering the rate of mutation of malaria parasites as being 10^{-8} per nucleotide site per generation, the chances of emergence of a parasite carrying mutations conferring resistance to both drugs simultaneously would be represented by the product of the mutation rate for each drug alone, therefore, 10^{-16} (White, 1999). For this reason, the use of ACTs was believed to nearly abolish the chances of appearance of parasites showing resistance to the combination.

In addition, considering the possibility of resistance to two drugs would be caused by two different mutations in two different genes, genetic recombination in the mosquito could break

the resistant haplotype apart, rendering the progeny sensitive to one of the two drugs of the combination (Dye and Williams, 1997). However, mutation rates may not be the limiting factor for the acquisition of resistance to two drugs in a combination. There is a phenomenon that suggests that parasites previously resistant to one or more drugs have an increased ability to acquire resistance to additional drugs. This is known as Accelerated Resistance to Multiple Drugs (ARMD) phenotype, and the mechanisms underlying this phenotype are still not fully understood (Rathod *et al,* 1997). Considering some areas where parasites already display high levels of resistance to one or more drugs, it would be expected that these parasites would more easily evolve resistance to any other drugs, including when administered as combinations.

In addition, the existence of multi-drug resistant parasites may consist of yet another problem: ACTs are often deployed in regions where parasites have already been extensively exposed to one of the drugs used in the combination. This implies that parasites showing high levels of resistance to one of the partner drugs would only need to develop resistance to the other drug of the combination.

Also, it is essential to bear in mind that in some cases the parasite may harbour mutations in genes related to unspecific efflux mechanisms (such as the *mdr1* gene), which are capable of blocking the action of chemically unrelated drugs by preventing their interaction with their intracellular target. In this case, if the two drugs of the combination are substrates of this mutated efflux pump, ACT would be nearly ineffective in the mutated parasites.

Finally, the exposure of parasites to sub-therapeutic doses of ACTs may also shorten their useful therapeutic life. In a similar fashion as previously described to other antimalarials, self-medication, incomplete treatment courses, sub-standard or counterfeit drugs, etc… may result in selection of a tolerant sub-population and eventually give rise to parasites showing high levels of resistance.

Unfortunately, increased tolerance to ACTs has already been described and its possible mechanisms will be presented below.

Mode of action

Artemisinin (ART) derivatives mode of action is still unclear and their intracellular target is still an object of great controversy. The presence of the endoperoxide bridge in ART's chemical structure was described as being essential for its antimalarial activity (Brossi *et al,* 1988). Some studies have shown that ARTs interact with the parasite's free intracellular iron (Meshnick *et al,* 1991; Haynes and Krishna, 2004), while other groups have found that ART reacts with haem (Robert *et al,* 2005) forming adducts that then alkylate the parasite's proteins and membranes, but not DNA (Meshnick, 1996; Kannan *et al,* 2005).

Due to its structural similarity to thapsigargin, another sesquiterpene lactone, a different mode of action has been proposed: ART would act as an inhibitor of thapsigargin's specific target, the orthologue of the human Sarco-endoplasmic Reticulum Ca^{++} ATPase (SERCA), known in Plasmodium as ATPase6. In fact, ART can inhibit the activity of ATPase6 in transfected *Xenopus laevis* oocytes (Eckstein-Ludwig *et al,* 2003), and a single aminoacid substitution in ATPase6 (L263E) is enough to prevent ART's inhibitory effects (Uhlemann *et al,* 2005). By replacing the wild-type version of *pfatpase6* gene for one carrying the L263E mutation in *P. falciparum* laboratory strains, a barely significant small reduction in the susceptibilities to ART and dihydroartemisinin, but not to ATN was observed when compared to the wild-type strain (Valderramos *et al,* 2010). A field study published in 2005 appeared to support ATPase6 as ART's intracellular target, by showing that the presence of a mutation in *pfatpase6* was associated with increased tolerance to artemether *in vitro* (Jambou *et al,* 2005).

Alternative intracellular targets have also been proposed for ART derivatives. For instance, Li and colleagues (2005) have found that ART seems to be activated by, and to

interfere with the components of the mitochondrial electron transport chain (Li *et al,* 2005). Also, ART's interaction with the translationally controlled tumor protein (TCTP) has been proposed in different malaria models (Bhisutthibhan *et al,* 1998; Walker *et al,* 2000).

ART derivatives have also been found to inhibit the endocytosis of macromolecules and the digestion of haemoglobin by malaria parasites (Hoppe *et al,* 2004). Also, the accumulation of ART within food vacuole-associated lipid bodies has been demonstrated (Hartwig *et al,* 2009). Taken together, these studies seem to indicate a role of ART in interfering with ingestion of nutrients by the parasite.

Resistance

A threshold for determining artemisinin (ART) *in vivo* or *in vitro* resistance has not yet been defined by the WHO. However, it is clear that the efficacy of these compounds in the treatment of malaria cases is slowly decreasing. This is reflected in various studies as an increase in parasite clearance time observed after treatment with artesunate (ATN) alone or Artemisinin Combination Therapy (ACT). Interestingly, in some cases, no increases in *in vitro* IC_{50} were reported (Noedl *et al,* 2009; Dondorp *et al,* 2009; Carrara *et al,* 2009; Anderson *et al,* 2010).

The majority of cases of increased parasite clearance time are found in the Thai-Cambodian border. In fact, in this region the parasite population was subjected to strong drug pressure during the past 30 years. In the 1980's, mefloquine (MF) monotherapy was deployed (ter Kuile *et al,* 1992), and later replaced by ATN monotherapy in some areas (Dondorp *et al,* 2009). In 1998, the ACT version ATN + MF was implemented as first-line treatment for uncomplicated malaria cases, reaching a cure rate of 100% (Nosten *et al,* 2000).

Previous selective pressure with each drug of the combination administered separately may have been associated with other factors which are characteristic of this region. First, South-

East Asia is a region where transmission rate is relatively low. This results in low levels of genetic recombination between different parasite strains (see Section I.1.3.2). As another consequence of low transmission rates, the affected population shows low levels of immunity. Thus, most malaria cases are symptomatic, and consequently treated (Luxemburger *et al,* 1997), adding up to the strong selective pressure exerted over the parasite population. Together, these factors may explain why this region was the first where the initial signs of ART resistance have appeared.

Nonetheless, increased tolerance to ART derivatives was not only found in the Thai-Cambodian border. In Central Africa, 15% of cases treated with ATN monotherapy have shown recrudescence of parasites (Menard *et al,* 2005). Similarly to South-East Asian cases, no change in *in vitro* IC_{50} over time was observed. On the other hand, parasites recrudescing after treatment showed a higher IC_{90} when compared with the parasites from cured infections (Menard *et al,* 2005).

Regardless of the increasing frequency at which therapy failure cases are reported, the mechanisms that may underlie increased ART-tolerance – and maybe ART-resistance in a near future – are still not known. A very recent study has attributed a high heritability level to increased clearance time after ART treatment, indicating genetic factors as underlying ART increased tolerance (Anderson *et al,* 2010).

Indeed, many genes have been implicated in resistance to this class of compounds. One important candidate is *pfatpase6* gene, described as a target for this drug in the previous section. The first study to implicate this gene in ART resistance showed that a substitution of a serine for an asparagine in position 769 (S769N) was found in six out of seven field samples showing lower *in vitro* sensitivity to artemether in parasites from French Guiana (Jambou *et al,* 2005). Many other studies, however, showed that the correlation between mutations in *pfatpase6* gene and ART tolerance is not always observed (Dahlstrom *et al,* 2008; Jambou *et al,*

2010). In fact, assuming *pfatpase6* has any role in ART resistance and in spite of the many polymorphisms found in this gene in different endemic regions, it has been suggested that, possibly, not enough ART pressure has been exerted over natural populations in order to select the most favourable genotypes (Jambou *et al,* 2010).

Amplification of the *pfmdr1* gene seems to be involved in ART resistance in many malaria models. In field studies, *pfmdr1* amplification has been associated with treatment failure following ACTs (Price *et al,* 2004; Alker *et al,* 2007; Lim *et al,* 2009; Rogers *et al,* 2009) and increased *in vitro* tolerance to ATN (Price *et al,* 1999; Pickard *et al,* 2003; Lim *et al,* 2009; Chaijaroenkul *et al,* 2010). Also in *P. falciparum* laboratory strains, selection of ART-resistant parasites has resulted in increased *pfmdr1* copy number, followed by increased mRNA and MDR1 expression levels (Chavchich *et al,* 2010). These results are in line with the work described by Sidhu and colleagues, where the disruption of one of the two *pfmdr1* copies present in a *P. falciparum* drug-resistant strain has led to increased sensitivity to ART (Sidhu *et al,* 2006).

Evolution of ART-resistance in the rodent model *P. yoelii*, also selected for parasites harbouring 2-to-3-fold increased *pymdr1* copy number (Ferrer-Rodriguez *et al,* 2004). In another rodent model, *P. chabaudi*, MF-resistant parasites carrying two copies of the *pcmdr1* gene show increased tolerance to ART and ATN (Borges, 2009).

Besides gene amplification, the presence of the Y184F polymorphism in *pfmdr1* was identified in parasites showing increased *in vitro* ART-tolerance (Pickard *et al,* 2003). In addition, in a genetic cross performed between a drug-sensitive and a drug-resistant *P. falciparum* clone, the inheritance of the *pfmdr1* drug-sensitive allele was associated with sensitivity to ART (Duraisingh *et al,* 2000). Also, analysis of field samples have shown the presence of selective sweeps around this locus indicating this gene is under great selective pressure associated with ACT use (Nair *et al,* 2007; Vinayak *et al,* 2010).

Other studies however suggest no implication of changes in the *mdr1* gene in ART resistance. In these studies, no correlation was found between ART *in vivo* or *in vitro* resistance and gene amplification or polymorphisms in *pfmdr1* sequence (Dondorp *et al,* 2009; Muangnoicharoen *et al,* 2009; Imwong *et al,* 2010). Also, in the rodent model *P. chabaudi*, selection for ART or ATN resistance did not result in parasites carrying mutations or amplification of *pcmdr1* gene (Afonso *et al,* 2006). Alternatively, the selection of ART resistance in the rodent model *P.yoelii* has shown changes in expression levels of TCTP. However, the resistance phenotype exhibited by this strain was unstable (Walker *et al,* 2000).

Stable ART and ATN resistance was first achieved experimentally in the rodent model *P. chabaudi* (Afonso *et al,* 2006). These parasites showed a mutation in a gene coding for a de-ubiquitinating enzyme (*pcubp1*) (Hunt *et al,* 2007)as will be presented below.

The appearance of ACT tolerance in natural *P. falciparum* populations, associated with data obtained using laboratory strains suggest that evolution of high levels of resistance to ACTs in the field may be not only likely, but also imminent. Therefore, it is important to unravel the mechanisms underlying resistance to these drugs.

II.1.2. THE RODENT MALARIA MODEL OF *PLASMODIUM CHABAUDI*

Malaria studies do not rely uniquely in the investigation of *P. falciparum*. As mentioned throughout the previous sections many other species are currently used for elucidating aspects of the disease, such as pathogen life cycle, host-pathogen interactions, aspects of transmission, response to drugs and drug resistance (Janse and Waters, 1995; Carlton and Carucci, 2002; Waters, 2002).

Amongst the many species affecting different vertebrate hosts, rodent models present many advantages including easy handling and maintenance of rodent hosts under laboratory conditions (Carlton *et al,* 2005).

One of the best models used for building up the current knowledge of malaria is the rodent parasite *P. chabaudi chabaudi*, referred here as *P. chabaudi* (Carlton *et al,* 2001). This species was first isolated from its original host, the African thicket rat and adapted to laboratory mice and rats (Landau and Chabaud, 1965). Its complete schizogonic cycle is synchronous, lasting lasts for 24h (Cambie *et al,* 1990). Apart from its synchronicity, *P. chabaudi* also shares other similarities with the human parasite *P. falciparum*: for example, it preferentially infects mature erythrocytes (Culleton, 2005) and some sequestration of mature trophozoites occurs.

One great advantage of working with *P. chabaudi* as a model for malaria studies is that it is possible to follow the parasite's complete life cycle, including sexual recombination in mosquitoes. This would be virtually impossible using *P. falciparum* due to ethical and logistical constraints.

In addition, *P. chabaudi* shows high level of gene synteny with *P. falciparum*, i.e. the organization of genes along chromosomes shows high degree of conservation when comparing rodent and human parasites (Kooij *et al,* 2005), allowing easy mapping of orthologous genes between the two species.

P. chabaudi's genome is 18.8Mb in size and has an A + T content of about 77%, which is similar to the A + T content observed in *P. falciparum* (Hall *et al,* 2005). Currently, the Welcome Trust Sanger Institute is focusing efforts in the sequencing and assembly of its complete genome. So far, 4391 genes were identified as having orthologues in *P. falciparum*, whereas 736 have none. Most of the genes with no orthologues in *P. falciparum* are located in chromosomic sub-telomeric regions (Hall *et al,* 2005).

It is important to note that in spite of the many similarities between Plasmodia species and technical advantages of working with rodent models, direct extrapolation to *P. falciparum* malaria must be done with caution. Similarly, results obtained from other models, such as *in vitro* cultured *P. falciparum* parasites – which represent a well accepted model – must also be

interpreted carefully. Although differing in their advantages and shortcomings, the different models used for the study of malaria do not faithfully reproduce the biological mechanisms, the population size and distribution, selective pressure, host conditions, etc. pertaining to *P. falciparum* natural populations, and therefore, can only help in providing insights for understanding the complex phenomena involving the evolution of malaria worldwide, and cannot act as a substitute for *in vivo* research with *P. falciparum*.

II.1.2.1. AS LINEAGE

There are many different strains of *Plasmodium chabaudi*, however, two of them deserve special attention within the context of the present work: AS and AJ. Both are sensitive to antimalarials used to treat the human pathogen *P. falciparum*, however, AJ is more virulent than AS (de Roode *et al,* 2005). These two parasites have different genetic backgrounds which make them easy to distinguish by simple molecular methods. This allowed their use in several studies on parasite virulence and fitness (Mackinnon *et al,* 2005; de Roode *et al,* 2005), as well as the development of analytical methods for the study of parasite genetics (Walliker *et al,* 1975; Grech *et al,* 2002; Culleton *et al,* 2005).

The AS strain has been historically used in the experimental evolution of drug resistance, resulting in a series of isogenic parasites which are resistant to many different drugs. These parasites were obtained over successive generations through exposure to different antimalarials (Figure 3). As a consequence, these parasites should have the same genetic background, with mutation in genes involved in resistance to the drug used for the selection of each clone. These parasites are part of what will be referred in this work as the AS lineage.

Initially, as the original drug-sensitive AS-SENS clone were inoculated into laboratory mice and exposed to one round of treatment with pyrimethamine (PYR). Parasites that recrudesced after treatment were cloned (Walliker *et al,* 1975) and showed the substitution of a

serine to an asparagine in position 106 (S106N) in the orthologue of *P. falciparum*'s *pfdhfr*, the *pcdhfr* gene (Hayton *et al,* 2002). This clone was named AS-PYR and was used for the selection of two different clones. One of them AS-50SP, was obtained by sub-inoculation of AS-PYR into mice followed by treatment with 4 daily doses of the combination sulphadoxine (SDX) and PYR (SP). AS-50SP is resistant to 25 mg/kg of SDX in combination with 1.25 mg/kg of PYR and shows no mutation in *pcdhps*, the orthologue of *P. falciparum*'s *pfdhps*, (Hayton *et al,* 2002). Instead, the critical mutation is K392Q in the *pcmdr2* gene (Martinelli unpublished results). The other clone generated from AS-PYR is resistant to low doses of chloroquine (CQ), and was named AS-3CQ (Rosario, 1976). In spite of being resistant to CQ, no mutation on *pcccg10* (orthologue of *P. falciparum*'s *pfcrt*) was found (Hunt *et al,* 2004). Recently a mutation has been identified in a gene known as *pcaat1*, coding for an aminoacid transporter, expressed in the parasite's food vacuole (Modrzynska, 2010).

AS-3CQ was submitted to further selection with CQ, generating parasites capable of surviving intermediate doses of CQ (Padua, 1981). These parasites, named AS-15CQ did not represent a clonal population, and, furthermore, underwent mosquito passaging, resulting in genetic recombination among the various parasites of the population. The unclonal nature of AS-15CQ parasites affected the genetic analysis of AS-15CQ and of the three clones derived from it.

One of the three clones derived from AS-15CQ is AS-30CQ, which is resistant to high levels of CQ (Padua, 1981). This clone was subsequently used for the generation of AS-ART, by being submitted to stepwise increasing doses of artemisinin (ART) (Afonso *et al,* 2006). No specific mutation was found in AS-ART in the candidate genes previously suggested as candidate determinants of resistance to this drug – *pcatp6*, *pctctp* and *pcmdr1* (Afonso *et al,* 2006). Instead, a valine to a phenylalanine in position 2728 (V2728F) of the *pcubp1* gene,

coding for a de-ubiquitinating enzyme (Hunt *et al,* 2007) was identified. However, this mutation was already present in AS-ART's parental AS-30CQ.

The second clone deriving from AS-15CQ was selected by exposure to stepwise increasing doses of mefloquine (MF). The selected clone was named AS-15MF and is resistant to four daily doses of 5 mg/kg/day of MF. AS-15MF carries two copies of gene *pcmdr1* (Cravo *et al,* 2003). *Pcmdr1*'s amplification was shown to be part of a large duplication event where the terminal segment of chromosome (chr) 12 underwent a duplication event followed by its translocation to the end of chr 4 (Cravo *et al,* 2003). The duplication of gene *pcmdr1* is involved in AS-15MF's resistance to MF, and also to ART and lumefantrine (Borges, 2009). Additionally, AS-15MF carries a substitution of a threonine to a proline in position 823 (T823P) in a gene coding for a putative lysine decarboxylase, whose role in resistance to MF or other drugs is still to be determined.

Finally, AS-15CQ served as progenitor for the generation of a third clone, AS-ATN (Afonso *et al,* 2006). This clone was selected by treatment with stepwise increasing doses of ATN, until parasites were capable of recrudescing after treatment with three daily doses of 60 mg/kg/day of ATN (Afonso *et al,* 2006). Similarly to AS-ART, AS-ATN does not show mutations in the candidate genes involved in resistance to ART derivatives in other models (Afonso *et al,* 2006). Instead, AS-ATN carries a different mutation in the *pcubp1* gene, the substitution of a valine for a phenylalanine in position 2687 (V2687F). Although in different positions (Hunt *et al,* 2007), both the V2728F mutation (present in AS-30CQ and AS-ART) and the V2687F mutation (found in AS-ATN) seem to be present at key functional sites of the enzyme coded by *pcubp1*. These mutations are postulated to cause changes in the enzyme's tri-dimensional structure, diminishing its affinity to its substrate (Hunt *et al,* 2007).

The summary of the AS lineage, including the mutations known so far is presented in Figure 3.

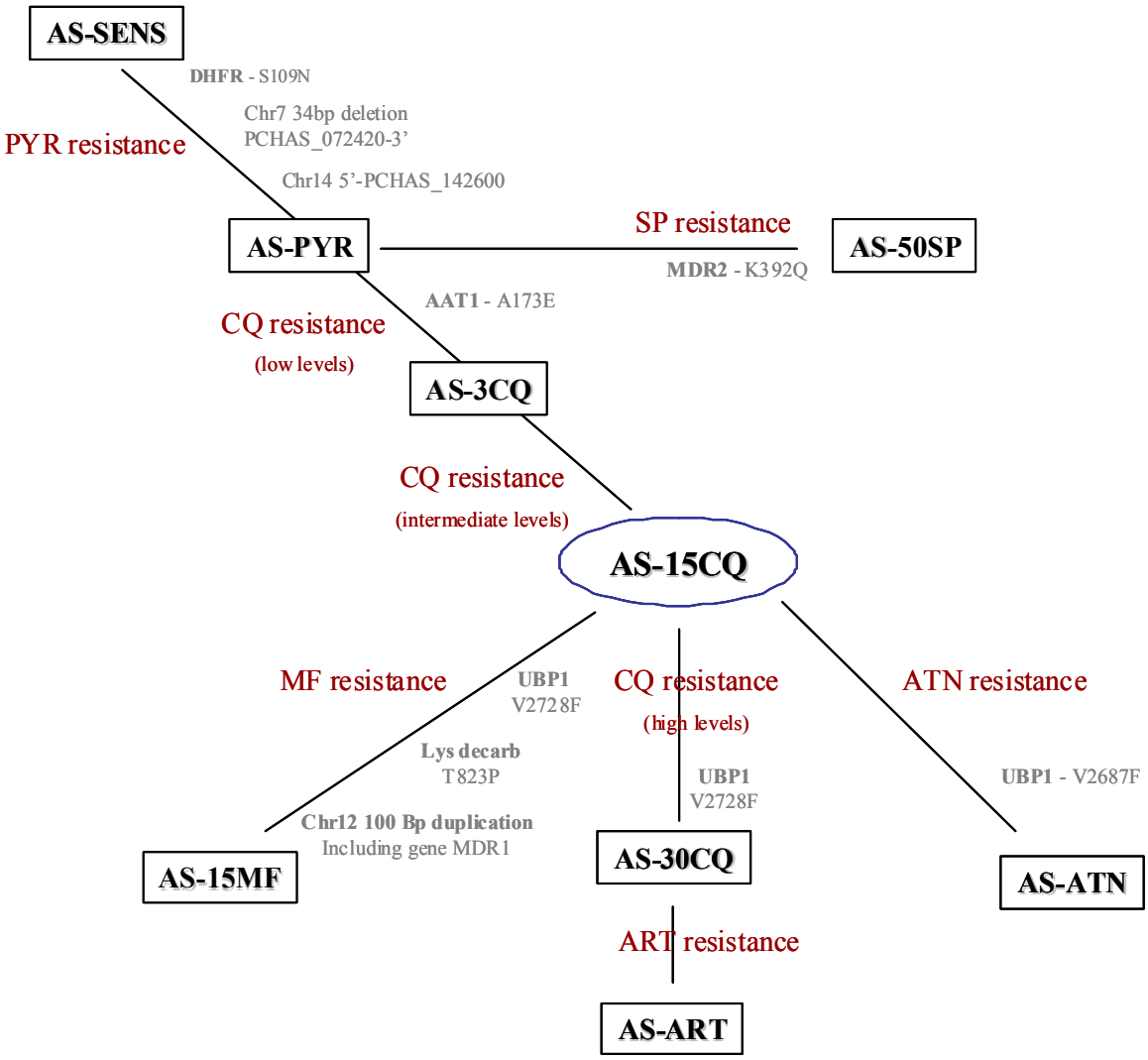


Figure 3 – Schematic representation of the AS lineage. Drugs used for the selection of each clone are highlighted in red: PYR – pyrimethamine; SP – sulphadoxine-pyrimethamine; CQ – chloroquine; MF – mefloquine; ART – artemisinin; ATN - artesunate. The mutations arising in each step are shown in gray.

In conclusion, the parasites belonging to *P. chabaudi* AS lineage described above have so far provided an excellent biological platform for studies on drug resistance. Although many studies aimed at the generation and investigation of resistance to drugs which are commonly used in ACTs, such as MF and ATN, selection of parasites showing high levels of resistance to both drugs simultaneously has never been attempted in *P. chabaudi* or, to my knowledge, in *P. falciparum* either.

II.1.3. AIMS

In this Chapter, parasites showing different resistant backgrounds were used in order to select resistance to the Artemisinin Combination Therapy (ACT) version artesunate + mefloquine by different approaches.

The parasites obtained here were analysed regarding the genetic variants involved in resistance to these two drugs. This may provide invaluable data for the surveillance of the efficacy of ACTs before the emergence and establishment of high levels of resistance in natural parasite populations.

II.2. RESULTS

II.2.1. SELECTION OF ARTESUNATE + MEFLOQUINE RESISTANCE

Resistance to the artesunate + mefloquine (ATN + MF) version of Artemisinin Combination Therapy (ACT) could emerge in natural *P.falciparum* populations in two different ways. The first is through the appearance of mutants exhibiting low levels of resistance to the combination due to inadequate drug treatment (patients receiving sub-optimal drug doses due to mal-absorption, vomiting, counterfeit medication, etc.) (White *et al,* 2009) that could then give rise to a population of parasites showing high levels of resistance. In addition the presence of parasites which are resistant to one of the two drugs of the combination could be present and this could facilitate the emergence of parasites that could withstand high levels of ATN and MF administered in combination, rendering this version of ACT ineffective.

An alternative way through which resistance to the ATN + MF combination could emerge is by genetic recombination of parasites bearing the genetic traits responsible for resistance to each component of the ACT separately, which applies to areas where each component was used as a monotherapy over a significant period of time.

In order to test these assumptions and in an attempt to mimic the conditions occurring in natural parasite populations, different approaches were attempted here for the generation of resistance to the ATN + MF version of ACT, using the rodent model *P. chabaudi*.

II.2.1.1. SELECTION OF ARTESUNATE + MEFLOQUINE RESISTANCE THROUGH DRUG PRESSURE

In order to select rare parasite mutants displaying high levels of resistance to the artesunate + mefloquine (ATN + MF) combination, two different parasite clones that had been exposed to antimalarials for many generations were used as starting material. One of them was initially resistant to mefloquine (MF) and was exposed to stepwise increasing doses of artesunate (ATN). The other one was initially resistant to ATN and was exposed to stepwise increasing

doses of the ATN + MF combination. In both cases, in each round of selection the drug-exposed parasite biomass was maximised by infecting individual mice with a very high parasite inoculum (10^7 parasitised red blood cells). In addition, the parasites were allowed to grow for two days before drug pressure was applied. Two clones derived from different genetic and phenotypic backgrounds were selected as will be described in the following sections.

II.2.1.1.1. EXPOSURE OF A MEFLOQUINE RESISTANT CLONE (AS-15MF) TO ARTESUNATE

SELECTION

In *Plasmodium falciparum* natural populations, resistance to mefloquine (MF) was first described in South-East Asia in 1990's, few years after its deployment as first line treatment for malaria (ter Kuile *et al,* 1992). In this region, MF treatment was replaced by artesunate (ATN) monotherapy (Dondorp *et al,* 2009) or for the combination of both drugs (Nosten *et al,* 2000). Recent reports indicate that in this area malaria parasites have developed increased tolerance to treatment with this drug combination (Carrara *et al,* 2009; Dondorp *et al,* 2009; Noedl *et al,* 2009; Anderson *et al,* 2010).

In *Plasmodium chabaudi*, Cravo and colleagues (2003) have generated the MF-resistant AS-15MF clone by exposure of a chloroquine-resistant clone (but MF-sensitive) to stepwise increasing doses of MF. This clone is resistant to 5 mg/kg/day administered to the host mice for three consecutive days (Cravo *et al,* 2003). Further analysis showed that these parasites also display some degree of resistance to artemisinin (ART) in spite of never being exposed to this drug previously (Borges, 2009).

In order to interrogate one of the ways through which resistance to artesunate + mefloquine (ATN + MF) in the field may arise, AS-15MF was exposed to stepwise increasing doses of ATN alone. Thus, mice were initially inoculated with 10^7 pRBC and were treated with

ATN on Days 3, 4 and 5 post-inoculum (pi). Parasitaemias were monitored and parasites surviving after treatment were further passaged into uninfected mice. This procedure was repeated for several consecutive rounds. In addition, the AS-15MF parental clone was inoculated into mice that were left untreated. These parasites were sub-inoculated in parallel to those that were being treated.

The treated group received an initial dose of 10 mg/kg/day of ATN and parasites were able to reach parasitaemias of 5.5% on Day 7 pi (Figure 4). This dose was maintained for a further week and at the third week, this was increased to 15 mg/kg/day (Figure 4). At that stage, parasites grew well and reached parasitaemias of about 13%, after which the ATN dose was increased once more, to 25 mg/kg/day of ATN (Figure 4). Following this round of selection, parasites were almost eradicated, being incapable of reaching 1% parasitaemia on Day 7 pi. For this reason, the ATN amount was reduced, allowing for improved parasite recovery. Subsequently, drug doses given to mice oscillated along the selection procedure, depending on the empirical evaluation of parasite recovery following treatment. The evolution of drug doses along time is shown in Figure 4. After thirty six weeks the parasites under drug pressure were already able to grow after treatment with 60 mg/kg/day of ATN.

The parallel line of parasites left untreated was also passaged thirty six times through mice and will be referred herein as AS-15MF36P.

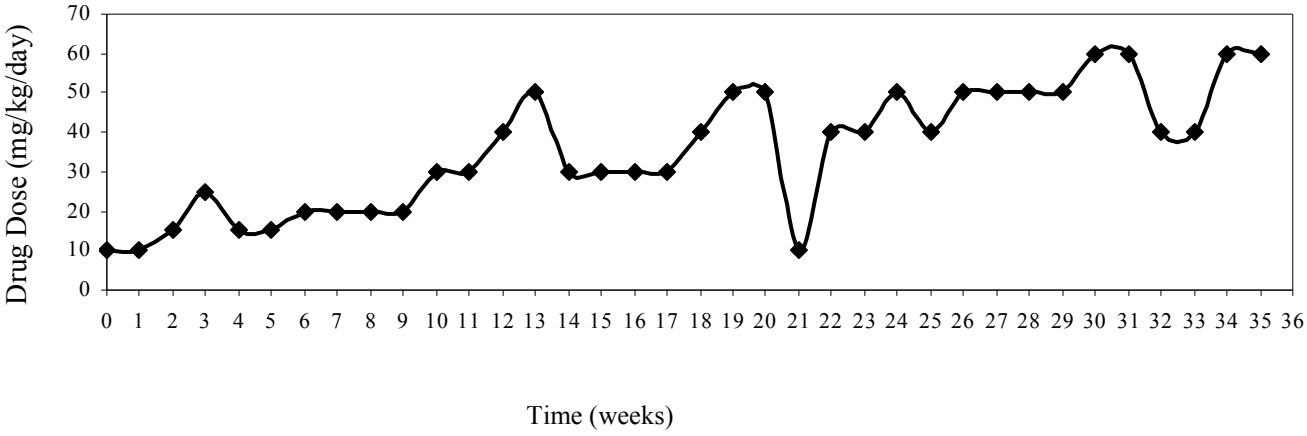


Figure 4 – Evolution of doses of artesunate given to mice infected with the AS-15MF clone along the course of thirty six weeks of selection.

CLONING

As shown in Figure 4 above, after thirty-six weeks under drug pressure, parasites were able to survive a dose of 60 mg/kg/day of ATN for 3 days. These parasites were then cloned by limiting dilution, as described on Material and Methods, Section 2.2.

After 15 days, one animal showed detectable parasitaemia in the group of twenty mice inoculated with 0.5 pRBC, and therefore, the other 19 (95%) of the mice receiving this inoculum were parasite-free (see Material and Methods Section 2.2). As such, it was assumed that the parasite population carried by this one mouse was clonal. This clone was named AS-MFATN-1 and stored in liquid nitrogen.

Similarly, out of twenty animals inoculated with the equivalent of one pRBC, four mice showed detectable parasitaemias after 15 days. In this case, 16 (80%) of the mice showed no detectable parasitaemia, and parasite-positive mice were also considered as carrying a clonal population. Therefore, the parasite clones were extracted from their respective host mice and named AS-MFATN-2, AS-MFATN-3, AS-MFATN-4, and AS-MFATN-5. AS-MFATN-5 displayed the fastest growth following the cloning procedure (Figure 5), and was selected for further analysis. The remaining clones were stored in liquid Nitrogen.

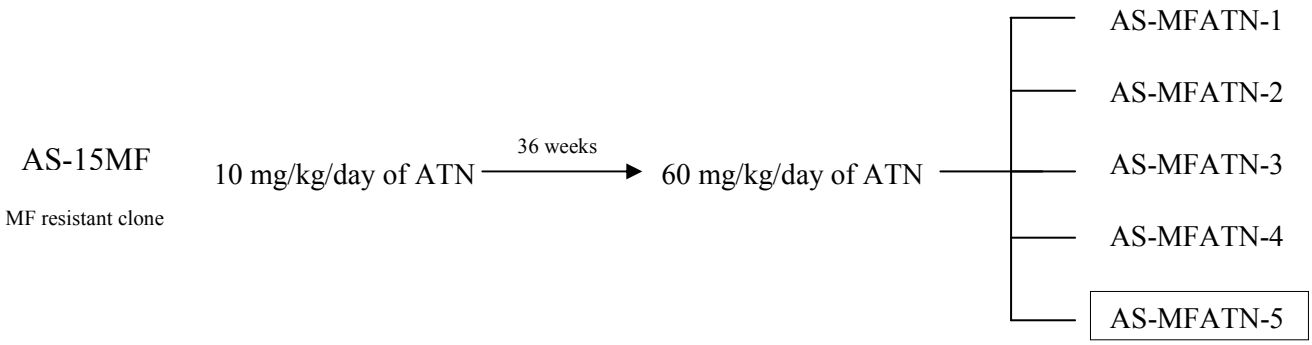


Figure 5 – Schematic representation of selection and cloning. The AS-15MF clone used here as starting material was submitted to increasing doses of artesunate (ATN) over thirty six weeks and cloned by limiting dilution. This process originated five clones. The AS-MFATN-5 clone was characterised further.

DRUG SENSITIVITY TEST

In order to ascertain whether drug resistance was retained after cloning, the growth of AS-MFATN-5 parasites was assessed under treatment with different drugs. The responses of the original parental, AS-15MF, the ATN-resistant AS-ATN clone, and the ATN and MF-sensitive AS-3CQ clone were also investigated. The response of the AS-15MF36P parasites (passaged in absence of drug treatment during the selection of AS-MFATN-5 clone) was assessed in separate experiments, in comparison to the original AS-15MF clone.

For all drug tests performed here, a group of mice was inoculated with each clone and left untreated as a control for parasite growth. The untreated parasites grew as expected, reaching peak parasitaemias of 40-to-60 % at Day 6 (Figure 6A and 7A).

The behaviour of the each clone after treatment will be described in detail below.

AS-MFATN-5 response to artesunate

The AS-MFATN-5 clone was obtained by the exposure of a MF-resistant clone to increasing doses of ATN alone. Therefore, it could be expected that this clone would be able to

survive after treatment with higher doses of this drug, when compared to its progenitor AS-15MF. Indeed, after treatment with 70 mg/kg/day of ATN, AS-MFATN-5 was able to establish a peak parasitaemia of about 20% on Day 8. The parental AS-15MF and AS-ATN showed a lower response (peak at 5% on Day 9 and 9% Day 10 p.i., respectively). In contrast, the sensitive AS-3CQ clone only reached peak parasitaemia around Day 15 (Figure 6B).

AS-MFATN-5 response to mefloquine

As mentioned previously, AS-MFATN-5 derives from AS-15MF which survives 5 mg/kg/day of MF for four days (Cravo *et al,* 2003).

As shown in Figure 6C, after treatment with 8 mg/kg/day of MF, the AS-MFATN5 clone was able to grow and reach peak parasitaemia of about 25%, whereas AS-15MF only showed a peak parasitaemia of about 3.5%, both on Day 9. This indicates that the AS-MFATN-5 clone selected here seems to have evolved an increase in the level of MF resistance when compared to its progenitor, AS-15MF. This dose was enough to prevent the ATN-resistant clone, AS-ATN, to recrudesce until about Day 12. The ATN and MF-sensitive AS-3CQ clone was almost completely eradicated by this dose (Figure 6C).

AS-MFATN-5 response to the combination of artesunate and mefloquine

We wished to investigate whether the increase in resistance of AS-MFATN-5 to ATN and MF when given separately would also be reflected as increased resistance to the combination.

When treated with doses of 7 mg/kg/day of MF in combination with 45 mg/kg/day of ATN, the AS-MFATN-5 clone recrudesced on Day 4, reaching peak parasitaemias of about 60% on Day 7. Mice inoculated with AS-15MF showed detectable parasitaemias on Day 4-5 but these animals reached peak of parasitaemias of only 13% on Day 7. AS-ATN recrudesced

on Day 10, reaching peak parasitaemia of 10.6% on Day 15. AS-3CQ recrudesced on Day 13, showing peak parasitaemia of 10% on Day 15 (Figure 6D).

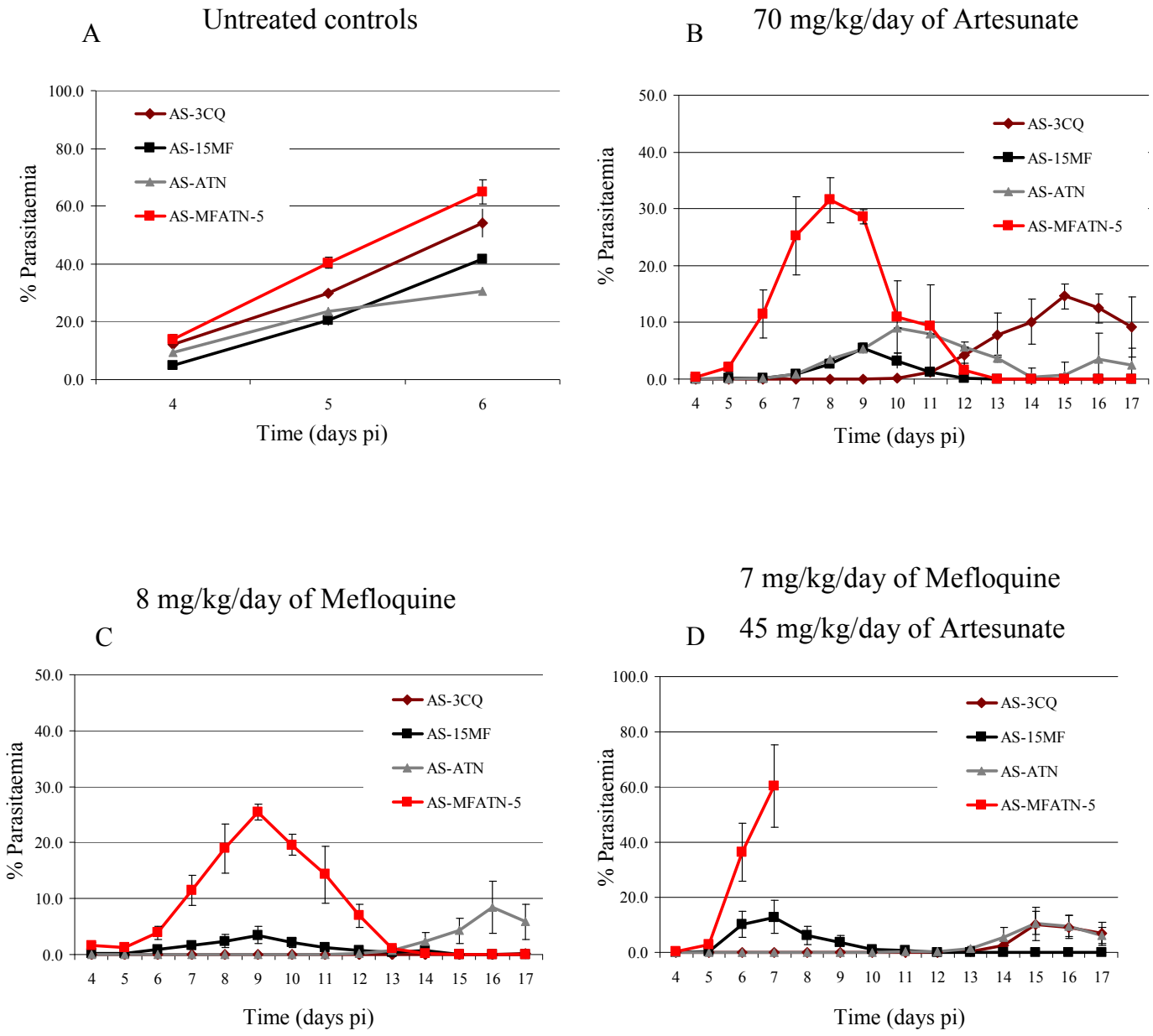


Figure 6 – Response to drug of different clones was assessed. (A) Untreated controls received DMSO vehicle. Clones were treated with different drugs as follows: (B) treatment with 70 mg/kg/day of artesunate (ATN); (C) treatment with 8 mg/kg/day of mefloquine (MF); and (D) treatment with 7 mg/kg/day of MF in combination with 45 mg/kg/day of ATN. Each point represents the mean percentage (%) parasitaemia of at least five mice determined daily. Red squares represent the clone selected here AS-MFATN-5; black squares represent the parental AS-15MF clone; gray triangles represent AS-ATN clone; burgundy diamonds represent AS-3CQ.

AS-15MF36P PHENOTYPE

As described above, a group of parasites was left untreated and was passaged through mice thirty six times simultaneously with the treated parasites that gave origin AS-MFATN-5. These parasites were named AS-15MF36P and their response to drugs was assessed and compared to the original AS-15MF clone.

The AS-15MF36P parasite population seems to have experienced a reduction in its level of resistance to ATN. The dose of 50 mg/kg/day was enough to prevent its growth until Day 8. After treatment with the same dose, the original AS-15MF recrudesced on Day 4, reaching peak parasitaemia of about 7%, on Day 7 (Figure 7B).

When treated with 5 mg/kg/day of MF, AS-15MF36P parasites were not able to grow beyond vestigial levels (about 0.5%) until Day 8. In accordance with previously published data (Cravo et al, 2003), the original clone, AS-15MF was able to grow after treatment with this dose of MF. Thus, AS-15MF showed parasitaemias of 1.5% on Day 4, with peak parasitaemia at about 14.5% on Day 7 (Figure 7C). In addition, AS-15MF36P was not able to grow after treatment with the ATN + MF combination. Treatment with 3 mg/kg/day of MF plus 20 mg/kg/day of ATN was enough to prevent AS-15MF36P parasite growth beyond vestigial levels. Under treatment with the same dose, the original AS-15MF reached peak parasitaemia at about 2% on Day 7 (Figure 7D).

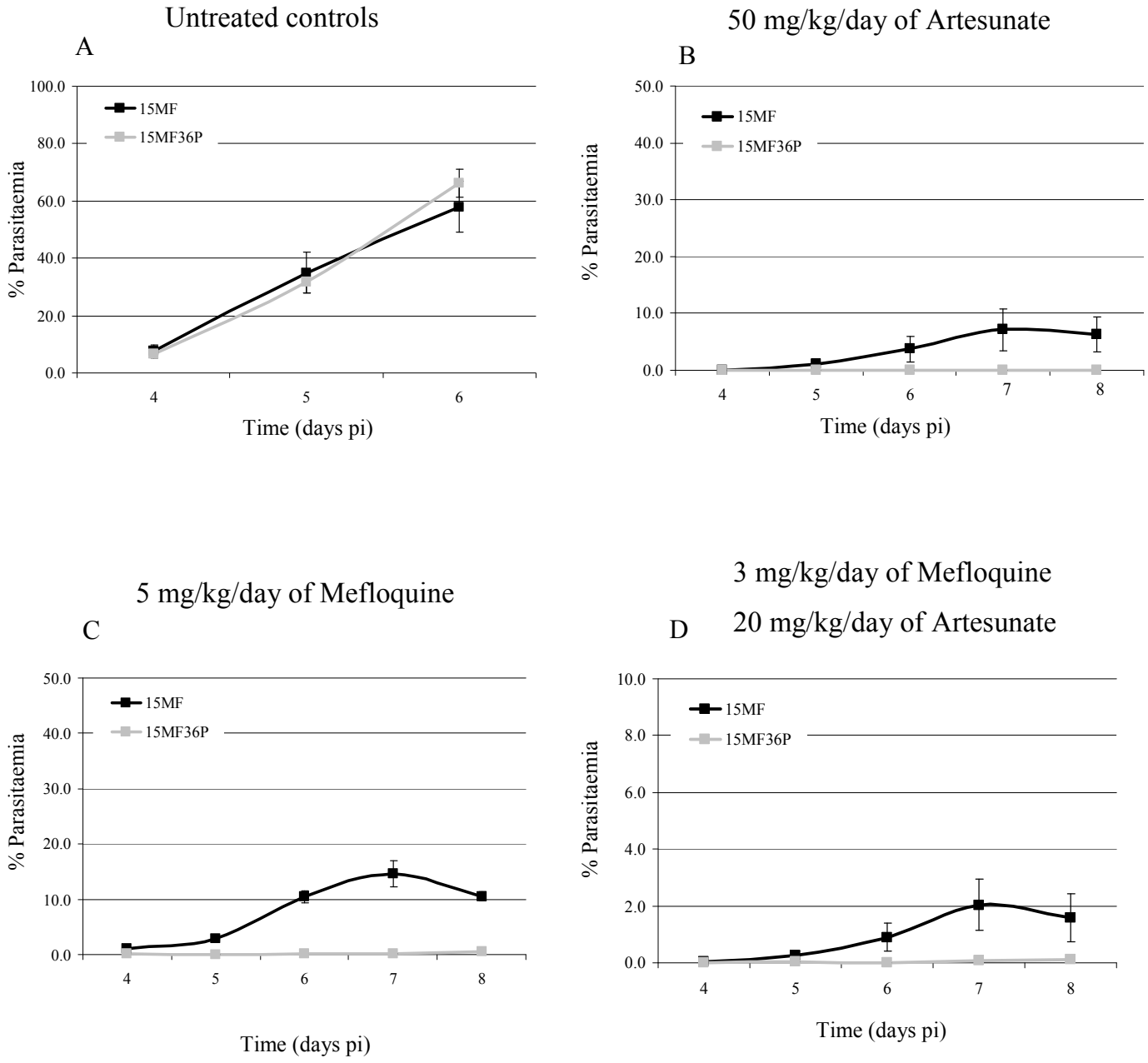


Figure 7 – Response to drug of different clones was assessed. (A) Untreated controls received DMSO vehicle. Clones were treated with different drugs as follows: (B) treatment with 50 mg/kg/day of artesunate (ATN); (C) treatment with 5 mg/kg/day of mefloquine (MF); and (D) treatment with 3 mg/kg/day of MF in combination with 20 mg/kg/day of ATN. Each point represents the mean percentage (%) parasitaemia of at least five mice determined daily. Black squares represent the parental AS-15MF and gray squares represent the population passaged thirty six weeks in absence of drug, AS-15MF36P.

SUMMARY

As described above the objective intended here was to select an artesunate + mefloquine (ATN + MF)-resistant clone by exposure of mefloquine (MF)-resistant parasites to stepwise increasing doses of artesunate (ATN) alone. Thus, five clones were obtained: AS-MFATN-1, -2, -3, -4, and -5. The AS-MFATN-5 clone was further analysed regarding its level of resistance to the combination ATN + MF, as well as to each drug alone.

Collectively, results showed that a resistant parasite was successfully generated, as AS-MFATN-5 displayed higher levels of resistance to ATN and the ATN + MF combination when compared to its progenitor AS-15MF. Interestingly, AS-MFATN-5 shows increased resistance to MF too, despite no further selection with this drug, suggesting that the genetic determinants of ATN resistance also mediate augmented MF resistance phenotypes.

In addition, the AS-15MF clone was passaged through mice in absence of drug pressure simultaneously with the treated parasites that generated AS-MFATN-5. After thirty six passages, the untreated parasites AS-15MF36P had their response to drug assessed. AS-15MF36P displayed a reduced level of resistance to ATN, MF and to the ATN + MF combination when compared to the original AS-15MF clone. Thus, these results suggest that the original AS-15MF resistance phenotype was reversed after prolonged *in vivo* passaging in the absence of drugs.

II.2.1.1.2. EXPOSURE OF THE ARTESUNATE RESISTANT CLONE AS-ATN TO ARTESUNATE + MEFLOQUINE

SELECTION

The previous sections described the selection of artesunate + mefloquine (ATN + MF) resistance using the mefloquine (MF)-resistant AS-15MF clone as starting material. In parallel, the AS-ATN clone, resistant to four daily doses of 60 mg/kg/day of artesunate (ATN) (Afonso *et al,* 2006) was used as starting material for selection of ATN + MF resistance. This clone was submitted to treatment with ATN in combination with MF, on Days 3, 4 and 5 post-inoculum (pi). On Day 7, the parasites surviving after treatment were passaged into uninfected mice. In addition to the treated parasites, a line of AS-ATN was sub-inoculated consecutively in parallel, but left untreated.

The starting selecting dose was 1 mg/kg/day of MF in combination with 5 mg/kg/day of ATN, and on Day 7 pi the parasites reached a parasitaemia of almost 30%. ATN was then increased to 10 mg/kg/day, whereas the MF dose was maintained (1 mg/kg/day). For the following two weeks, parasites seemed to adapt well, and accordingly drug doses were increased to 2 mg/kg/day of MF plus 20 mg/kg/day of ATN. However, after two weeks, this drug dose appeared to have caused the complete eradication of the parasite population in the host mice, forcing a restart of the process from the previous round of selection. The dose of 2 mg/kg/day of MF plus 20 mg/kg/day of ATN was thus maintained for a further four weeks, until parasites were able to reach high parasitaemias on Day 7 (around 24%). ATN amounts were then increased to 25 mg/kg/day, but this was met with limited success, as parasites did not recover well after treatment. In order to avoid losing the parasites for a second time, ATN was lowered again. From this stage onwards, parasites started to display a slightly improved recovery (Figure 8).

At the end of the 27th week of selection, parasites were able to survive a treatment of 3.5 mg/kg/day of MF in combination with 30 mg/kg/day of ATN.

As mentioned at the beginning of the section, AS-ATN was also inoculated into mice that were left untreated and were passaged to new mice in parallel with the treated group for twenty seven times. This parasite population will be herein referred to as AS-ATN27P.

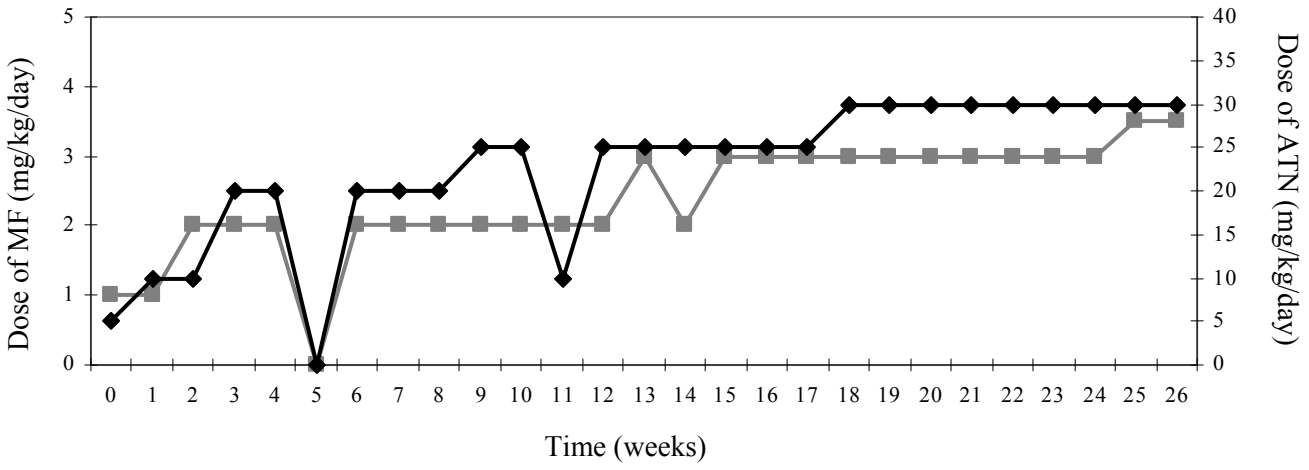


Figure 8 – Evolution of doses of mefloquine (MF) (gray squares) and artesunate (ATN) (black diamonds) administered in combination to mice infected with AS-ATN along the course of twenty seven weeks of selection.

CLONING

After twenty-seven weeks the parasites selected by drug pressure as described above were cloned by limiting dilution. Thus, blood of mice infected with drug selected parasites was used for inoculating new mice as described in Material and Methods, Section 2.2.

On Day 11, eight animals from the group of twenty mice inoculated with the equivalent of one pRBC showed detectable parasitaemias, which means that 60% of mice were not infected. On the other hand, after eleven days, only five out of twenty mice inoculated with the equivalent of 0.5 pRBC were parasitized, i.e. 75% of mice did not show detectable parasitaemias. Considering that in both cases the percentage of uninfected mice was higher than 37%, theoretically both one and 0.5 pRBC inoculums resulted into successful cloning (see Material and Methods, Section 2.2). However, only the parasites present in the five mice

inoculated with 0.5 pRBC were stored for further analysis, since these are statistically more likely to be clonal. These clones were named AS-ATNMF-1, AS-ATNMF-2, AS-ATNMF-3, AS-ATNMF-4, and AS-ATNMF-5 (Figure 9), with AS-ATNMF-1 clone exhibiting the fastest growth rate.

AS-ATNMF-1 was further characterised regarding its phenotype and genetic background, whereas the other four clones were only inspected for the presence of mutations identified by Solexa whole genome re-sequencing analysis below.

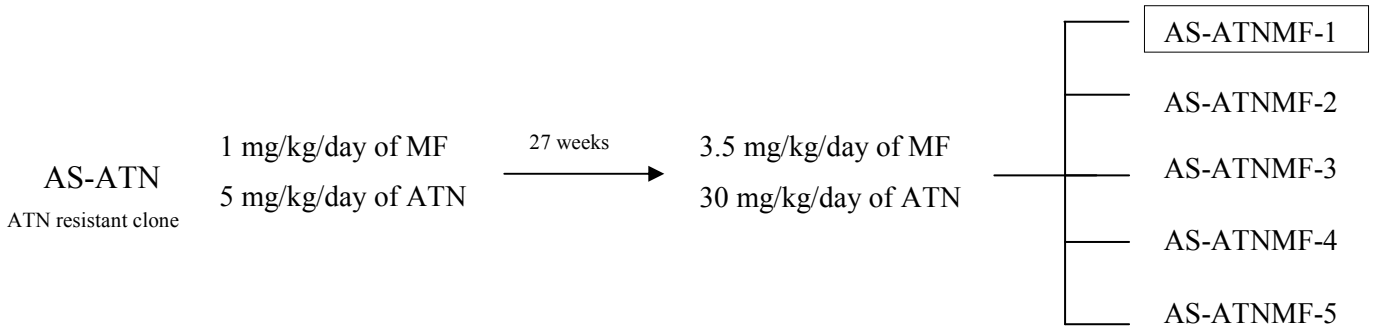


Figure 9 – Schematic representation of selection and cloning. The AS-ATN clone used here as starting material was submitted to increasing doses of mefloquine (MF) in combination with artesunate (ATN) over twenty seven weeks, after which was cloned by limiting dilution. This process originated five clones. The AS-ATNMF-1 clone is the one exhibiting the fastest growth rate and will be further characterised.

DRUG SENSITIVITY TEST

After twenty seven weeks of being passaged under treatment with ATN + MF combination and following cloning in the absence of drug, the level of resistance of AS-ATNMF-1 to both drugs given separately or in combination was assessed. The responses of the original parental AS-ATN, the parasites passaged in absence of drug treatment AS-ATN27P, the MF-resistant AS-15MF clone and the ATN and MF-sensitive AS-3CQ clone were also investigated.

Five groups of mice were inoculated with each clone and left untreated. These parasites grew normally and reached peak parasitaemias of 40-to-80% at Day 5 or 6 post-inoculum (pi) (Figure 10).

The responses of each clone to the different drug treatments will be described in detail in the following sections.

AS-ATNMF-1 response to artesunate

In order to assess whether the evolution of ATN + MF resistance could have caused increased resistance to ATN given alone, AS-ATNMF1 was tested for its response to this drug in comparison to its AS-ATN progenitor.

After treatment with 80 mg/kg/day of ATN, AS-ATNMF-1 recrudesced earlier than its progenitor (Figure 10B). AS-ATNMF-1 recrudesced on Day 4 while AS-ATN recrudesced one day later (Day 5).

The untreated parasite AS-ATN27P displayed an increased growth delay, recrudescing on Day 8 only, and showing peak parasitaemias of 26% on Day 12 (Figure 10B).

Interestingly, ATN did not cause significant delay in recrudescence and growth of the MF-resistant parasite AS-15MF in comparison to AS-ATNMF-1 (Figure 10B). By observing the first seven days of the drug test, AS-15MF appears to display a phenotype resembling that of AS-ATNMF-1, as both clones show recrudescence on Day 4, and approximately similar parasitaemias by Day 7 (12% and 16%, respectively). However, there was a noticeable persistence in the infection for AS-ATNMF-1 and AS-ATN until at least Day 14 p.i. which was not observed for AS-15MF. The ATN and MF-sensitive AS-3CQ recrudesced on Day 6. However, it was never able to establish parasitaemias above 4%.

AS-ATNMF-1 response to mefloquine

In order to assess AS-ATNMF-1's response to MF administered alone, AS-ATNMF-1's growth was compared to the progenitor AS-ATN, and also to the MF-resistant AS-15MF clone and to the ATN and MF-sensitive AS-3CQ clone after treatment with three daily doses of 8 mg/kg/day of MF.

AS-ATNMF-1 is more resistant to MF alone than its progenitor, AS-ATN, when treated with 8 mg/kg/day of MF. Mice infected with AS-ATNMF-1 were able to show detectable parasitaemias on Day 4 pi, reaching peak parasitaemia of about 21% on Day 8 pi. In contrast, AS-ATN was not able to recrudesce until Day 11 pi. The parasites derived from AS-ATN, passaged through mice twenty seven times in the absence of drug pressure (AS-ATN27P), showed similar growth as AS-ATN (Figure 10C).

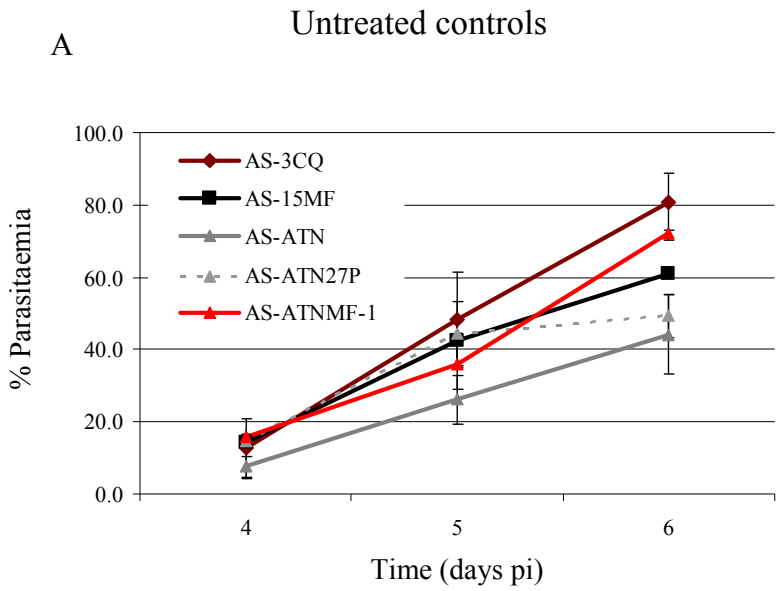
The MF-resistant AS-15MF clone displayed parasitaemias of about 2.5% on Day 4 pi. However, this clone was never able to establish high parasitaemias, reaching its peak on Day 8 at 5.5%. AS-3CQ was not able to recrudesce after treatment (Figure 10C).

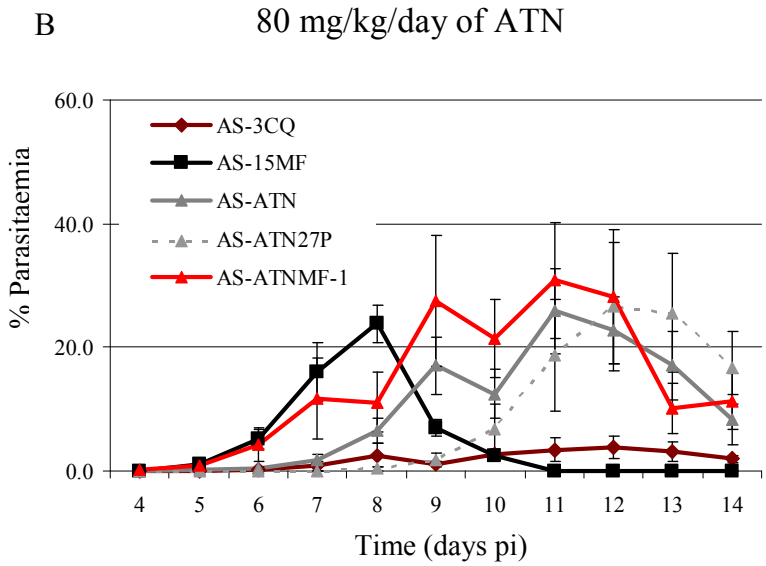
AS-ATNMF-1 response to the combination of artesunate and mefloquine

AS-ATNMF-1 was selected by treatment with increasing doses of the combination ATN + MF. In order to assess if selection resulted in parasites displaying high levels of resistance to these two drugs in combination, AS-ATNMF-1's growth was compared with AS-ATN, AS-15MF and AS-3CQ, after 3 daily doses of 4 mg/kg/day of MF in combination with 40 mg/kg/day of ATN.

Under the above mentioned conditions AS-ATNMF-1 was able to recrudesce on Day 7 and reach peak parasitaemia of about 47% on Day 11. When treated with the same dose, its progenitor, AS-ATN was able to recrudesce on Day 8, reaching parasitaemias of about 20% on Day 14 (Figure 10D). Interestingly, the AS-ATN27P population derived from AS-ATN after

twenty seven weeks in absence of drug treatment took longer to recrudesce than AS-ATN (around Day 12). Similarly, the MF-resistant AS-15MF clone was also able to recrudesce on Day 12, whilst mice infected with the ATN and MF-sensitive AS-3CQ clone never showed detectable parasitaemias during the follow-up period (Figure 10D).





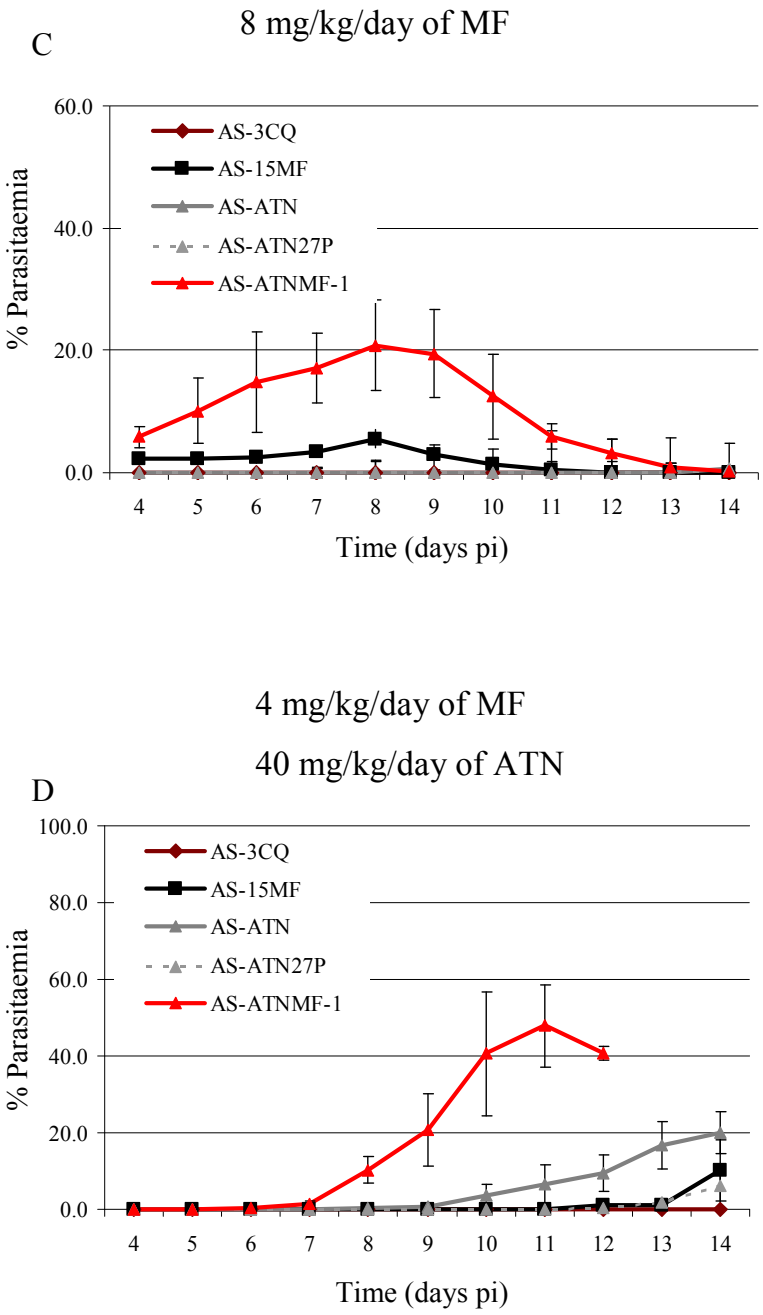


Figure 10 – Response to drug of different clones was assessed. Clones were treated with different drugs as follows: (A) untreated controls (DMSO vehicle); (B) treatment with 80 mg/kg/day of artesunate (ATN); (C) treatment with 8 mg/kg/day of mefloquine (MF); and (D) treatment with 4 mg/kg/day of MF in combination with 40 mg/kg/day of ATN. Each point represents the mean percentage (%) parasitaemia of at least five mice determined daily. Red triangles represent the clone selected here AS-ATNMF-1; gray triangles represent the parental AS-ATN clone; gray diamonds and dashed line represente the parasites passaged twenty seven weeks in absence of drug; black squares represent AS-15MF; and burgundy diamonds represent AS-3CQ.

SUMMARY

The objective was to select and clone parasites resistant to the artesunate + mefloquine (ATN + MF) combination using a clone showing limited resistance to artesunate (ATN) as starting material. To that purpose, the AS-ATN clone was exposed to stepwise increasing doses of ATN and mefloquine (MF) administered simultaneously. Five clones were obtained: AS-ATNMF-1, -2, -3, -4, and -5. AS-ATNMF-1 was further analysed regarding its response to the ATN + MF combination, as well as to ATN and MF administered separately.

Collectively, the results showed that by following this approach, a resistant parasite was generated, as AS-ATNMF-1 displayed higher levels of resistance to the ATN + MF combination, as well as to MF administered alone when compared to its progenitor, AS-ATN, and to the MF-resistant clone, AS-15MF. In addition, AS-ATNMF-1 shows a slight increase in its level of resistance to ATN administered alone when compared to its progenitor AS-ATN, but not to the MF-resistant AS-15MF.

In addition, during the selection procedure, the AS-ATN clone was passaged through mice in absence of drug treatment in parallel with the treated parasites. After twenty seven sub-inoculations, the response to drug treatment with the ATN + MF combination as well as each drug alone of the AS-ATN27P parasites was assessed. Our observations indicated that after consecutive sub-inoculations in the absence of drug, AS-ATN27P shows a slight decrease in its level of resistance to ATN + MF combination, as well as to ATN and MF administered separately, when compared to the original AS-ATN clone, suggesting that the resistance phenotype was reversed in the absence of continuous drug pressure.

II.1.1.2. SELECTION OF ARTESUNATE + MEFLOQUINE RESISTANCE THROUGH GENETIC CROSSING

It has been postulated that one of the advantages of the administration of artemisinin derivatives in combination with drugs with a distinct mode of action is that the appearance of resistance to both drugs simultaneously would require the appearance of parasites bearing mutations in at least two different genes belonging to each of the pathways targeted by each drug (White, 1999). However, in the case of the use of each component of the combination as monotherapy, resistance to each drug could appear separately, and therefore, two different sub-populations of parasites could exist. If a single host were infected with each of these sub-populations and then, gametes generated by these sub-populations were to mate in the mosquito, a "double-resistant" parasite could rise from the cross progeny, due to genetic recombination of the two parental groups of parasites. The "double-resistant" offspring would carry resistant alleles of genes underlying resistance to each drug, and as a consequence, be resistant to the two drugs administered simultaneously.

In order to test this hypothesis, a mosquito cross between the ATN-resistant AS-ATN clone and the MF-resistant AS-15MF clone was attempted. Two genetic markers originating from each of the two parental clones were analysed. This analysis revealed that only the AS-ATN parental alleles were present in the resulting cross progeny. There are two possible explanations to this finding: i) no self-fertilisation of AS-15MF gametes or cross-fertilisation of AS-15MF gametes with AS-ATN gametes took place; or ii) cross-fertilisation did take place between AS-ATN and AS-15MF clones, however, the AS-15MF alleles of markers analysed here were present in the cross-progeny in small amounts, and remained undetected.

As this genetic cross did not produce clear results, the analysis of the parasites generated here was discontinued. The full description of the results obtained for this experiment are presented as an Appendix (Appendix I).

SECTION SUMMARY

Two different clones with distinct genetic backgrounds were used for the selection of resistance to the combination ATN + MF. In this context, AS-ATNMF-1 and AS-MFATN-5, were generated from AS-ATN and AS-15MF, respectively.

In addition, the above parasites displayed increased tolerance to each of the drugs of the combination when administered separately.

In parallel, the parental AS-15MF and AS-ATN were passaged by consecutive sub-inoculations in mice, and two groups of parasites were obtained: AS-15MF36P and AS-ATN27P. Whereas AS-ATN27P seems to have a very slight decrease in the level of sensitivity to ATN and MF compared to AS-ATN, AS-15MF36P displayed reduced levels of resistance to these drugs when compared to the original AS-15MF.

II.2.2. GENETIC CHARACTERISATION OF RESISTANT CLONES

As described above, two distinct clones were successfully selected by drug pressure. One of them derives from the mefloquine (MF)-resistant AS-15MF clone, and was named AS-MFATN-5. The other one derived from the artesunate (ATN)-resistant AS-ATN clone, and was named AS-ATNMF-1. In spite of their different backgrounds, both clones display considerably high levels of resistance to the artesunate + mefloquine (ATN + MF) combination, as was the first objective intended here.

Two main approaches were employed to determine the genetic basis of the phenotype displayed by the multi-drug resistant clones generated in the course of this study; a candidate gene approach and a '*de novo*' genetics/genomics approach. Firstly, the sequences of candidate genes which were previously related to resistance to ATN and MF were investigated. Thus, the coding sequences of the *pcatp6* and *pcubp1* genes were determined for both AS-ATNMF-1 and AS-MFATN-5 clones and compared with their progenitors. The copy number, mRNA expression and protein amounts of MDR1 was also determined in resistant clones.

Secondly, a genome-wide approach (Linkage Group Selection, LGS) was applied in order to locate the locus or loci where phenotypically relevant mutations have occurred. For this purpose, a genetic cross between the AS-ATNMF-1 clone and the sensitive and genetically unrelated AJ clone, under treatment with different drugs was produced and analysed.

Complementary to LGS, the whole genome of the AS-ATNMF-1 clone was re-sequenced using a second generation genome re-sequencing method, known as Solexa (Illumina, Inc.), and some of the mutations found using this methodology were validated through traditional di-deoxy sequencing.

II.2.2.1. GENETIC ANALYSIS OF RESISTANT CLONES

In order to assess if the evolution of resistance to artesunate + mefloquine (ATN + MF) had selected particular genetic mutations, a candidate gene approach was used. The involvement of genes previously described as putatively involved in resistance to artesunate (ATN) and mefloquine (MF) were analysed as described below.

pcatpase6

SERCA type Ca^{++} ATPase is considered a possible intracellular target of artemisinin (ART) derivatives (Eckstein-Ludwig *et al,* 2003). In *P. chabaudi*, the homologue of this protein is coded by the *pcatpase6* gene (PCHAS_020540). Overlapping fragments covering the gene's coding sequence were amplified by PCR and sequenced for both AS-ATNMF-1 and AS-MFATN-5.

No changes were found when comparing each clone with the reference sequence retrieved from the Welcome Trust Sanger Institute website (see Material and Methods Section 3.7.1).

pcubp1

When inducing increased ATN resistance, Hunt and colleagues (2007) identified a mutation in a gene denoted *pcubp1* (PCHAS_020720). This gene codes for a deubiquitinating enzyme, which is involved in protein turn-over pathways. The G8089T mutation found in AS-ATN is exclusive to this clone and codes for the substitution of a valine to a phenylalanine in aminoacid 2697 (V2697F) (Hunt *et al,* 2007). This mutation is believed to be involved in the expression of the ATN-resistant phenotype. *Pcubp1* is also mutated in other clones of the AS lineage, i.e. AS-15MF, AS-30CQ and AS-ART. However, in these clones, a G to T mutation is found in position 8182 and codes for a valine to phenylalanine substitution in aminoacid 2728

(V2728F). This mutation is also believed to have a role in resistance to ART derivatives (Hunt *et al,* 2007).

Similarly to what was described above for *pcatpase6*, overlapping fragments were amplified and sequenced for both AS-ATNMF-1 and AS-MFATN-5, as well as for their respective progenitors, AS-ATN and AS-15MF. Only the final 3500 bp of *pcubp1* gene were sequenced. This is because this work was carried out prior to a new annotation which merged this gene with the preceding gene (full length gene is 8766 bp, as currently annotated in GeneDB)

The analysis of the final 3500 bp fragment of *pcubp1* revealed no changes when comparing the sequences obtained for each of the clones selected for ATN + MF resistance and their respective progenitors. Therefore, AS-ATNMF-1 carries the G8089T mutation, also present in its progenitor AS-ATN, and AS-MFATN-5 shows the G8182T mutation, which is also borne by it progenitor AS-15MF.

As will be presented below, the complete genome sequence of AS-ATNMF-1 clone was obtained by Solexa whole genome sequencing technique, and no additional mutations were identified in the initial 5266 bp segment of *pcubp1* gene.

The whole genome analysis of AS-MFATN-5 is scheduled to be performed in the near future; alternatively, the overlapping fragment technique could be applied for the initial fragment of *pcubp1*, allowing the determination of additional mutations appearing in this portion of the gene.

pcmdr1

Copy number variation

Amplification of the *mdr1* gene is responsible for increased resistance to MF and also to ART derivatives both in human parasites (either field or laboratory strains) (Price *et al,* 1997;

Price *et al,* 1999; Pickard *et al,* 2003; Price *et al,* 2004; Alker *et al,* 2007; Lim *et al,* 2009; Chaijaroenkul *et al,* 2010; Chavchich *et al,* 2010) and in rodent models (Gervais *et al,* 1999; Cravo *et al,* 2003; Ferrer-Rodriguez *et al,* 2004; Borges, 2009). As such, *pcmdr1* copy number was determined for AS-MFATN-5 and AS-ATNMF-1 clones. Three clones were used as controls, as follows: AS-SENS and the two progenitors AS-15MF and AS-ATN. Note that AS-15MF is known to carry two copies of the *pcmdr1* gene (Cravo *et al,* 2003); AS-ATN and AS-SENS, each harbouring a single copy of this gene (Afonso *et al,* 2006). The data normalised against AS-SENS and AS-ATN is presented in Table 1 below.

The MF-resistant AS-15MF clone was confirmed to carry 2 copies of the *pcmdr1* gene relative to the AS-ATN clone (1.5 ± 0.12 ($p<0.00001$), and the AS-SENS clone (1.6 ± 0.12 ($p<0.001$). This gene amplification is in line with previous work that indicates that the *pcmdr1* gene is duplicated in AS-15MF (Cravo *et al,* 2003).

Importantly, AS-MFATN-5 has acquired a further copy of *pcmdr1* relative to its progenitor AS-15MF ($p<0.0002$). Mdr1 gene copy number is estimated to be 2.8 (± 0.85, $p<0.001$) and 2.7 (± 0.44, $p<0.00001$) relative to the negative controls AS-SENS and AS-ATN, respectively) (Table 1).

For AS-ATNMF-1 too, there was an increase in *pcmdr1* copy number; to 1.8 (± 0.37, $p<0.001$) and 1.8 (± 0.09, $p<0.00001$) relative to AS-SENS and the progenitor AS-ATN, respectively (Table 1).

Interestingly, the AS-15MF36P parasite population shows the loss of one of the two copies originally present in the original unpassaged AS-15MF clone (0.9 ± 0.09 relative to AS-ATN, $p<0.0001$) (Table 1).

In agreement with previous work (Afonso *et al,* 2006), the analysis carried out here showed that the ATN-resistant AS-ATN clone harbours a single copy of *pcmdr1* gene (1.0 ± 0.20 relative to AS-SENS) (Table 1). Similarly, the AS-ATN27P parasite population passaged

twenty seven times in absence of drug remained unchanged when comparing to the original AS-ATN. Thus, AS-ATN27P also shows a single copy of *pcmdr1* (0.7 ± 0.08 relative to AS-ATN).

mdr1 Copy Number

	AS-SENS				AS-ATN			
	mean		SE		mean	SE		
AS-SENS	-				1,0	± 0,09		
AS-15MF	1,6	±	0.12	*	1,5	± 0.05	**	†
AS-MFATN-5	2,8	±	0.38	*	2,7	± 0.20	**	†
AS-15MF36P	0,9	±	0,1		0,9	± 0,04		
AS-ATN	1,0	±	0,09		-			
AS-ATNMF1	1,8	±	0.17	*	1,8	± 0.04	**	
AS-ATN27P					0,7	± 0,04		

Table 1 – Copy number of *pcmdr1* gene. Genomic DNA of different clones was analysed by Real-Time PCR. The values were normalized against the drug-sensitive AS-SENS clone or the artesunate-resistant AS-ATN clone, as indicated. Results are expressed as means of five experiments ± standard error (SE) of the mean. (*) $p< 0.001$; (**) $p<0.00001$; (†) $p<0.0002$.

RNA expression

Changes in copy number of *pcmdr1* gene have been previously associated with modifications in the level of RNA expression (Wilson *et al,* 1989; Peel *et al,* 1994; Cowman *et al,* 1994; Chavchich *et al,* 2010).

In order to assess whether this is the case for the ATN + MF-resistant AS-ATNMF-1 clone, RNA was extracted from blood infected with the AS-3CQ, AS-15MF, AS-ATN, AS-ATN27P, and AS-ATNMF-1 clones. cDNA was synthesised, as described in Material and Methods, Sections 3.3 and 3.4, and subsequently analysed by Real-Time PCR. Here, AS-3CQ

was used as a control instead of AS-SENS. Similarly to AS-SENS, used for the determination of *pcmdr1*'s copy number, AS-3CQ is also ATN and MF-sensitive and carries a single copy of *pcmdr1* gene.

AS-ATNMF-1 showed increase expression of the *pcmdr1* gene as indicated in Table 2. In line with an increase of genomic copies, AS-ATNMF-1 also displayed higher levels of *pcmdr1* RNA expression relative to its progenitor, AS-ATN clone and to AS-3CQ (2.6 ± 0.03, $p<0.00002$ and 3.4 ± 0.22, $p<0.00002$, respectively). AS-15MF (which contains two copies of this gene) presented similar levels of RNA expression when compared to AS-ATNMF-1 (2.5 ± 0.39, $p<0.00002$ and 2.1 ± 0.34, $p<0.01$, normalised against AS-3CQ and AS-ATN, respectively). Additionally, in agreement with the results obtained for copy number, the AS-ATN clone and the untreated population derived from it, AS-ATN27P (both shown to carry a single copy of this gene) have similar levels of RNA expression when normalised against AS-3CQ (1.4 ± 0.06 and 1.2 ± 0.22, respectively).

One surprising feature worth highlighting is the difference in expression found when comparing AS-ATN and AS-3CQ. Although both clones only carry one copy of *pcmdr1* gene, AS-ATN seems to have statistically significant increased level of RNA expression relative to AS-3CQ (1.4 ± 0.006, $p<0.0003$) (Table 2).

mdr1 RNA Expression

	AS-3CQ			AS-ATN		
	mean		SE	mean		SE
AS-3CQ	-			0.7	±	0.08 *
AS-ATN	1.4	±	0.06 **	-		
AS-ATNMF1	3.4	±	0.22 ***	2.6	±	0.17 ***
AS-ATN27P	1.2	±	0.07	0.9	±	0.03
AS-15MF	2.5	±	0.39 *	2.1	±	0.34 †

Table 2 – RNA expression of *pcmdr1* gene determined by Real-Time PCR. cDNA was synthesised from RNA and analysed. Values were normalised against the artesunate and mefloquine-sensitive AS-3CQ clone and the artesunate-resistant AS-ATN clone, as indicated. Results are expressed as means of five experiments ± standard error (SE). (†) $p<0.01$, (*) $p<0.004$, (**) $p<0.0003$, (***) $p<0.00002$.

Protein expression

The *mdr1* gene codes for the homologue of the human P-gp1, an ATP-dependent efflux pump known in Plasmodium as MDR1 (Foote *et al,* 1989; Wilson *et al,* 1989).

In order to ascertain whether an increase in copy number and higher levels of RNA expression is also reflected in the level at which MDR1 protein is expressed, Western Blot analyses were carried out. In addition to AS-ATNMF-1, MDR1 expression was quantified in AS-ATN, AS-ATN27P, AS-15MF and AS-3CQ (Figure 11).

AS-ATNMF-1 revealed a 4-to-6-fold increase in MDR1 expression, relative to AS-3CQ and to AS-ATN (5.8 ± 0.44, $p<0.005$ and 4.5 ± 0.78, $p<0.05$ respectively) (Figure 11B). These observations are in agreement with the duplication of *pcmdr1* and the higher levels of RNA expression displayed by AS-ATNMF-1. However, while there seems to be a duplication in both gene copy number and RNA levels, the levels of MDR1 expression are four-to-five-fold higher

when comparing AS-ATNMF-1 and AS-ATN. This disproportional increase may be indicative of some type of post-transcriptional or post-translational regulation.

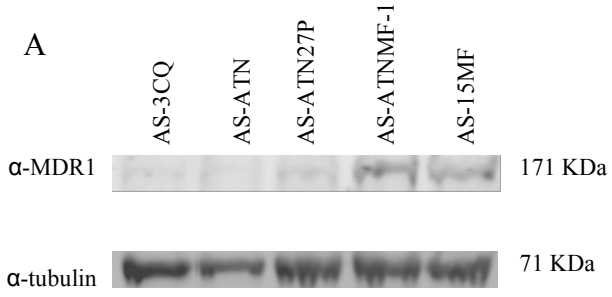
Also, in line with copy number and RNA expression of *pcmdr1*, the AS-15MF clone seems to have similar levels of MDR1 expression of to those of AS-ATNMF-1 (6.2 ± 0.88, $p<0.005$ and 5.1 ± 0.28, $p<0.005$ normalised against AS-3CQ and AS-ATN, respectively). Again, in AS-15MF, MDR1 expression levels show a disproportional increase (five-to-six-fold) when compared to *pcmdr1* gene copy number and RNA expression (about two-fold).

When comparing AS-3CQ and AS-ATN, both carrying a single copy of *pcmdr1*, there seems to be a slight difference between the two. AS-ATN shows 1.39 ± 0.17 expression of MDR1 when compared to AS-3CQ ($p<0.05$) (Figure 11B). These results seem to be in agreement with the level of RNA expression displayed by these clones, where AS-ATN seems to show a slightly higher level of RNA expression of *pcmdr1* than AS-3CQ.

Interestingly, in spite of having only one copy of *pcmdr1* and showing similar levels of RNA expression as AS-ATN, the untreated parasites AS-ATN27P seem to have an almost two-fold increase in expression of MDR1 when compared to the original AS-ATN (1.8 ± 0.16, $p<0.05$ and 2.4 ± 0.32, $p<0.01$, normalised against AS-ATN and AS-3CQ, respectively).

MDR1 Protein Expression

A



B

	AS-3CQ			AS-ATN		
	mean		SE	mean		SE
AS-3CQ	-			0.7	±	0.09
AS-ATN	1.4	±	0.17 *	-		
AS-ATNMF1	5.8	±	0.44 ***	4.5	±	0.78 *
AS-ATN27P	2.4	±	0.32 **	1.8	±	0.16 *
AS-15MF	6.2	±	0.88 ***	5.1	±	0.28 ***

Figure 11 - *pcmdr1* expression (MDR1 expression) determined by Western Blot. Protein extracts were prepared from erythrocyte stage parasites and analysed. Values were normalised against AS-3CQ and AS-ATN, as indicated. Results are expressed as means of five experiments ± standard error (SE). (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.005$.

A summary of all the results obtained for copy number, RNA and protein expression of *pcmdr1* gene are depicted in Figure 12. The values were normalised against AS-3CQ (or AS-SENS, for copy number) (Figure 12A) or AS-ATN (Figure 12B).

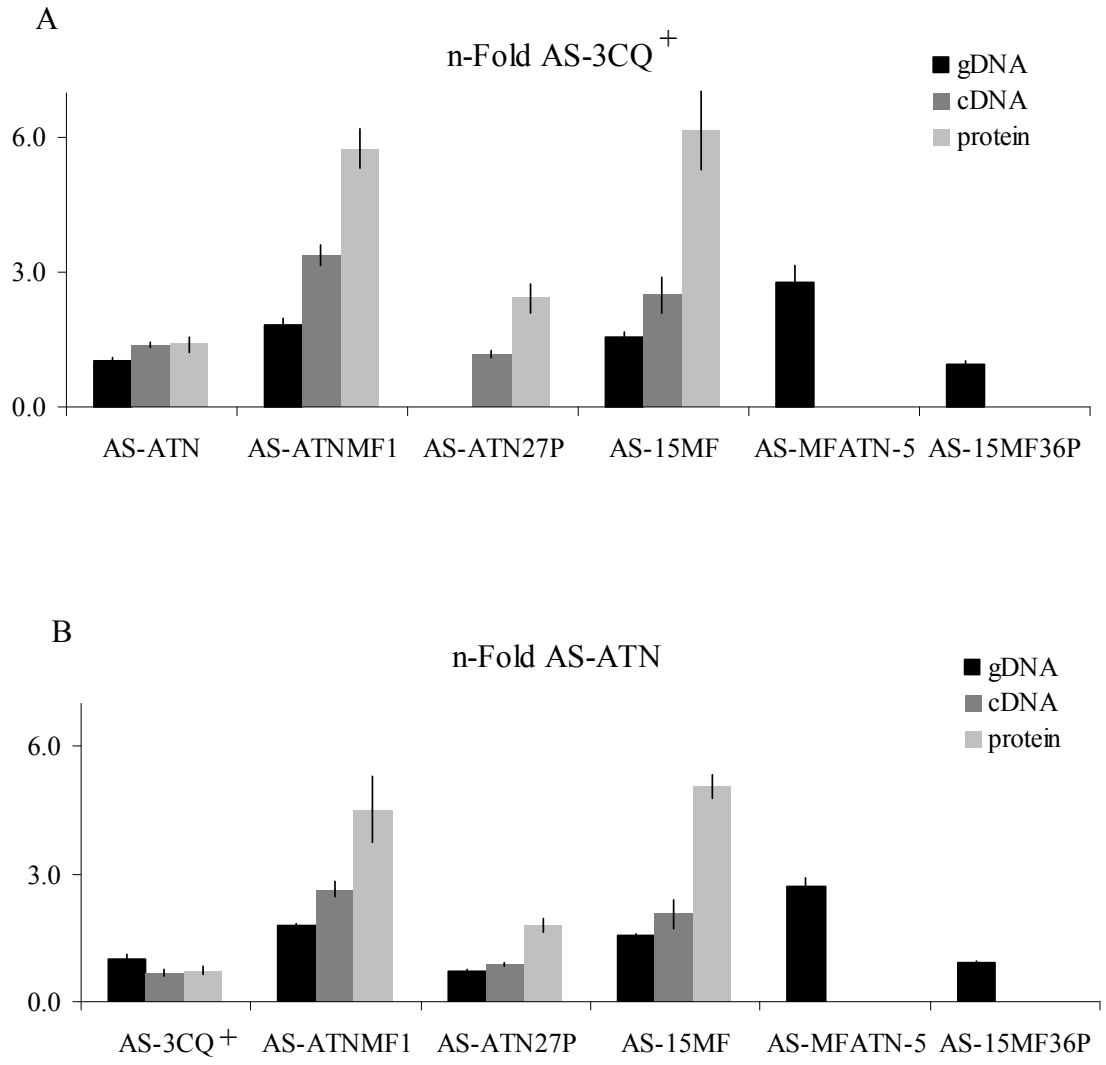


Figure 12 – Summary of data obtained for copy number, RNA and protein expression of *pcmdr1* gene. Copy number (gDNA, for genomic DNA) is shown as black bars; RNA expression (cDNA, for complementary DNA), as dark gray bars; and protein expression, as light gray bars. The bars represent the mean of at least five experiments and standard errors are represented by black lines. (A) Samples were normalised against AS-SENS (copy number) or AS-3CQ (RNA and protein expression). (B) Samples were normalised against AS-ATN. (+) AS-3CQ or AS-SENS.

SUMMARY

In an attempt to identify genetic changes underlying the artesunate + mefloquine (ATN + MF)-resistant phenotype exhibited by AS-MFATN-5 and AS-ATNMF-1, the *pcatp6*, *pcubp1* and *pcmdr1* genes, previously described as involved in resistance to mefloquine and/or artesunate (ATN) were analysed. When comparing AS-MFATN-5 and AS-ATNMF-1 with their respective progenitors, no mutations were found in the whole coding sequence of the *pcatpase6* gene. Similarly, the final 3500 bp of gene *pcubp1* presented no mutations in AS-MFATN-5 and AS-ATNMF-1 clones when compared with their respective progenitors AS-15MF and AS-ATN.

The analysis of AS-MFATN-5 indicated that exposure of AS-15MF to consecutive rounds of selection with ATN selected parasites which underwent further amplification of *pcmdr1* gene, as AS-MFATN-5 harboured three copies of *pcmdr1*. On the other hand, consecutive passaging through mice in absence of drug has induced the loss of one of the two copies originally present in AS-15MF, as AS-15MF36P carries a single copy of *pcmdr1* gene.

The induction of ATN + MF resistance using AS-ATN clone as starting material selected parasites with a duplicated copy of *pcmdr1*, in AS-ATNMF-1. An increase in RNA and protein (MDR1) expression were also observed in this clone. However, whereas the RNA level seems to be slightly increased when compared to copy number (about two-to-three-fold higher in AS-ATNMF-1 when compared to AS-ATN), the level of MDR1 expressed in AS-ATNMF-1 seems to be five-to-six-fold increased when compared to the parental AS-ATN. In addition, AS-ATNMF-1 and AS-15MF, both carrying a duplication of *pcmdr1* gene, show similar levels of RNA and protein expression.

In spite of carrying a single copy of *pcmdr1*, AS-ATN shows increased levels of RNA and protein expression when compared with AS-3CQ. AS-ATN27P, which also harbours a single

copy of *pcmdr1* gene and has similar levels of RNA expression as the original unpassaged AS-ATN, shows increased levels of MDR1 protein expression when compared with AS-ATN.

II.2.2.2. LINKAGE GROUP SELECTION

In order to map the regions of the genome containing genes conferring the resistance phenotypes, a genome wide approach known as Linkage Group Selection (LGS) was carried out. This analysis consisted of performing a genetic cross between AS-ATNMF-1 and the genetically unrelated AJ clone. The cross progeny obtained here was then selected using four different drugs or drug combinations: i) artesunate, ii) mefloquine, iii) the combination of these two drugs, and iv) artemisinin. However, these experiments met with limited success, as drug treatment seemed to be insufficient to allow the detection of relevant *loci*. The data is presented in detail in the Appendices section (Appendix II).

II.2.2.3. GENOME-WIDE MUTATION DETECTION

With the purpose of identifying additional mutations arising after selection for artesunate + mefloquine (ATN + MF) resistance, the complete genome sequence of the AS-ATNMF-1 clone was investigated.

The analysis carried out in this work was made using the Illumina/Solexa platform. AS-ATNMF1 was sequenced using 50 bp paired-end reads and mapped to the reference genome (AS-WTSI) using the SSAHA2 and MAQ programs, as described in the Material and Methods (Section 3.7.1).

23,732,208 reads were produced for AS-ATNMF-1, of which 91% (21,816,439 reads) and 87.2% were mapped to the genome by SSAHA2 and MAQ softwares, respectively. 86% (20,423,970 reads) of the total reads were found to map uniquely in SSAHA2 and 84% in MAQ. The average coverage of the whole genome was ~50-fold for SSAHA2 and ~49-fold for

MAQ. Furthermore, according to SSAHA2 (similar data was not readily available from MAQ), 43% of the genome had a coverage of at least 40-fold, 92% was covered by at least 10 reads, whereas <0.7% of genome was not covered by any read.

The reads obtained for AS-ATNMF-1 were mapped against the reference genome (AS-WTSI), and a list of mutations was generated. This list of mutations was then compared to similar lists obtained previously for the AS-SENS and AS-50SP clones. Thus, non-clone specific mutations were filtered out (see Materials and Methods, section 3.7.1), and as such a total of 214 mutations were identified, which consisted of 21 potential SNPs (Appendix III) and 193 potential indels or CNVs (Appendix IV). It has to be noted that the quality of the reads was not as high as obtained in previous studies (Hunt *et al*, 2010). This affected the quality of the mutation calls, particularly SNPs. As a consequence, no filtering of SNPs based on quality scores could be performed, with all SNPs needing to be treated as potentially genuine mutations pending verification by di-deoxy sequencing. In the case of small and large indels, the vast majority is still expected to be false positive calls (Hunt *et al*, 2010) and limited di-deoxy sequencing indeed appears to suggest so (see *False positives*).

A sub-set of twenty three mutations was chosen for validation by di-deoxy sequencing or Real-Time PCR analysis of AS-ATNMF-1. These included thirteen SNPs, nine indels and one CNV. The criteria used for selecting this sub-set of mutations was the potential biological role attributed to the proteins coded by the genes where mutations were present or, in the case of intergenic mutations, the role of genes immediately close (either upstream or downstream) to the identified mutations. In addition, since the list of mutations present in AS-ATNMF-1 was made by comparison with AS-SENS or AS-50SP, di-deoxy sequencing analysis was also carried out for the other clones of the AS lineage in order to trace back the origin of each mutation. In total, thirteen mutations (nine SNPs, three indels and one CNV) were confirmed by di-deoxy sequencing or Real-Time PCR (Appendices V and VI, highlighted in green) and are

described in detail in the following sub-sections. Of the rejected SNPs, three were actually proxies for large deletions (*False positives*).

Furthermore, two additional potential indels that were not verified in this study were considered as real mutations due to their size, location and evidence from other sequenced clones (see *Unverified high-confidence mutations*).

Finally, one previously known deletion was missed by our analysis. This was due to the use of AS-50SP clone, instead of AS-SENS for the identification of large indels, due to technical issues. This is described in detail below.

Thus, in total, fifteen mutations were considered genuine, of which thirteen were actually confirmed directly or indirectly by di-deoxy sequencing or Real-Time PCR. A summary of these mutations will be presented in the end of this section, in Figure 16.

It is important to note that the list of mutations for ATNMF-1 provided here is not yet final and more verification is pending, particularly for the remaining eight potential SNP calls (Appendix III, highlighted in orange).

VERIFICATION OF MUTATIONS

In total, twenty three mutations were analysed either by di-deoxy sequencing or Real-Time PCR. They consisted of thirteen SNPs, nine indels, and one CNV (Table 3). Each of these mutations and their potential biological functions are described in the following sub-sections. In total, thirteen mutations were confirmed and ten rejected as false positives. A graphic summary of the verified mutations across the AS lineage is presented in Figure 16.

For all mutations analysed here, the AS-MFATN-5 clone and the parasites AS-15MF36P showed the same genotype as its progenitor AS-15MF. Similarly, the uncloned parasites AS-ATN27P showed the same genotype as AS-ATN.

						AS-WTSI			AS-SENS			ATNMF-1						
	Chromosome	Type	Analysis	Start (indels only)	End (indels only)	Base	Read depth	Main Base	Relative coverage	Comparative coverage	Relative coverage	Base	Read Depth	SSAHA/MAQ Quality	Gene ID	Nearest gene	Aminoacid Substitution	Annotation
1	2	SNP	MAQ/SSAHA		217,047 C	C	56	C		0.2	0.3	A	13	62	PCHAS_020720		**V2697F**	deubiquitinating enzyme
2	6	SNP	MAQ/SSAHA		636,862 A	A	19	A		0.6	0.3	G	14	70		3-PCHAS_061710		seryl-tRNA synthetase
3	7	SNP	MAQ/SSAHA		994,546 G	G	67	G		0.4	0.7	A	34	99	PCHAS_072830		**S109N**	dihydrofolate reductase
4	11	SNP	MAQ/SSAHA		996,332 G	G	56	G		1.1	1.6	T	80	99	PCHAS_112780		**A173E**	aminoacid transporter
5	13	SNP	MAQ/SSAHA		805,658 A	A	31	A		0.1	0.1	T	3	15	PCHAS_132020		**K998***	conserved plasmodium protein
6	13	SNP	MAQ/SSAHA		805,659 A	A	32	A		0.1	0.1	T	3	15	PCHAS_132020		**K998L**	conserved plasmodium protein
7	13	SNP	MAQ/SSAHA		1,322,938 G	G	35	G		1.9	1.7	A	85	99	PCHAS_133430		**E738K**	26S proteasome subunit
8	14	SNP	SSAHA		936,945 T	T	13	T		0.2	0.1	G	4	20		5-PCHAS_142600		conserved plasmodium protein
9	14	SNP	MAQ/SSAHA		1,155,448 C	C	46	C		1.4	1.7	A	84	99	PCHAS_143160		**Y560D**	transferase, ARM, WD
10	7	SNP	MAQ/SAAHA		876,917 A	A	54	A		0.2	0.3	G	16	80		PCHAS_072420-3		conserved malaria protein
11	7	SNP	MAQ/SSAHA		876,919 C	C	54	C		0.2	0.3	A	17	85		PCHAS_072420-3		conserved malaria protein
12	12	SNP	SSAHA		566,547 A						0.3	G/A	14	27	PCHAS_121630			40S ribosomal protein S3A
13	13	SNP	MAQ		56,840 G	G/A	52	G		0.1	0.1	A	6	45	PCHAS_130140			HAD hydrolase, putative
1	**3**	Deletion	SSAHA	472,273	472,275									36/43	PCHAS_031370		**103I**	conserved Plasmodium protein
2	**13**	Deletion	SSAHA	56	60,867		25			0.0	0.0		0		PCHAS_130140			60 Kb deletion
3	**12**	CNV	SSAHA	1,382,067	1,462,751		43			2.0	1.9		96		PCHAS_123820			
4	**2**	indel	SSAHA	301,741	301,767		20			0.1	0.1		5		PCHAS_020900			ubiquitin carboxyl-terminal hydrolase
5	**3**	indel	SSAHA	523,111	523,127		21			0.2	0.1		3		PCHAS_031520			vacuolar protein-sorting protein VPS45
6	**3**	indel	SSAHA	524,946	524,964		26						5		PCHAS_031520			vacuolar protein-sorting protein VPS45
7	**7**	indel	SSAHA	103,522	103,536										PCHAS_070250			erythrocyte binding protein 1
8	**8**	indel	SSAHA	1,355,069	1,355,081											5'-PCHAS_083770		haloacid dehalogenase-like hydrolase
9	**9**	Deletion	SSAHA	919,771	919,771									2/3		5'-PCHAS_092710		26S proteasome regulatory complex subunit
10	**7**	Deletion	SSAHA	876,894	876,927											PCHAS_072420-3'		conserved malaria protein (34 bp deletion)

Table 3 – List of mutations identified by Solexa in AS-ATNMF-1 and verified by di-deoxy sequencing. False positives are highlighted in red. In green, genuine mutations. In blue, false negative confirmed by di-deoxy sequencing as present in AS-ATNMF-1.

Verified point mutations

Nine mutations are described below. Seven of them encode non-synonymous substitutions while the remaining two are positioned in intergenic regions.

a) G217,047T SNP on chr 2

A G to T substitution was found on position 217,047 of chr 2 by Solexa in AS-ATNMF-1 (Table 3). Di-deoxy sequencing confirmed this mutation was also present in AS-ATNMF-1's parental clone, AS-ATN. This mutation falls into the coding region of PCHAS_020720 gene, which codes for the pcUBP1 (coding for a deubiquitinating enzyme) enzyme. It was already known to be present in AS-ATN (Hunt *et al,* 2007) and probably appeared in AS-15CQ under CQ selection. It represents a non-synonymous mutation, on aminoacid 2697, where a valine was replaced by phenylalanine (V2697F). As previously described, this position is close to the protein's catalytic site and is likely to reduce the activity of the pcUBP1 enzyme (Hunt *et al,* 2007).

No additional mutations were found in the coding sequence of this gene in AS-ATNMF-1.

b) A636,862G on chr 6

An A to G substitution was identified in position 636,862 on chr 6 by Solexa in AS-ATNMF-1 (Table 3). Di-deoxy sequencing confirmed this mutation was also present in AS-ATN. It may have appeared in AS-ATN under ATN selection. This nucleotide substitution falls within an intergenic region near the 3'-end of PCHAS_061710 gene. PlasmoDB analysis indicated that this gene codes for a seryl t-RNA synthetase which mediates the attachment of a serine residue to a specific transfer RNA (t-RNA). Assuming the mutation is placed in what could be a regulatory region, it could induce changes in the expression level of

PCHAS_061710, thus possibly interfering with the synthesis of other proteins. However, at present there is insufficient data to interpret the role of this mutation, if any.

c) G994,546A on chr 7

A G to A substitution in position 994,546 on chr 7 was found by Solexa in AS-ATNMF-1 (Table 3). Di-deoxy sequencing confirmed the presence of this mutation in AS-ATNMF-1 and also in all preceding clones up to AS-PYR (*i.e*. only AS-SENS has wild-type allele). As described previously, this represents a known mutation, identified in AS-PYR after the selection of AS-SENS for pyrimethamine (PYR) resistance (Walliker *et al,* 1975). This nucleotide substitution falls within the coding region of the *pcdhfr* gene (dihydrofolate reductase, PCHAS_072830), coding for the substitution of a serine to an asparagine in residue 106 (S106N). This mutation is homologous to the S108N substitution observed in *P. falciparum*, and known as underlying PYR resistance (Wang *et al,* 1997) and is involved also with resistance to this drug in *P. chabaudi* (Walliker *et al,* 1975; Hayton *et al,* 2002).

d) G996,332T on chr 11

A G to T substitution in position 996,332 on chr 11 was found by Solexa in AS-ATNMF-1 (Table 3). Its presence first appears in AS-3CQ clone under CQ selection. It was therefore expected to be present in AS-ATNMF-1. This mutation falls in the coding region of the PCHAS_112780 gene (coding for a putative amino acid transporter, *pcaat1*), and results in the replacement of an alanine to a glutamic acid in aminoacid 173 (A173E). This mutation appears to be involved in CQ resistance in *P.chabaudi* (Modrzynska, 2010).

e) A805,658T on chr 13

An A to T substitution was identified by Solexa in position 805,658 on chr 13 in AS-ATNMF-1 (Table 3). The presence of this mutation was confirmed by di-deoxy sequencing and found to first appear in the AS lineage in AS-15CQ parasites. Di-deoxy sequencing analysis of the three clones derived from AS-15CQ revealed that this substitution is also present in AS-ATN, but not in AS-15MF or AS-30CQ.

This mutation falls in the coding region of the PCHAS_132020 gene (coding for a hypothetical protein), and the prediction of the protein sequence coded by this gene shows that this mutation causes the introduction of a STOP codon at position 998 (K998*). This will result in a truncated version of the protein, possibly affecting the intracellular processes where it is involved.

However, the function of this protein is unknown and its aminoacid sequence shows no similarity to any conserved domains. PROSITE only identified the presence of two asparagine-rich (ANS-rich) regions (aminoacids 301 to 371; and 1120 to 1335) and one lysine-rich (LYS-rich) region (aminoacids 538 to 593). In order to find more information, the DNA and protein sequences of the *P. falciparum* orthologue of PCHAS_132020 (PF14_0506) were retrieved from PlasmoDB. PF14_0506 gene is described as possibly belonging to a RNI-like superfamily. BLAST analysis of the protein coded by PF14_0506 also showed the presence of a synthase/transferase motif.

f) A805,659T on chr 13

An A to T substitution was also found at position 805,659 adjacent to that described in the previous section (A805,658T) on PCHAS_132020 gene (Table 3). This mutation appeared between AS-ATN and AS-ATNMF-1 and is predicted to 'rescue' the previous STOP mutation. It encodes a K998L mutation relative to the wild-type allele.

The population passaged twenty seven times in absence of drug, AS-ATN27P and the other four clones selected by drug pressure with AS-ATNMF-1 (AS-ATNMF-2, AS-ATNMF-3, AS-ATNMF-4 and AS-ATNMF-5) were also analysed by di-deoxy sequencing. AS-ATN27P carried the same allele as AS-ATN, as did two of the four AS-ATNMF clones (AS-ATNMF-4 and AS-ATNMF-5). Conversely, the other two AS-ATNMF clones (AS-ATNMF-2 and AS-ATNMF-3) also showed the additional base substitution detected in AS-ATNMF-1. This suggests that the second mutation (*998L) was only partially selected (by ATN + MF) in the uncloned line, and that therefore the function of this protein is not crucial to parasite survival in the untreated or drug-treated lines.

Further analyses are required in order to determine the importance of these mutations for ATN + MF resistance.

g) G1,322,938A on chr 13

A G to A substitution was identified at position 1,322,938 on chr 13 of AS-ATNMF-1 (Table 3). Di-deoxy sequencing showed that this mutation was also present in AS-ATNMF-1's progenitor, AS-ATN. This nucleotide substitution is located inside the coding region of PCHAS_133430 gene, which encodes a 26S proteasome subunit.

The translation of the mutated sequence shows that this substitution causes a non-synonymous mutation in aminoacid 738, where a glutamic acid is replaced by a lysine (E738K), thus a polar negatively charged aminoacid is replaced by a polar positively charged one.

By performing a BLAST search for the aminoacid sequence of this protein, it was possible to identify a region homologous to many conserved domains involved in protein transport and turnover, and also a proteasome/cyclosome repeat, which is described as an anaphase-promoting repeat, indicating the involvement of this protein with cell cycle regulation. The prediction of these domains in PCHAS_133430 protein sequence is consistent with the function

attributed to this gene. The mutation E738K falls into the putative proteasome/cyclosome repeat region and this could possibly have an effect on the activity of this protein. Its role in resistance to ATN is currently being studied.

h) T936,945G on chr 14

A T to G substitution was found in position 936,945 on chr 14 of AS-ATNMF-1 (Table 3). This mutation was first identified in AS-PYR and for that reason it was also expected to be present in AS-ATNMF-1, although it displayed poor read coverage. It falls in an intergenic region, close to the 5'-end of PCHAS_142600 gene, which has an unknown function. Analysis of PCHAS_142600 protein sequence by HMMTOP indicated the absence of putative transmembrane domains. BLAST indicated the presence of a conserved domain in the N-terminal region of the protein coded by this gene (aminoacids 4 to 125) whose function is still unknown. The relation between this intergenic mutation and PYR resistance is still to be determined.

i) G1,155,448T on chr 14

A G to T substitution at position 1,155,448 on chr 14, was found by Solexa in AS-ATNMF-1 (Table 3) and was confirmed by di-deoxy sequencing as being unique to this clone (arising between AS-ATN and AS-ATNMF-1). The parasite population AS-ATN27P carries the wild type allele of this gene. Interestingly, di-deoxy sequencing of the other four clones selected along with AS-ATNMF-1 revealed that none of them carry this mutation, suggesting that the selection on this mutation during passage under ATN + MF treatment was not very strong.

This nucleotide substitution is placed in the coding region of the PCHAS_143160 gene, and the predicted sequence of the mutated protein shows the substitution of an aspartic acid for a tyrosine, in position 560 (D560Y).

As indicated in PlasmoDB, the function of the protein coded by this gene is unknown. However, the analysis of its aminoacid sequence by BLAST reveals the presence of 3 putative conserved domains. One of them represents a Glycosyl transferase super-family A (GT-A) domain (aminoacids 20 to 274) which is involved in the synthesis of oligosaccharides, polysaccharides, and glycoconjugates. Another conserved domain identified by BLAST belongs to the Left-handed parallel beta-Helix (LbetaH) superfamily (aminoacids 387 to 462), and is usually present in enzymes showing acyltransferase activity, and in some cases being related to ion transport and translation initiation. The third one consists of a Glutaryl Co-A Dehydrogenase (GCD1) domain (aminoacids 266 to 455) which is present in enzymes involved in lipopolysaccharide biosynthesis, or in translation and ribosomal structure and biogenesis. In addition, PROSITE identified a W2 domain near the N-terminal of the protein (aminoacids 854 to 1022), and a glutamate-rich (GLU-rich) region (aminoacids 488 to 765) as well as a casein kinase II phosphorylation site (aminoacids 558 to 561). HMMTOP identified the presence of two putative transmembrane domains (aminoacids 448 to 456 and 894 to 911).

It is worth noticing that the D560Y mutation is located in the putative casein kinase II phosphorylation site contained inside the GLU-rich region, both identified by PROSITE. Nevertheless, further analysis still remains necessary to determine the significance of this mutation.

Verified indels/CNVs

Five high confidence indels/CNVs were identified by Solexa in AS-ATNMF-1. Three were analysed and confirmed either by di-deoxy sequencing or Real-Time PCR. Each confirmed mutation will be described below.

a) 3bp deletion on chr03

A 3 bp deletion from position 472,273 to 472,275 on chr 3 was identified by Solexa in AS-ATNMF-1 (Table 3). The presence of this mutation was confirmed by di-deoxy sequencing as first appearing in the AS lineage in AS-15CQ, and is also present in two of the three clones deriving from it, AS-ATN and AS-15MF, while being absent in the third one, AS-30CQ.

The deletion of this triplet (AAT) is placed inside the coding region of PCHAS_031370 gene. The predicted translation of the mutated protein indicated a deletion of an isoleucine in position 103 (I103).

PlasmoDB describes this gene as a conserved Plasmodium protein, and PlasmoDB and HMMTOP indicated the presence of twelve transmembrane domains, with small divergences about their precise locations. Additionally the analysis of its predicted translation by BLAST search identified a region homologous to a Paraquat Inducible protein A (PqiA) domain (aminoacids 52 to 210). This domain is present in proteins belonging to a family of integral membrane proteins and its expression is promoted by superoxide radical generating agents. It is worth noticing that the I103 deletion falls inside this predicted domain.

The *P. falciparum* orthologue of this protein (PFB0675w) is believed to target the membrane of the digestive vacuole (P. Moura and D. Fidock, pers. comm.). Additionally, another mutation (T707N) in the same gene occurs in AS-30CQ. This mutation is believed to confer high-level CQ resistance (Modrzynska, 2010).

However, the biological relevance of this mutation still remains to be assessed.

b) 60 Kb deletion on chr13

A 60 Kb deletion spanning from the very beginning of chr 13 to approximately position 60,867 was identified by Solexa in AS-ATNMF-1 (Table 3).

As described previously, indels were identified by comparing the coverage between AS-ATNMF-1 and AS-50SP, both sequenced by 50 bp paired-end reads. The comparative coverage between AS-ATNMF-1 and AS-50SP along chr 13 is shown in Figure 13, where an area of reduced coverage can be visualised as the peaks in the beginning of the chromosome using the Artemis sequence viewing tool. Along the rest of chr 13, a flat line is observed, indicating there is no significant difference between the comparative coverage of the two clones in this region.

Figure 13 – Schematic representation of comparative coverage between AS-ATNMF-1 and AS-50SP clones visualised on chr 13 using Artemis Software. Peaks in the beginning of the chromosome (forward and reverse strands are represented in grey) indicate a difference in comparative coverage between AS-ATNMF1 and AS-50SP. In this case the difference indicates drastically reduced coverage, while the flat line indicates no difference between the two clones. Yellow, pink and orange horizontal bars represent different genes along the chromosome.

According to SSAHA2 values, this deletion spans approximately 60Kb and includes approximately fifteen genes from PCHAS_130010 to PCHAS_130150, most of which represent members of a sub-telomeric gene family, the *P. chabaudi* interspersed repeats proteins or CIR proteins (Table 4).

GENE	DIRECTION	FUNCTION	CONSERVED PROTEIN DOMAINS	ORTHOLOGS
PCHAS_130010	W	phospholipase, putative		
PCHAS_130020	C	CIR protein	1 transmembrane domain	BIR protein, putative (P.berghei), putative yir1, yir2, yir3, and yir4 protein (P.yoelii)
PCHAS_130030	C	CIR protein	1 transmembrane domain	pc-FAM 4 protein (P.chabaudi)
PCHAS_130040	C	conserved P.chabaudi protein	1 transmembrane domain	
PCHAS_130050	C	CIR protein	1 transmembrane domain	pc-FAM 4 protein (P.chabaudi)
PCHAS_130060	C	pc-FAM 6 protein	1 transmembrane domain	interspersed repeat antigen, putative (P.falciparum), Drosophila melanogaster CG5228 gene product (P.yoelii)
PCHAS_130070	C	CIR protein	1 transmembrane domain	pc-FAM 4 protein (P.chabaudi)
PCHAS_130080	W	CIR protein	2 transmembrane domains	pc-FAM 4 protein (P.chabaudi), pc-FAM 6 protein (P.chabaudi)
PCHAS_130090	C	pc-FAM 6 protein	2 transmembrane domains	interspersed repeat antigen, putative (P.falciparum), Drosophila melanogaster CG5228 gene product (P.yoelii)
PCHAS_130100	W	pc-FAM 6 protein	2 transmembrane domains	pc-FAM 4 protein (P.chabaudi)
PCHAS_130110	C	pc-FAM 2 protein	2 transmembrane domains	CIR protein (P.chabaudi)
PCHAS_130120	W	pc-FAM 6 protein	2 transmembrane domains	pc-FAM 4 protein (P.chabaudi)
PCHAS_130130	W	pc-FAM 1 protein	2 signal peptides	
PCHAS_130140	C	HAD hydrolase, putative	2 signal peptides	
PCHAS_130150	W	pc-FAM 1 protein	2 signal peptides	

Table 4 – List of genes inside the 60 Kb deletion in the beginning of chr 13 identified in AS-ATNMF-1 by Solexa. This deletion is also present in other clones of AS lineage, and first appeared in AS-15CQ.

In order to obtain indirect evidence of this deletion, oligonucleotide primers were designed for PCR amplification of a fragment of PCHAS_130140 gene, which is located inside of the deleted region. A PCR product will be amplified unless the clone under analysis carries a deletion of this region. In this case no product will be seen.

All clones belonging to the AS lineage were investigated. Under the same conditions, some samples presented a single band (approximately 500 bp), while other samples presented two bands (the previous 500 bp and another one of 400 bp) (Figure 14A and 14B). These results were reproducible and each band obtained for each clone was analysed by di-deoxy sequencing separately.

A careful analysis of the sequences obtained for the two different bands was made by performing a BLAST search against *P.chabaudi* genome. This analysis revealed that each band corresponded to a different region on the parasite's genome. The smaller band (~400 bp) represented the predicted fragment of the target gene PCHAS_130140, which is present on chr 13 and codes for a putative Haloacid Dehalogenase (HAD) hydrolase. On the other hand, the larger band (~500 bp) represented a fragment of a different gene (PCHAS_144300), present on chr 14. PCHAS_144300 codes for a putative Hydrolase/phosphatase protein, which belongs to the same superfamily as that encoded by PCHAS_130140.

Therefore, whenever a clone carried both genes, two regions would be amplified by PCR and two bands were observed. On the other hand, clones bearing the deleted fragment on chr 13 had only one region amplified (on chr 14) and therefore, only a single band would be seen.

As shown in Figure 14, the deletion seems to be present in AS-ATNMF-1, and seems to have first appeared in the AS lineage in AS-15CQ parasites. The deletion also seems to be present in AS-ATN. Interestingly, the other two clones derived from AS-15CQ, AS-30CQ and AS-15MF carry two bands, i.e. do not have a deletion at the beginning of chr 13.

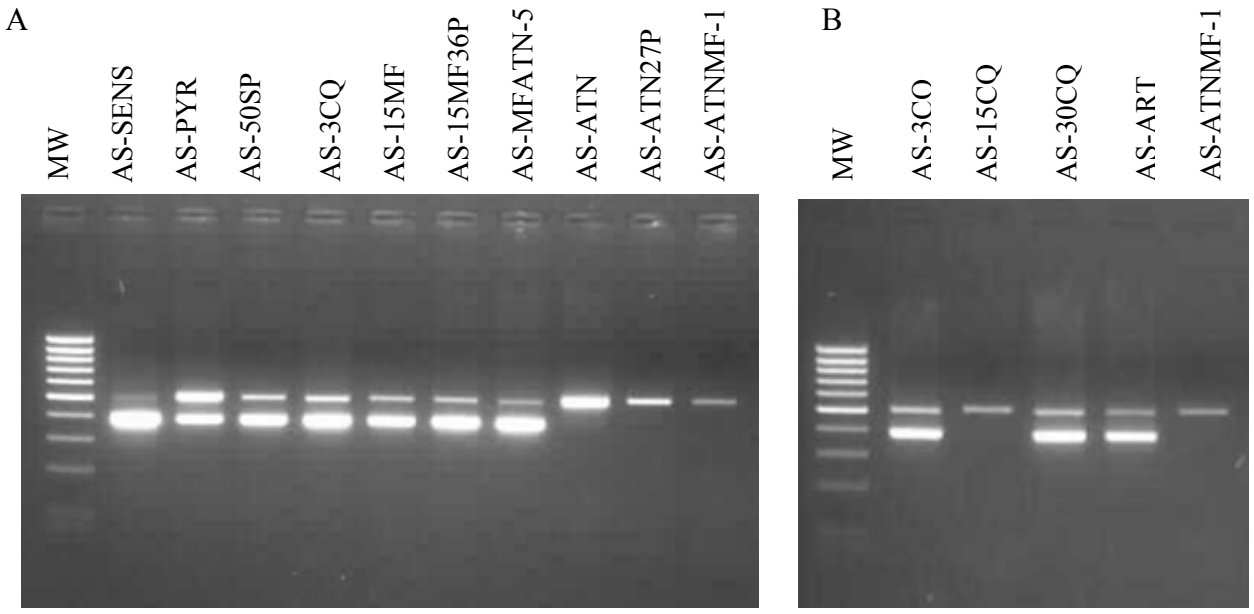


Figure 14 – (A) and (B) PCR products resolved in agarose gel. It is proposed that wild-type clones show two bands, whereas clones bearing the 60 Kb deletion in chr 13 only show one band.

c) 80 Kb amplification on chr12

An 80 Kb segment spanning from position 1,382,067 to 1,462,751 on chr 12 seemed to be amplified in AS-ATNMF-1 (Table 3).

By using a similar analysis as the one performed for the identification of differences in comparative coverage along chr 13 described in the previous section, it was possible to visualise the comparative coverage between AS-ATNMF-1 with AS-50SP along chr 12 (Figure 15). In this case, however, the peaks correspond to an increase in coverage along a region spanning over approximately 80 Kb in AS-ATNMF-1.

When analysing this region in more detail, it could be verified that it encompasses about twenty three genes, from PCHAS_123760 to PCHAS_123980 (Table 5), inclusive. Most of these genes code for proteins of unknown function, with a few exceptions. The full list of the genes and their proposed functions is presented on Table 5.

GENE	DIRECTION	FUNCTION	CONSERVED PROTEIN DOMAINS	ORTHOLOGS
PCHAS_123760	W	conserved plasmodium protein		
PCHAS_123770	W	mitochondrial ribosomal L17 precursos, putative	signal peptides	
PCHAS_123780	W	conserved plasmodium protein	8 transmembrane domains, signal peptide	
PCHAS_123790	W	iron sulphur assembly protein, putative	1 trasnmembrane domain, signal peptide	
PCHAS_123800	C	G10 protein, putative		
PCHAS_123810	W	conserved plasmodium protein	zinc finger domain (P.falciparum)	erythrocyte membrane protein PFEMP3 (P.yoelii)
PCHAS_123820	W	**multidrug resistance protein, putative**	11 transmembrane domains	
PCHAS_123830	C	mitochondrial processing peptidase alpha subunit, putative		
PCHAS_123840	W	conserved plasmodium protein		
PCHAS_123850	C	conserved plasmodium protein		
PCHAS_123860	W	DNAJ protein, putative	2 tranmembrane domains	
PCHAS_123870	C	conserved plasmodium protein		outer arm dynein lc3,putative (P.falciparum)
PCHAS_123880	W	conserved plasmodium protein		
PCHAS_123890	C	conserved plasmodium protein		
PCHAS_123900	W	transporter, putative	12 transmembrane domains	metal ion transporter (P.vivax)
PCHAS_123910	C	conserved plasmodium protein	2 tranmembrane domains	
PCHAS_123920	W	karyopherin beta, putative		
PCHAS_123930	W	conserved plasmodium protein		
PCHAS_123940	C	conserved plasmodium protein	4 transmembrane domains	
PCHAS_123950	C	conserved plasmodium protein		
PCHAS_123960	C	cytosolic preribosomal GTP-binding protein,putative		
PCHAS_123970	W	conserved plasmodium protein		
PCHAS_123980	W	organelle ribosomal L7/L12 precursor, putative	1 transmembrane domain	mitochondrial ribosomal L12 precursor (P.falciparum, P.chabaudi, P.berghei)

Table 5 – List of genes contained in the 80 Kb region that was amplified on chr 12, in AS-ATNMF-1. The amplification was detected by Solexa, and indirect confirmation is given by the results obtained by Real Time PCR, indicating that the clone AS-ATNMF-1 has two copies of gene MDR1 (PCHAS_123820 – highlighted in bold).

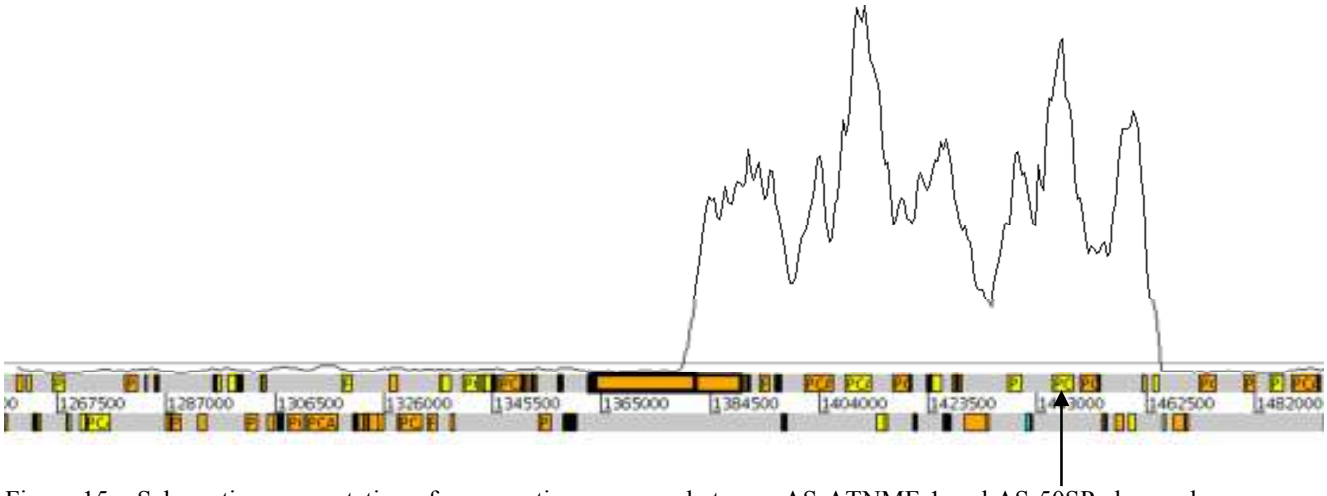


Figure 15 – Schematic representation of comparative coverage between AS-ATNMF-1 and AS-50SP clones along chr 12 visualised using Artemis Software. Peaks indicate a difference in comparative coverage between AS-ATNMF1 and AS-50SP, in this case, an increase in coverage on a region spanning over approximately 80 Kb. Yellow and orange horizontal bars represent different genes along the chromosome. Black arrow indicates gene PCHAS_123820, the *pcmdr1* gene (yellow bar).

One gene present in this region is PCHAS_123820, coding for *pcmdr1* coding for the MDR1 ABC transporter. Real-Time PCR analysis carried out in this work confirmed that this gene is duplicated in AS-ATNMF-1, whereas its parental AS-ATN only carries one copy (Section II.2.2.1, *pcmdr1* – copy number variation). This provides an indication that this whole segment of chr 12 has been amplified resulting in the duplication of gene *pcmdr1* along with many other genes. Due to technical constraints, the other four AS-ATNMF clones were not inspected for the presence of the *mdr1* duplication.

False positives

Ten out of the twenty three mutations analysed in this study were false positives (four SNPs and six indels) (Table 3, highlighted in red). In one case, SSAHA2 identified position 566,547 on chr 12 (gene PCHAS_121630, coding for a 40S ribosomal S3A) as heterozygous G/A (Table 3). However, di-deoxy sequencing indicated AS-ATNMF1 as showing an A in this position, i.e. same as the AS-WTSI reference. Three false positive SNPs were actually found to

act as “proxies” for deletions. These were: an A to G substitution in position 876,917 and a C to A substitution in position 876,919 both on chr 7, representing proxies for the 34 bp deletion previously known to be present in this region (see *False negatives*, below) and a G to A substitution in position 56,840 on chr 13, also a proxy for a large deletion event, the 60 Kb deletion described above.

False negatives

A previously known 34bp deletion from position 876894 to 876927 on chr 7 was not detected in AS-ATNMF-1 by Solexa (Table 3, highlighted in blue). This was due to the use of AS-50SP as a filter for the identification of indels. This clone also carries the same deletion on chr 7. Instead, two SNPs very close to each other were identified (A to G substitution in position 876,917 and a C to A substitution in position 876,919), presumed to be deletion ‘proxies’. It is worth noting that SNP identification was made using AS-SENS as filter, instead of AS-50SP, and for that reason, these mutations were identified.

Di-deoxy sequencing analysis was carried out in order to verify the presence of this deletion in AS-ATNMF-1. The alignments of the fragments obtained for AS-ATNMF-1 were compared with AS-50SP, AS-PYR, and AS-SENS, showing that of these four clones, three carry the deletion, whilst AS-SENS shows the wild type allele (Appendix V).

This mutation appeared after the exposure of AS-SENS to one dose of PYR (Walliker *et al,* 1975), generating the PYR-resistant clone, AS-PYR. It is thus expected to be present in all clones selected from AS-PYR. Indeed this mutation is present in AS-50SP (originated directly from AS-PYR) and also in all the other clones of the AS lineage, including AS-ATNMF-1’s parental, AS-ATN.

The presence of this false negative raises the issues of other potential indels having been overlooked due to the use of AS-50SP as a filter. However, as referred above, the extensive

knowledge of the other clones belonging to the AS lineage minimizes the probability of other false negatives.

UNVERIFIED HIGH-CONFIDENCE MUTATIONS

Two high confidence indels (Appendix IV, highlighted in yellow) identified by the analysis carried out here could not be verified: i) a ~2.4 Kb deletion predicted between positions 682,682 to 685,106 on chr 5; and ii) a ~30 Kb deletion predicted between position 305,928 and 339,914 of an artificial chromosome made up of unassigned contigs ("bin") that seems to involve part of contig 11844.

Although no di-deoxy sequencing analysis was carried out for confirming the presence of these two mutations in AS-ATNMF-1, there is strong evidence indicating they consist of true mutations. When performing coverage analysis it is possible to note that large deletions leave a strong signature. In addition, the deletion on chr 5 was identified as present in other clones of the AS lineage in previous work (Hunt *et al*, 2010). The large deletion on contig 11844, on the other hand, may be a continuation of the sub-telomeric 60 kb deletion on chr13, since contig 11844 contains many features associated with subtelomeric and telomeric chromosomal regions.

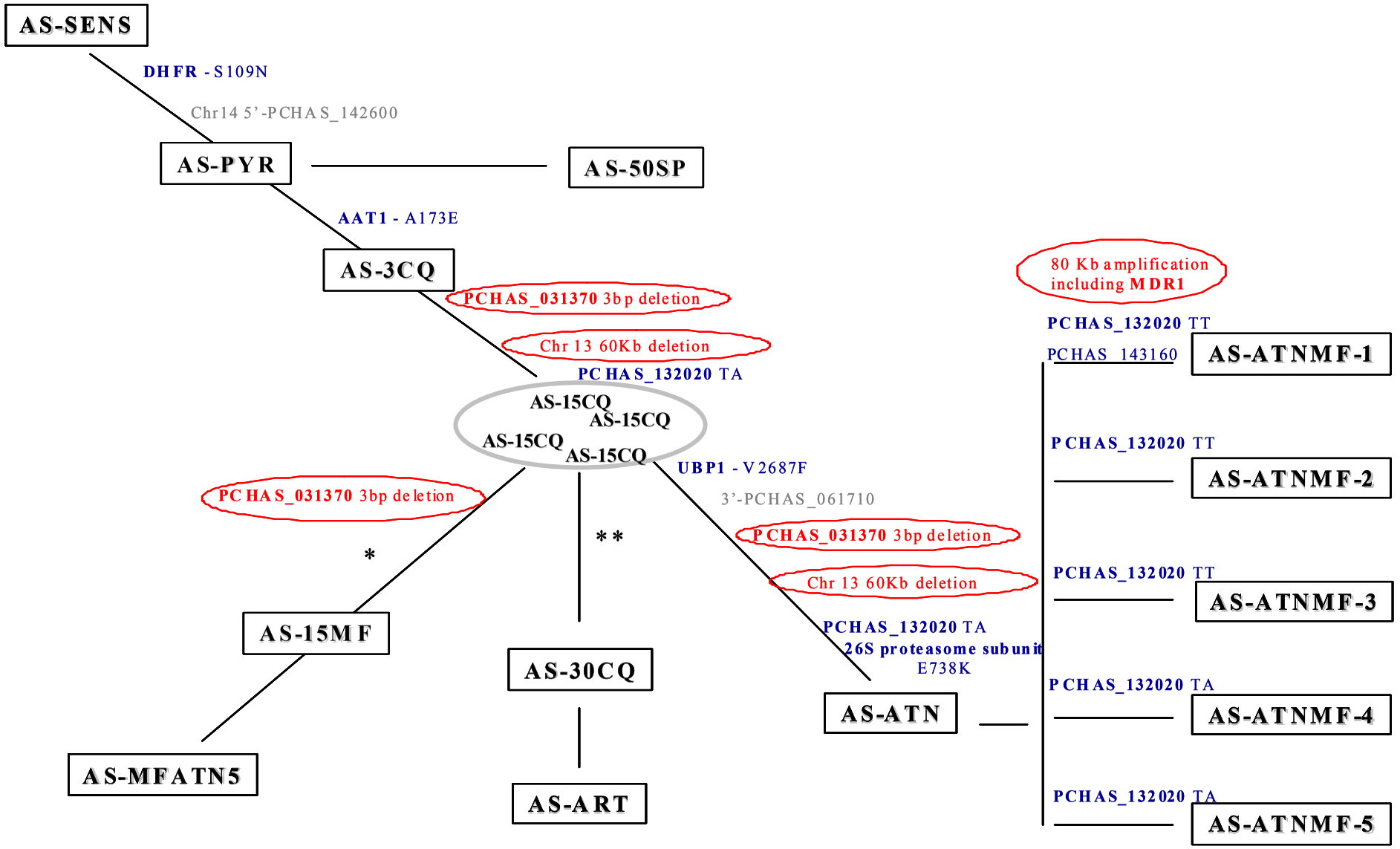


Figure 16 – Summary of the mutations identified by Solexa and verified by di-deoxy sequencing as present in AS-ATNMF-1. Mutations are indicated as they first appeared in the AS lineage. The three mutations first appearing in AS-15CQ are present in AS-ATN, deriving from it. On the other hand, the other clones originated from AS-15CQ are different from their parental: * AS-15MF only bears the 3bp deletion on Chr3 (PCAS_031370), and (**) AS-30CQ shows none of them. Non-coding mutations are shown in gray; SNPs are shown in blue and indels are represented in red.

SUMMARY

By using Solexa whole genome sequencing of AS-ATNMF-1 a total of 214 mutations were identified. Further analysis indicated twenty five mutations (nine SNPs, five indels and one CNV) which were confirmed to be genuine.

Ten of these mutations were detected for the first time in this study and will be grouped below according to the clone where they were selected:

a) three mutations were identified as first appearing in AS-15CQ: i) a 3bp deletion on chr 3, on PCHAS_031370 gene; ii) a A805,658T substitution on chr 13, on PCHAS_132020 gene, which causes the introduction of a STOP codon in position 998 of its predicted protein sequence; iii) a 60 Kb deletion of the initial segment of chr 13, which may also include the 30 Kb deletion identified in contig 11844.

All three mutations identified in AS-15CQ are present in AS-ATN and, on the other hand, none of them were observed in AS-30CQ. Only the 3bp deletion on chr 3, on PCHAS_031370 gene is present in AS-15MF;

b) two previously unknown mutations appeared in AS-ATN: i) an A636,862G substitution near the 3'end of PCHAS_061710 gene, on chr 6; ii) a non-synonymous substitution E738K on PCHAS_133430 gene (chr 13) coding for the 26S proteasome subunit.

c) the following mutations appeared in various clones of the AS-ATNMF population after ATN +MF selection: i) an 80 Kb amplification on chr 12, including *pcmdr1* among other genes, in conformity with the results of the previous section, where the duplication of *pcmdr1* gene was identified by Real-Time PCR analysis. The other four AS-ATNMF clones were not inspected for the duplication of *mdr1* gene; ii) a K998L substitution on PCHAS_132020 gene (chr 13), replacing the STOP codon present in the progenitor AS-ATN clone (originally found in AS-15CQ) for a leucine. This mutation was also observed in AS-ATNMF-2, and -3, but not in AS-ATNMF-4 and -5, the other clones selected along with AS-ATNMF-1; and iii) a D560Y

substitution on PCHAS_143160, on chr 14, which is found exclusively in AS-ATNMF-1 but none of the other AS-ATNMF clones.

The last novel mutation identified in this work is the ~2.5 Kb deletion on chr 5. The presence of this mutation could not be confirmed by di-deoxy sequencing, and as consequence, its origin in the AS lineage remains unknown.

It is important to highlight that the list of mutations discovered in AS-ATNMF-1 presented here may not be complete, since only a sub-set of all mutations identified was confirmed or rejected by di-deoxy sequencing or Real-Time PCR.

SECTION SUMMARY

The ATN + MF-resistant AS-MFATN-5 and AS-ATNMF-1 clones were analysed in order to identify the genetic mutations selected by consecutive rounds of treatment with ATN or ATN + MF, respectively.

AS-MFATN-5 showed a further amplification of *pcmdr1* gene (three copies) when compared to its progenitor AS-15MF, previously known as carrying two copies of this gene. In addition, consecutive sub-inoculations of AS-15MF clone into mice seems to have caused the loss of one of its two *pcmdr1* copies, as AS-15MF36P harbours a single copy of *pcmdr1*.

Similarly, when compared to its parental AS-ATN, AS-ATNMF-1 has also undergone an amplification of the *pcmdr1* gene, and carries two copies of this gene. Gene amplification was followed by an increase in RNA and protein expression. However, protein levels were disproportionally higher (about six-fold) when comparing to gene (two-fold) and RNA transcripts (two-to-three-fold).

Other mutations were found in AS-ATNMF-1 through whole-genome sequencing, and were located within genes PCHAS_132020, on chr 13, and PCHAS_143160, on chr 14. However, the function of these genes is still unknown.

Together, these results obtained for AS-MFATN-5 and AS-ATNMF-1 indicate that *pcmdr1* seems to have a central role in ATN + MF resistance. However, the presence of other mutations appearing in AS-ATNMF-1 after selection with ATN + MF suggest that *pcmdr1* may not be the sole determinant of this phenotype.

II.3. DISCUSSION

The results obtained in the previous sections of this Chapter will be discussed below. In order to facilitate comprehension the different implications of the results obtained here will be divided as follows. First, the implications of emergence of parasites which are resistant to artesunate and mefloquine (ATN + MF) administered in combination will be discussed under the perspective of the advantages and pitfalls of the administration of Artemisinin Combination Therapy as antimalarial treatment.

Secondly the genetic changes accompanying the evolution of resistance to these drugs in our model will be discussed. This section will be subdivided as to first present the implications of acquisition of an extra copy of *mdr1* gene in both ATN + MF-resistant clones selected here. Then, overexpression of MDR1 and the possible interaction with other genes (mutated or wild-type) will be debated, as well as the novel mutations identified in AS-ATNMF-1.

Finally, other results obtained in this work will also be discussed, such as the implications of mutations disclosed by whole-genome sequencing of AS-ATNMF-1, which were identified as arising prior to AS-ATNMF-1 selection in the chloroquine-resistant AS-15CQ and artesunate-resistant AS-ATN.

II.3.1. ARTESUNATE + MEFLOQUINE COMBINATION DOES NOT PREVENT EVOLUTION OF RESISTANCE

At present, widespread drug resistance represents the greatest set-back in the control of malaria cases in endemic areas. Indeed, the last standing force against resistant parasites are the Artemisinin Combination Therapies, which consist of the administration of an artemisinin (ART) derivative in combination with a chemically unrelated antimalarial, such as mefloquine (MF). At the time of its implementation, it was believed that administration of combination therapy would prevent the evolution of resistance to ART derivatives by malaria parasites. However, cases of clinical failure after treatment with ART compounds have already been reported, particularly in South-East Asia, where the combination of artesunate (ATN) and MF has been used for the past 15 years (Wongsrichanalai and Meshnick, 2008).

In this context, the main goal of this work was to generate parasites which were resistant to the artesunate + mefloquine (ATN + MF) combination, aiming at: i) providing experimental evidence that it is biologically possible for a parasite to evolve resistance to ATN + MF and ii) identifying genetic mutations associated with the resistant phenotype evolved by these parasites, before resistance becomes established in natural *P. falciparum* populations. In order to meet these goals, parasite clones initially resistant to one of the two drugs of the combination were exposed to sub-therapeutic drug treatment as follows: i) a MF-resistant *Plasmodium chabaudi* clone was selected with ATN alone and ii) a *P. chabaudi* clone displaying moderate ATN resistance was exposed to ATN + MF combination. Resistance to the combination of the two drugs was successfully selected in both cases.

One important question is whether selection for ATN + MF-resistance could have been attempted by using fully sensitive parasites as starting material. This can be argued for, as the use of the drug sensitive AS-SENS clone would allow inferring the importance of newly arising mutations and their immediate association with resistance to these two drugs administered

simultaneously, without the confounding background of the various mutations accumulated along time due to repeated passaging through mice or exposure to other drugs, such as pyrimethamine and chloroquine (CQ). However, the main goal of this work was not only to identify the genetic mutations conferring drug resistance, but also to create a model that could help understand the mechanisms of ACT resistance as they would appear in the field. As such, the first reason for not choosing AS-SENS as stating material is that currently fully sensitive parasites simply do not exist in natural populations. In most endemic areas, resistance to CQ is at near-fixation level. In addition, MF-resistant parasites are also widespread, as is the case for instance in Western Thailand where MF monotherapy was used for almost 10 years, contributing for the emergence of parasites with high levels of resistance to this drug (Carrara *et al,* 2006). Also, ATN monotherapy has been used for almost 30 years in the Thai-Cambodian region. This is regarded as being the most important factor contributing for the evolution of tolerance to ART derivatives by *P. falciparum* parasites in this area (Dondorp *et al,* 2009). Use of ACTs for treatment of malaria cases has reached very high cure rates (Nosten *et al,* 2000). However, parasites with previous ATN-resistant or MF-resistant background showed a delayed clearance time after ATN + MF combination (Alker *et al,* 2007; Carrara *et al,* 2009; Dondorp *et al,* 2009; Noedl *et al,* 2009), indicating ACT's efficacy may be waning. Since South-East Asia is historically the cradle of resistance to most antimalarial drugs, it is particularly interesting to try and model the context of the natural parasite populations of this region.

For the above reasons, the use of the AS-15MF and AS-ATN clones of the *P. chabaudi* AS lineage for the selection of ATN + MF resistance was an adequate choice.

The successful selection for ATN + MF resistance shows that even the highly efficacious drug artesunate (ATN), when administered in sub-optimal doses may induce resistance (as observed for the selection of AS-MFATN-5). Additionally, in case of the administration of ATN + MF combination, the quick action of ATN combined with the long-term action of MF is

not enough to prevent the appearance of resistant parasites if the two drugs are present in sub-therapeutic levels (as observed for the selection of AS-ATNMF-1). These results are therefore relevant for the application of ACTs for treatment of malaria cases. Indeed, although health authorities of each endemic country may apply rigid control over the indiscriminate use of ACTs, self-medication is still a reality, as well as counterfeit drugs which contain lower doses of one or both of ACT components can also easily be found. As such, parasites would be exposed to sub-therapeutic drug doses in the host's bloodstream increasing the chances of selection of resistance.

One of the premises determined by WHO for malaria treatment is the administration of combination therapies involving chemically unrelated drugs as partners of ART derivatives. ATN and MF are chemically unrelated and supposedly have two distinct modes of action. This would be one of the supposed advantages of the administration of the combination of these two drugs. It was postulated that the number of asexual parasite forms present in a single infection would not be sufficient to allow enough replication cycles for the appearance of parasites harbouring mutations in genes involved in resistance to both drugs (White, 1999). However, the evolution of ATN + MF resistance described here may indicate that the mutation rates of the parasite have been underestimated, and the appearance of mutations in different genes may be more frequent than expected. Moreover, both parasites used as starting material in this work already showed a multiple-drug resistant phenotype (AS-15MF is resistant to MF, chloroquine (CQ) and pyrimethamine (PYR), and AS-ATN is resistant to ATN, CQ and PYR). Therefore, there is a possibility that the progenitor clones used here may have evolved the Accelerated Resistance to Multiple Drugs (ARMD) phenotype (Rathod *et al,* 1997) along the course of selection throughout the AS lineage, and this could facilitate the evolution of ATN + MF-resistance as shown here. Other possiblility is that, pre-existing resistance to one of the partner drugs may have facilitated the evolution of resistance to the combination, as a parasite exposed

to ATN + MF must only acquire mutation(s) in genes modulating sensitivity to the other partner drug, and not to both simultaneously, as postulated previously (White, 1999).

ACTs are the last standing force against multi-drug resistant malaria and this work provides evidence that resistance may evolve in parasites with different genetic backgrounds of resistance to one of the two drugs of the combination when exposed to sub-therapeutic doses of drug treatment.

II.3.2. GENETICS OF RESISTANCE TO ARTESUNATE + MEFLOQUINE

Many genes have been previously proposed as involved in resistance to artesunate (ATN) and mefloquine (MF) separately. However, no definitive intracellular target has been identified to these drugs so far. In addition, the genetic mechanisms behind the delayed clearance time after ATN + MF treatment displayed by tolerant parasites have not been identified.

Genetic analysis targeting the previously proposed ATN resistance *pfatpase6* and *pcubp1* genes revealed that, independent of genetic background borne by progenitor clones, no mutations have followed the acquisition of resistance to ATN + MF.

Mutations in the *pfatpase6* gene have been implicated in ART resistance in artificial models: i) in transfected Xenopus oocytes (Eckstein-Ludwig *et al,* 2003; Uhlemann *et al,* 2005); or ii) in transfected *P. falciparum* cultured strains (Valderramos *et al,* 2010). In *P. falciparum* natural populations however, apart from one work (Jambou *et al,* 2005) no mutations in this gene have been further reported as implicated in ART increased tolerance (Dahlstrom *et al,* 2008; Jambou *et al,* 2010).

The *pcubp1* gene was also investigated. However, the genotype identified corresponded to the same as the previously borne by the respective progenitor in case of both ATN + MF resistant clones. Mutations in this gene were first identified in *P. chabaudi* clones selected for ATN and ART resistance (Hunt *et al,* 2007), but no correlation between mutations in *pcubp1*

gene and resistance to artemisinin derivatives have has been reported in the field (Imwong *et al,* 2010).

II.3.2.1. MDR1 AMPLIFICATION

Amplification of *mdr1* gene seems to play a determinant role in resistance to ART derivatives or MF, as was suggested by many studies using laboratory adapted strains as well as parasites from natural populations from different geographic endemic regions. In these studies, *mdr1* amplification is associated with increased *in vitro* resistance or delayed parasite clearance after treatment with ATN and MF either alone or in combination (Price *et al,* 1997; Price *et al,* 1999; Pickard *et al,* 2003; Price *et al,* 2004; Alker *et al,* 2007; Wongsrichanalai and Meshnick, 2008; Lim *et al,* 2009; Rogers *et al,* 2009; Chaijaroenkul *et al,* 2010; Chavchich *et al,* 2010). Other studies indicate the locus containing *mdr1* gene as being under strong selection. These studies attribute this selective pressure to the continued use of ACTs (Nair *et al,* 2007; Vinayak *et al,* 2010).

The two different clones selected for ATN + MF resistance, AS-MFATN-5 and AS-ATNMF-1, harbour a further amplification of *pcmdr1* gene. This provides indication that in our model, amplification of this gene also plays a central role in resistance to the combination of these drugs.

Additional evidence comes from the AS-15MF clone which was repeatedly sub-inoculated into untreated mice for thirty six weeks. These parasites, referred to as AS-15MF36P, seemed to be more tolerant to ATN and MF (either administered alone or in combination) than the original AS-15MF clone. Interestingly, *pcmdr1* gene copy number was decreased in AS-15MF36P parasites (a single copy instead of the two copies harboured by the original AS-15MF clone). Collectively, these results seem to indicate that prolonged *in vivo* passaging in the absence of drugs may lead to a deamplification of *pcmdr1* copy number and, as a consequence

a decrease in the parasite's tolerance to ATN and MF either administered alone or in combination.

The instability of the *mdr1* gene's amplification in the absence of drug pressure (Barnes *et al,* 1992; Chen *et al,* 2010) and the ability of malaria parasites to amplify it presence of drugs (Price *et al,* 1997; Cravo *et al,* 2003; Price *et al,* 2004; Alker *et al,* 2007; Lim *et al,* 2009; Rogers *et al,* 2009; Chavchich *et al,* 2010) was already observed in previous studies.

In addition, great variation has been identified in the size of amplified fragments involving *mdr1* gene and resistance to drug treatment. The size of the amplified region in AS-ATNMF-1 clone selected throughout in this work is of approximately 80 Kb, whereas size of the amplicon carried by AS-15MF is of 392 Kb (Borges, 2009). Chavchich and colleagues have observed an amplification of an approximately 100 Kb locus containing the *mdr1* gene, following prolonged *P. falciparum* culturing in presence of the ART derivative artelinin acid (Chavchich *et al,* 2010). In field samples, the presence of amplicons containing *mdr1* were found to vary between 15 to 49 Kb, containing two to eleven genes (including *mdr1*) (Nair *et al,* 2007). Other study has indicated the presence of a locus spanning over more than 100 Kb (Chaiyaroj *et al,* 1999).

Some studies indicate that amplifications occur at high rates, occurring once in 10^8 parasites in laboratory strains. This rate falls to 1 in 1000 parasites in case of an increase from two to three copies of the same region (Preechapornkul *et al,* 2009).

In our model, amplification of *pcmdr1* gene was observed in three distinct independent events: i) AS-15MF; ii) AS-ATNMF-1; and iii) AS-MFATN-5. AS-15MF was selected by consecutive sub-inoculation of approximately 10^6 parasites in each host mouse and drug was administered 3 hours later (Cravo *et al,* 2003). The other two clones were inoculated at a higher inoculum (10^7 parasites were inoculated in each mouse). Mice were treated three days after receiving the inoculum, which allowed parasite replication and increased the parasite biomass exposed to drug treatment. In all cases, enough parasites were exposed to drug pressure in order

to allow the spontaneous appearance of parasites harbouring an amplified locus containing *pcmdr1* and their subsequent selection by drug pressure.

The MF-resistant AS-15MF harbours two copies of *mdr1* gene and shows some level of resistance to ART, without ever being exposed to this drug (Borges, 2009). It could be observed here that AS-15MF actually shows increased tolerance to ATN when compared to AS-ATN clone, which only carries a single copy of *mdr1* gene. This could imply a role of *mdr1* amplification in increased resistance to ATN administered alone.

In accordance with this idea, AS-ATNMF-1, which has gained an extra copy of *mdr1* gene, shows a slight increase in its level of tolerance to ATN than its progenitor AS-ATN. On the other hand, AS-ATNMF-1 parasites are also more resistant to the ATN + MF combination and to MF administered alone when compared to AS-ATN and AS-15MF. In AS-15MF the presence of two copies of this gene is not enough to confer resistance to the combination of the two drugs, only some degree of resistance to ATN and MF separately. This may indicate that acquisition of the second copy in AS-ATNMF-1 may not be the only factor conferring resistance to the ATN + MF combination.

Repetitive treatment of AS-15MF with ATN has selected parasites showing ATN + MF resistance and an additional copy of *pcmdr1*. In *P. falciparum* cultured parasites selection with ART derivatives have originated parasites with increased *mdr1* copy number (Chavchich *et al,* 2010). However, in our model, previous selection with ART and ATN had not resulted in parasites harbouring an extra copy of *pcmdr1 (*Afonso *et al, 2006)*. The acquisition of an extra *pcmdr1* copy in AS-MFATN-5 was followed by an increase in the levels of tolerance to the combination but also to each drug administered separately. On the other hand, loss of an *mdr1* copy in AS-15MF36P has led to increased sensitivity to ATN + MF either alone or in combination. This indicates that in a MF-resistant background, drug pressure may dictate the status of *mdr1* gene (either amplified or not) and that in this case, the acquisition of an extra

copy is enough to render these parasites resistant to the combination as well as to the two drugs administered alone.

In our model, amplification of *pcmdr1* gene may play the most important role in resistance to ATN + MF combination. These findings raise an important issue. Although ATN and MF are chemically unrelated and have apparent distinct physiologic modes of action, the evolution of a generic mechanism of defence against drugs by the parasite, such as amplification of *mdr1* gene might render ACTs treatment ineffective.

II.3.2.2. MDR1 OVEREXPRESSION AND ITS POSSIBLE INTERACTION WITH OTHER MUTATIONS

Mdr1 gene codes for an efflux pump present in the parasite's food vacuole (Cowman, 1991; Peel, 2001; Duraisingh and Cowman, 2005) and in this case, may be responsible for keeping ATN and MF from reaching their respective intracellular targets, through overexpression. That mechanism has been widely described in cancer cells, where P-gp1 (the human homologue of MDR1) has been shown to mediate the extrusion of chemically unrelated drugs. Amplification and/or overexpression of human *mdr1* gene in cancer cells have been reported to be responsible for high levels of resistance to multiple drugs used in cancer chemotherapy (Baker and El-Osta, 2004; Perez-Sayans *et al,* 2010).

As mentioned above, AS-ATNMF-1 has a duplication of a segment of approximately 80 Kb on chr 12, containing the locus of *pcmdr1* gene (along with other twenty three genes). In addition, the amplification of *pcmdr1* gene in AS-ATNMF-1 is followed by an increased expression at RNA (two-to-three-fold increase) and protein (about five-to-six-fold increase) levels. This disproportional increase in RNA and MDR1 protein levels may reflect some particular transcriptional and/or posttranscriptional regulation of this gene.

Treatment with ATN can induce an increase in *mdr1* transcription in *P. falciparum* cultured strains (Natalang *et al,* 2008), indicating the existence of a regulatory pathway that can alter transcription of this gene upon stimulation. In addition, other study also indicated that amplification of a locus containing *mdr1* gene and other thirteen genes was shown to influence the level of 269 transcripts (Gonzales *et al,* 2008). As such, the amplification of *pcmdr1* gene in association with the other genes in the amplified region in AS-ATNMF-1 may be followed by some type of constitutive upregulation of transcription of *pcmdr1* and other genes throughout the genome.

It was demonstrated in cancer cells that P-gp1 (coded by *mdr1* gene in humans) is targeted to degradation by the proteasome and that increased ubiquitination results in reduction of P-gp1 function (Zhang *et al,* 2004). As mentioned previously, the AS-ATN clone harbours a V2697F mutation in *pcubp1* gene, which is believed to affect the function of this enzyme, reducing de-ubiquitination of different proteins, which would probably increase protein degradation via the 26S proteasome (Hunt *et al,* 2007). AS-ATNMF-1 has inherited this mutation in *pcubp1* gene and in case protein turnover in Plasmodium presents the same mechanisms as described for cancer cells, it would be expected that reduced function of *pcubp1* product would cause an increase in MDR1 degradation, therefore, being responsible for a reduction of MDR1 expression. The almost six-fold increased MDR1 expression following gene duplication observed in AS-ATNMF-1 suggests that MDR1 expression may be regulated by an alternative pathway.

In parallel with the selection of AS-ATNMF-1 by ATN + MF drug pressure, the AS-ATN clone was consecutively sub-inoculated into mice for twenty seven weeks, resulting in the AS-ATN27P parasites. Overall, AS-ATN27P shows a slight decrease in its level of tolerance to both drugs, either administered alone or in combination, indicating consecutive passaging in

absence of drug was responsible for a small reduction in drug-resistance when compared to the original AS-ATN.

AS-ATN27P harbours no mutations when compared to the unpassaged AS-ATN clone. Also, although AS-ATN27P showed no changes in *pcmdr1* copy number (single copy) or RNA levels, it displayed an increased level of expression of MDR1 protein when compared to AS-ATN. This is an intriguing observation. In case our previous assumptions regarding the role of *mdr1* amplification and consequent overexpression in drug-resistance are correct, then, AS-ATN27P should show a slight decrease in its level of sensitivity to ATN and MF treatment. On the contrary, AS-ATN27P is less tolerant to treatment with these drugs than AS-ATN. Nonetheless, it is important to consider that the increase in MDR1 protein level accompanying *pcmdr1* gene duplication in AS-ATNMF-1 was about five-to-six-fold, whereas the increase in MDR1 observed for AS-ATN27P nears two-fold. Therefore, in order to display high levels of resistance to ATN and MF (alone or in combination), it may be necessary a much higher increase in expression of MDR1 than what was observed in AS-ATN27P. In this case, the two-fold increase in MDR1 protein may simply reflect normal fluctuations in intracellular levels of this protein, or even an experimental artefact.

Most studies that describe *mdr1* amplification as determining resistance to ATN and MF, either alone or in combination had their focus placed exclusively on *mdr1* ((Price *et al,* 1997; Price *et al,* 1999; Gervais *et al,* 1999; Pickard *et al,* 2003; Cravo *et al,* 2003; Price *et al,* 2004; Alker *et al,* 2007; Lim *et al,* 2009; Chaijaroenkul *et al,* 2010; Chavchich *et al,* 2010). On the other hand, the characterisation of *pcmdr1* amplification in *P. chabaudi* AS-15MF clone has taken a whole genome approach and, although in this clone a region containing approximately one hundred and twelve genes was amplified, linkage analysis has implicated *pcmdr1* and its duplicated locus as the most influential when parasites were treated with MF or ART (Borges, 2009).

However, it is important to remember that in AS-ATNMF-1 other twenty two genes have also been amplified with *pcmdr1* and they may be acting in concert with *pcmdr1* amplification, for instance by influencing the level at which other genes are transcribed (Gonzales *et al,* 2008).

In AS-ATNMF-1 apart from the duplication of *pcmdr1* locus, two additional SNPs were identified: i) the A805,659T substitution on PCHAS_132020 gene in chr 13; and ii) the G1,155,448T on PCHAS_143160 gene in chr 14. AS-15CQ harbours the A805,658T mutation in the PCHAS_132020 gene. This mutation is also present in AS-ATN and was inherited by AS-ATNMF-1. In addition, a second mutation has appeared in AS-ATNMF-1, just next to the first mutation borne by these clones (A805,659T). Whereas the first mutation in AS-15CQ and AS-ATN result in the introduction of a STOP codon in position 998 of the protein coded by this gene (K998*), the second mutation causes another codon change, and in this case, the 998 codon encodes a leucine (K998L) in AS-ATNMF-1. Whilst the former mutation is very likely to result in a truncated version of this protein in AS-15CQ and AS-ATN, the latter mutation identified in AS-ATNMF-1 probably restores protein function, either entirely or partially.

As described above, apart from AS-ATNMF-1, four other clones resulted from the exposure of AS-ATN to ATN + MF treatment. By di-deoxy sequencing analysis it was possible to determine that the two mutations in PCHAS_132020 gene were present in AS-ATNMF-2 and AS-ATNMF-3, whereas only the first one was identified in AS-ATNMF-4 and AS-ATNMF-5. As discussed above, prior to cloning the parasite population selected with increasing doses of ATN + MF could survive after treatment with higher doses of the combination when compared with the AS-ATN clone used as starting material. After cloning, AS-ATNMF-1 has retained its drug-resistant phenotype, and is more resistant to ATN + MF than its progenitor. No phenotype analysis was carried out for the other clones, and assessing their level of resistance to ATN + MF either in combination or administered separately could help in estimating the importance of the acquisition of the second mutation in PCHAS_132020

gene for this phenotype. On the other hand, the appearance of a second mutation in PCHAS_132020 gene and the consequent recovery of protein function (as speculated above) may have some compensatory effect or even interfere with aspects of parasite's biology, other than drug resistance. Other possibility is that PCHAS_132020 gene has no major function in the erythrocyte stage of parasite life cycle. As such, mutations in this gene have no major consequences in this stage of development and as therefore, no importance for modeling of drug resistance, as attempted here.

The absence of data regarding PCHAS_132020 biological role in PlasmoDB and the inability to identify putative conserved domains in its protein sequence by the bioinformatics tools used here limit our ability to further speculate any biological function for these mutations regarding resistance to drugs and other cellular aspects.

The T936,945G mutation on chr 14 is placed in the coding region of PCHAS_143160 gene and encodes an non-synonymous substitution, in position 560 (D560Y). Bioinformatics analysis of the protein sequence coded by gene PCHAS_143160 suggests that the D560Y mutation falls in a putative casein kinase II phosphorylation site contained inside a GLU-rich region. In mammalian cells casein kinase II catalyses the phosphorylation of at least 307 different substrates and is involved in many cellular processes, such as apoptosis and t-RNA and r-RNA synthesis (Meggio and Pinna, 2003). In *P. falciparum*, erythocytic casein kinase II mediates the phosphorylation of parasite's proteins and by doing that participates in the regulation of cytoadherence of the infected red blood cells (Hora *et al,* 2009). In addition, the presence of casein kinases has been identified in the *P. falciparum*'s genome (Holland *et al,* 2009) suggesting different possible regulatory roles for casein kinase II mediated phosphorylation. In such context, the presence of the D560Y mutation in the protein coded by gene PCHAS_143160 may be affecting its phosphorylation state and therefore, its function.

Although the casein linase II phosphorylation site and other putative conserved domains were identified by the different bioinformatics tools used here, no function was attributed for the PCHAS_143160 gene in PlasmoDB, and again, our ability to predict the consequences of the D560Y mutation to ATN + MF resistance is limited.

Di-deoxy sequencing analysis of the other four AS-ATNMF clones demonstrated the D560Y mutation is only present in AS-ATNMF-1, and absent from AS-ATNMF-2, -3, -4, and -5. Again, phenotypic analysis of the other four clones could shed light on the importance of this mutation for ATN + MF resistance. However, considering that prior to cloning the parasite population selected under ATN + MF pressure was more resistant to drug treatment than the original AS-ATN, the absence of this mutation in the other AS-ATNMF clones may suggest that the D560Y substitution in the protein coded by PCHAS_143160 may not play a crucial role in resistance to these drugs.

Nonetheless, the role of the SNPs identified in PCHAS_132020 and PCHAS_143160 genes should be investigated. Amplification of *mdr1* gene has been reported as causing an increased biological cost for parasites (Preechapornkul *et al,* 2009). In such context, maybe the mutations identified in PCHAS_132020 and PCHAS_143160 genes could act as compensatory mutations, improving the effectiveness of their respective proteins. In addition, no mutations were identified in these PCHAS_132020 and PCHAS_143160 genes in AS-MFATN-5. Therefore we suggest that these two mutations may be result from a stochastic process where random variations in genome sequence are selected by chance and therefore, have no consequence in drug resistant phenotype displayed by AS-ATNMF-1.

II.3.2. OTHER ASPECTS INVESTIGATED IN THIS WORK

Throughout the course of this work, Solexa whole genome re-sequencing was used for obtaining the complete genome sequence of the AS-ATNMF-1 clone. Later, the presence of

high confidence mutations on AS-ATNMF-1 was confirmed by di-deoxy sequencing analysis. Di-deoxy sequencing was also used for determining the presence of these mutations in other clones, and as such their origin was traced back in the AS lineage. The only exceptions were two high confidence indels which could not be confirmed by di-deoxy sequencing due to technical difficulties. However, strong experimental evidence suggests that these two consist of genuine mutations. Since no di-deoxy sequencing confirmation was carried out, it was not possible to identify the clone in the AS lineage where they were originated, and therefore, it is not possible to correlate their presence with drug resistance.

On the other hand, five verified mutations present in the artesunate + mefloquine (ATN + MF)-resistant AS-ATNMF-1, were also present in the ancestral clones, prior to the selection of AS-ATNMF-1: i) three appeared after selection for increased chloroquine (CQ) resistance in AS-15CQ; and ii) two were selected by artesunate (ATN) pressure which originated AS-ATN.

The above mentioned mutations and their putative roles in drug resistance are going to be discussed separately in the sub-sections below.

II.3.2.1. MUTATIONS ARISING AFTER SELECTION FOR INCREASED LEVELS OF CHLOROQUINE RESISTANCE IN AS-15CQ

AS-15CQ was selected from AS-3CQ clone, which displays resistance to low doses of chloroquine (CQ) treatment. AS-15CQ parasites were later used for the selection of three distinct clones: i) AS-30CQ was selected for high-level of CQ resistance; ii) AS-15MF was selected for mefloquine (MF) resistance, and iii) AS-ATN was selected for artesunate (ATN) resistance.

In this work, three novel mutations were identified as first appearing in AS-15CQ.

The first consists of a SNP, where an A805,658T substitution was identified on chr 13. This mutation falls in the coding region of PCHAS_132020 gene, which is described by

PlasmoDB as coding for a hypothetical protein. The prediction of the mutated protein sequence indicates that this mutation introduces a STOP codon at position 998 (K998*). By di-deoxy sequencing it was possible to confirm the presence of this mutation in AS-ATN, and its absence in AS-15MF or AS-30CQ.

The second mutation first appearing in AS-15CQ clone is a 3 bp deletion from position 472,273 to 472,275 on chr 3. This triplet (AAT) is placed inside the coding region of PCHAS_031370 gene, and prediction of the protein sequence indicates this mutation results in a deletion of an isoleucine in position 103 (I103). BLAST analysis identified a domain which is present in proteins whose expression is induced by superoxide radical generating agents. The I103 deletion falls inside this putative domain, and as such may affect the function of this protein. This deletion is present in AS-ATN and AS-15MF, while being absent in AS-30CQ.

The third and last mutation identified in AS-15CQ consists of a 60 Kb deletion spanning from the very beginning of chr 13 to approximately position 60,867. This region encompasses approximately fifteen genes, most of which code for variable surface antigens which belong either to the CIR or pc-FAM family. This deletion is present in AS-ATN, being absent in AS-15MF and AS-30CQ.

The changes in protein expression and function caused by the three mutations presented above should entail drastic changes in parasite cellular machinery. However, the role of these mutations in CQ resistance remains elusive. First because most of genes implicated here have yet no known function, and it is therefore hard to predict the effect of polymorphisms or non-expression of the respective proteins on parasite's biology. Secondly and maybe more importantly, AS-30CQ harbours none of these three mutations. Regardless, AS-30CQ shows high level of CQ resistance, indicating other loci may be determining resistance, whereas these three mutated loci present in the parental AS-15CQ clone are involved with phenotypes not related to drug resistance.

One very interesting observation resulting from this analysis is that unlike expected, the clones deriving from AS-15CQ did not always inherit the mutations borne by this clone. How is it possible that AS-ATN has inherited all the mutations identified here as appearing in AS-15CQ, whilst AS-15MF harbours the 3 bp deletion on chr 03 and AS-30CQ carries none?

Whilst gene amplification and deamplification are very common events, the reversal of a mutated gene (SNPs or deletions) into its wild type version is very unlikely. Therefore, the presence/absence of mutations identified in AS-15CQ in the clones derived from it requires an alternative explanation.

In fact, the genetics of AS-15CQ and the clones deriving from it has a primary intriguing feature: AS-ATN carries the V2697F mutation in *pcubp1* gene while both AS-15MF and AS-30CQ harbour the V2728F mutation in this gene. This would have the following implications: considering neither of the two above mentioned mutations on *pcubp1* gene appeared in AS-15CQ, this would imply that the V2728F mutation has appeared independently in two different clones (AS-15MF and AS-30CQ) which is very unlikely to occur. So, one possibility is that the V2728F mutation should be present in AS-15CQ, and then was inherited by AS-15MF and AS-30CQ. However, in this case, it would mean AS-ATN has reversed the V2728F mutation and acquired a new mutation in *pcubp1* (V2697F). A third option would then be to consider both mutations were present in AS-15CQ prior to selection of AS-ATN, AS-15MF and AS-30CQ. And in order to be inherited separately by AS-15CQ progeny two parasite populations should be present, i.e. AS-15CQ is not representative of a clonal population.

The discovery of the three mutations identified in this work adds up to the puzzle. For clarity purposes, focus will be placed specifically on the the presence of the 3 bp deletion on chr 3 (PCHAS_031370 - I103) on AS-ATN and AS-15MF inherited from AS-15CQ, and its absence in AS-30CQ. Considering the 3 bp deletion on chr 3 and the two different genotypes of *pcubp1* discussed above, three haplotypes are present in the clones deriving from AS-15CQ: i)

presence of 3bp deletion on PCHAS_031370 and the V2697F mutation on *pcubp1* gene, as in AS-ATN; ii) presence of 3bp deletion on PCHAS_031370 and the V2728F mutation on *pcubp1* gene, as in AS-15MF; and iii) absence of 3bp deletion on PCHAS_031370 and the V2728F mutation on *pcubp1* gene, as in AS-30CQ.

A simple explanation for this mixture of alleles may lie in the selection procedure which has generated AS-15CQ. Briefly, AS-3CQ was exposed to treatment with CQ, generating parasites that showed increased level of resistance to this drug. These parasites then passaged through mosquitoes (Padua et al, 1980). However, inefficient cloning of the newly generated resistant parasites may have left a parasite population consisting of a few clones where the mutations in PCHAS_132020 gene, the 3 bp deletion in chr 03 and the 60 Kb deletion in chr 13 were either present or absent. In addition, the two different mutations on *pcubp1* gene would have appeared also upon AS-15CQ selection, in two distinct events. When passaged through mosquitoes, genetic recombination would have generated a multi-clonal population that after further selection and posterior cloning would have been converted into three different clones, carrying three different haplotypes, as seen in AS-ATN, AS-15MF and AS-30CQ.

This hypothesis may be not very far-fetched if one considers cloning methodology. According to statistics, by inoculating the equivalent of 1 parasite in each mouse, a clonal population is obtained in each animal showing parasite growth if at least 37% of all animals have no detectable parasitaemia. However, it is not possible to guarantee only one parasite was indeed inoculated in the acceptor mouse, or that, although more parasites could have been present in the first moment, only a single parasite multiplies in order to form the infecting population.

The appearance of two distinct mutations on *pcubp1* gene during the selection of AS-15CQ might indicate that CQ pressure, rather than ATN or artemisinin (ART), is the major factor influencing the evolution of mutations in this gene in our model. Indeed, work carried out

by our group has indicated that parasites harbouring *pcubp1* mutations are selected by CQ treatment (Mordzynska, 2010). Nevertheless, in a genetic cross between AS-15MF and AJ, treatment with ART, MF and lumefantrine was also found to select parasites bearing the V2728F mutation in *pcubp1* (Borges, 2009).

Elucidation of the role of the *pcubp1* gene in regulation of protein expression in drug-sensitive and drug-resistant parasites of the AS lineage may shed light in these questions. Additionally, identifying the functions and the changes imposed by mutations on the other genes identified as appearing in AS-15CQ will certainly help to clarify the mechanisms underlying the complex genetics of CQ resistance. However, it is important to retain that AS-15CQ does not consist of a clonal population, and as such, an accurate genetic analysis of these parasites may not be possible.

II.3.2.2. MUTATIONS ARISING AFTER SELECTION FOR ARTESUNATE RESISTANCE IN AS-ATN

AS-ATN was selected from AS-15CQ by consecutive sub-inoculations in mice treated with stepwise increasing doses of ATN. Genetic analysis of this clone carried out in this work have confirmed the presence of the V2697F mutation in *pcubp1* gene, whose role in ATN resistance has been discussed elsewhere (Hunt *et al,* 2007) and identified the presence of two previously unknown mutations: i) the A636,862G substitution near the 3'-end of PCHAS_061710 gene on chr 6; and ii) a mutation in gene PCHAS_133430 on chr13, resulting in an E738K substitution in a 26S proteasome subunit.

As indicated by PlasmoDB, the PCHAS_061710 gene on chr 6 codes for a seryl t-RNA synthetase, which is responsible for the attachment of the correct aminoacid to its cognate transfer RNA (t-RNA) during protein synthesis (Bhatt *et al,* 2009). In human cells, untranslated regions placed upstream the 3'-end of a gene were described as being involved in translational

regulation of this gene (Chatterjee and Pal, 2009). In addition, low complexity regions were described in Plasmodium as regulators of folding of the newly synthesized proteins (Frugier *et al,* 2010). Mutations in the upstream region near the 3'-end of gene PCHAS_061710 may therefore cause changes in the levels of expression or misfolding of seryl t-RNA synthetase, and as a consequence may cause serious effects in parasite protein synthesis machinery.

However, as the seryl t-RNA synthetase belongs to a pathway of major importance, there is a chance that protein synthesis would be maintained at its normal levels by other proteins with similar function. Functional redundancy has been already observed as present in other pathways in Plasmodium (Omara-Opyene *et al,* 2004) and could be responsible for counteracting the role of the mutations identified here.

The second mutation indentified in AS-ATN is a non-synonimous (E738K) mutation in gene PCHAS_133430, which codes for a 26S proteasome subunit. The E738K mutation falls in the putative proteasome/cyclosome repeat region identified by BLAST analysis of this gene. The 26S proteasome is described as involved in degradation of different types of ubiquitin-tagged proteins (van Leuken *et al,* 2008). In additon, cell cycle regulatory proteins can also be degraded by the 26S proteasome, and it has been indicated that cyclin degradation by the 26S proteasome is necessary for cell cycle progression (van Leuken *et al,* 2008).

In *P. falciparum* cultured strains, evolution of ART resistance has been described as mediated by a quiescence mechanism, in which ART-tolerant parasites remain arrested at ring stage in the presence of ART treatment. When the drug is removed from the medium, the parasites re-enter cell cycle, developing into schizonts and undergoing replication (Witkowski *et al,* 2010). In this context, the E738K mutation identified in AS-ATN could be affecting the replication machinery of this clone, by causing partial or complete disruption of 26S proteasome function.

In addition, it is possible that the V2697F mutation in *pcubp1* gene is acting in concert with the E738K mutation in the 26S proteasome in AS-ATN parasites, since de-ubiquitinating enzyme and the 26S proteasome are both part of the protein degradation pathway. Indeed, as suggested by Hunt and colleagues, the mutation V2697F would be responsible for a reduction in the de-ubiquitinating activity of the protein coded by *pcubp1* gene. This would increase protein degradation via the 26S proteasome. In this case, the E738K mutation identified in the 26S proteasome subunit identified here could have one of the two possible roles: i) decreasing the 26S proteasome activity, and acting as compensation for the V2697F mutation in *pcubp1* gene; ii) increasing the 26S proteasome activity and in this case, this would have an enhanced impact in protein turnover.

Given the high degree of redundancy identified in cell cycle components in other eukaryotes, it is possible, as hypothesized for the mutation in the region near the 3'-end of gene PCHAS_061710 above, that the E738K mutation on the 26S proteasome subunit may be compensated to some extent by other mechanisms, resulting in little or no effect in overall cell cycle progression.

The role of the two novel mutation identified in AS-ATN regarding ATN resistance needs to be further investigated.

CHAPTER III

FITNESS COST OF RESISTANCE TO ARTESUNATE + MEFLOQUINE

This Chapter describes the competition assays to which the ATN + MF-resistant AS-ATNMF-1 clone selected and characterised in Chapter II was subjected. These assays aimed to check if the selection procedure has resulted in changes in AS-ATNMF-1 fitness when compared to the progenitor AS-ATN. This Chapter will be subdivided in three sections: i) Introduction – a brief review of the literature involving parasite fitness and its consequences for the spread and maintenance of drug-resistant strains in parasite populations will be presented; ii) Results – firstly, optimisation of the protocol for the determination of proportions of individual strains in parasite mixtures will be described, and secondly, data relative to the within host competitive growth of the ATN + MF-resistant AS-ATNMF-1 and its AS-ATN progenitor or the untreated parallel line AS-ATN27P, will be described.; iii) Discussion – the results obtained in the previous section will be discussed.

III.1. INTRODUCTION

III.1.1. FITNESS COST OF DRUG RESISTANCE

As postulated in Charles Darwin's classical book The Origin of Species (Darwin, 1859), the evolution of a species is directly connected to its ability to survive and propagate in a certain environment. In very general terms, this means that individuals must compete not only with others belonging to different species (Richardson *et al,* 2009; Staves and Knell, 2010), but also that intra-specific competition shapes the characteristics of a population (Wargo *et al,* 2007; Baquero and Lemonnier, 2009; Staves and Knell, 2010; Mackinnon and Marsh, 2010). Therefore, in any given circumstance there is a tendency that the less fit individuals are displaced from the population by the best adapted ones (Wargo *et al,* 2007; Richardson *et al,* 2009). However, being extremely well adapted to a certain environment does not mean an individual is adapted to *any* environment (Hoffmann, 2010), and in fact, changes in surrounding conditions may subvert what was formerly an advantage into a disadvantage (Anderson, 2005; Bottger and Springer, 2008).

The malaria parasite interacts with different environments throughout its life cycle. It goes through two different hosts, from the mosquito's midgut through its haemolymph to its salivary glands, and then in the mammalian host from hepatocytes through the blood stream to the erythrocyte. Together, all these factors help to shape the parasite's genome and, consequently, its phenotype (Mackinnon and Marsh, 2010).

With regards to the mammalian host bloodstream only, it is clear that the malaria parasite has to face many obstacles in order to establish and maintain a successful infection. The most obvious challenge for the parasite's survival is the host's immune system, against which the parasite has developed a very efficient evasion strategy. Another aspect of parasite's adaptation would be its intrinsic ability to invade, explore, proliferate, and develop within the erythrocyte. Finally the parasite's investment in sexual stages can also be a determinant of fitness, as it allows the parasites to undergo genetic recombination and also explore new hosts.

III.1.1.1. EMERGENCE OF RESISTANCE: AN INCREASED BURDEN

In most cases, symptomatic malaria patients are treated with drugs (Luxemburger *et al,* 1997). As mentioned previously, intensive use of antimalarials has led to the appearance and dispersal of parasites harbouring increased tolerance and even high levels of resistance to one or multiple drugs. In some areas, parasite strains harbouring either drug sensitive or drug resistant alleles can be observed. Depending on the strength with which a drug was used in this population or the transmission intensity of the region (Mackinnon, 1997), the resistant allele(s) may be at very high frequency or close to fixation in the parasite pool (Price *et al,* 1999; Lopes *et al,* 2002; Price *et al,* 2004; Anderson *et al,* 2005).

Studies in many different organisms have shown that the acquisition of mutations conferring drug resistance is frequently deleterious in absence of drug pressure, being accompanied by a reduction in the ability of the resistant strain to compete with a sensitive one in untreated hosts (Anderson, 2005; Bottger and Springer, 2008). This is known as fitness cost and was largely described in bacteria (Gagneux, 2009; Andersson and Hughes, 2010).

Many groups have investigated the fitness cost of resistance in malaria parasites. In 1994, one study found that in the rodent malaria parasite *Plasmodium berghei* a pyrimethamine (PYR)-sensitive strain had transmission advantage over a PYR-resistant one (Shinondo *et al,* 1994). This finding is in accordance with previous work on another rodent model, *P. chabaudi* (Rosario *et al,* 1978). In this work a PYR-resistant strain was outgrown by its sensitive progenitor in two out of three experiments. In the same study the cost incurred by chloroquine (CQ)-resistant parasites was also investigated, by using a similar approach. However, in this case, the sensitive strain was outgrown by the resistant parasites indicating resistance may not always be followed by reduction in fitness of a parasite strain (Rosario *et al,* 1978).

In *in vitro* cultured *P. falciparum* strains, Hayward and colleagues (2005) have identified that polymorphisms on the *pfmdr1* gene that confer resistance to CQ can be deleterious in the

absence of drug pressure. Similarly, in a mefloquine (MF)-resistant strain, the amplification of *pfmdr1* was associated with a decrease in fitness when compared to the sensitive counterpart. In this study, additional increase in copy number was associated with increased detrimental effects on parasite's survival (Preechapornkul *et al,* 2009).

In addition to studies using laboratory strains, field analysis also indicates that in the absence of drug pressure, the frequency of mutated alleles related with drug resistance tends to decrease in a geographic region (Thaithong *et al,* 1988; Schwenke *et al,* 2001; Nair *et al,* 2003; Abdel-Muhsin *et al,* 2004; Ord *et al,* 2007; Babiker *et al,* 2009). The most important field study corroborating fitness cost of resistance is the case of CQ use and its subsequent withdrawal in Malawi (Laufer *et al,* 2006). In this country, CQ was used as first line therapy against *P. falciparum* malaria, and cases of resistance were first reported in the 1970's. In 1993, clinical failure after CQ therapy reached about 50%, accompanied by an 85% prevalence of the mutation K76T in the *pfcrt* gene (Kublin *et al,* 2003). CQ was then replaced by sulphadoxine + pyrimethamine (SP) as the first-line treatment. Over time, reports indicated that the presence of the mutated *pfcrt* K76T marker in the parasite population decreased to about 17% in 1998, and finally to 2% in 2000 (Mita *et al,* 2003). Clinical trials were carried out and the resurgence of CQ sensitivity was reported (less than 1% clinical failure in 2005) (Laufer *et al,* 2006).

The re-emergence of CQ sensitivity in Malawi was shown to have taken place due to the re-emergence of sensitive parasite populations, reflected by the re-expansion of parasites carrying *pfcrt* sensitive alleles, rather than by back-mutation in this gene (Mita *et al,* 2004). This provides strong indication that CQ-sensitive parasites were fitter than resistant ones, and therefore when CQ pressure was removed, resistant parasites were slowly removed from the parasite pool.

The case of Malawi might have serious implications for therapy policy in the field: the reversal of CQ resistance reported in Malawi provides evidence supporting the idea that

abandoned drugs, such as CQ, could be re-introduced in case of emergence of resistance to currently used therapies.

However, the case of reversal of CQ resistance in Malawi is so far unique. In Kenya, the substitution of CQ for SP, led to a decrease in the prevalence of the K76T mutation from 94% in 1993 to 63% in 2006, but not the elimination of the mutated allele, which remained correlated with low susceptibility to CQ *in vitro* (Mwai *et al,* 2009). Also, in Gabon there was a decrease in *in vitro* CQ resistance from 100% in 1992 to 45% in 1996 (Schwenke *et al,* 2001). However, *in vivo* studies showed 100% clinical failure after CQ therapy and the presence of K76T mutated *pfcrt* in all samples analysed (Borrmann *et al,* 2002).

It is important to reinforce the idea that successful reintroduction of a drug would only be possible if resistance to this drug imposes a burden to parasite's survival in the absence of drug pressure. Additionally, treatment with the drug must be completely ceased, and the newly deployed treatment cannot be based on drugs that share mechanisms of action with the replaced drug. Finally, it is also crucial that the resistant allele has not reached near-fixation levels, i.e, enough sensitive parasites must remain in the population so they can compete and displace the resistant ones (Hayward *et al,* 2005).

III.1.1.2. COMPENSATORY MUTATIONS

In some cases, the acquisition of resistance is accompanied by subsequent mutations that compensate for the burden caused by the changes conferring a disadvantageous phenotype. These "compensatory mutations" can then restore parasite's fitness either partially or completely, allowing resistant parasites to remain in the population even in the absence of selective pressure. This phenomenon has been reported in bacteria, where compensatory mutations can occur either in the gene where previous mutations have led to the acquisition of drug resistance or in a different one (Maisnier-Patin and Andersson, 2004; Nilsson *et al,* 2006).

In bacteria, some studies describe the appearance of compensatory mutations after serial passages of the resistant strain in the absence of drug pressure (Kugelberg *et al,* 2005; Nilsson *et al,* 2006).

In malaria parasites it is also possible that consecutive passaging through mice may increase parasite fitness. For instance, in a study carried out in *P. chabaudi*, a low-fitness (relative to the sensitive progenitor) PYR-resistant clone underwent consecutive passages through mice and then through mosquitoes in absence of drug. This clone eventually outgrew its sensitive progenitor in untreated hosts (Walliker *et al,* 2005). As the increased fitness shown by the passaged parasites was not reverted by transmission through mosquitoes, it is possible that consecutive sub-inoculations into mice was responsible for the appearance of mutations (genetically stable changes, which can be therefore transmitted through mosquitoes), and that these mutations may act as compensation for the deleterious effects of PYR resistance, increasing parasite's fitness in absence of drug.

In fact, other studies have shown that, some mutations were implicated as playing a role in compensating for the burden imposed by the acquisition of drug resistance in malaria parasites. Amplification of the *pfgch1* gene, coding for the GTP-cyclohydrolase I, was reported to be prevalent in field samples from areas where high levels of anti-folate drugs were used (Nair *et al,* 2008). This enzyme is involved in folate metabolism (Dittrich *et al,* 2008) and the amplification of this gene, and consequently increased protein expression, is believed to function as compensation for the mutations in *pfdhfr* and *pfdhps* genes in pyrimethamine (PYR)-resistant parasites (Nair *et al,* 2008). Osman and colleagues (2007) described that multiple mutations in genes associated with resistance to chloroquine (CQ) and PYR impose a high fitness cost, expressed as lower parasite density in the host blood. However, this trait was accompanied by an increased gametocyte production, which the authors believe to act as compensation for the reduction in asexual forms.

The presence of compensatory changes in expression pattern of different transporters has also been described as adaptation to CQ resistance conferred by the K76T mutation in the *pfcrt* gene (Jiang *et al,* 2008).

III.1.2. AIMS

In this chapter, results assessing the potential fitness cost associated with the acquisition of resistance to the ATN + MF are presented. As described in Chapter II, the ATN + MF-resistant AS-ATNMF-1 clone was selected after serial sub-inoculation into host mice treated with the ATN + MF combination. The AS-ATN clone was used as starting material for the selection of AS-ATNMF-1 and was also passaged through mice in the absence of drug treatment, generating the A-ATN27P parasite line.

We have then performed experiments to evaluate how the within-host growth of parasites resistant to combination treatment compares to that of sensitive ones. Thus the next section will outline competition assays designed to determine the biological cost of the acquisition of resistance to ATN + MF by AS-ATNMF-1. This clone was grown in competition with the original parental AS-ATN and also with AS-ATN27P.

III.2.RESULTS

III.2.1. COMPETITION ASSAYS

Increased resistance to a drug is commonly associated with reduced growth of resistant parasites when compared with drug-sensitive ones in the absence of drug pressure (Rosario *et al,* 1978; Shinondo *et al,* 1994; Peters *et al,* 2002). This biological cost (or fitness cost) could therefore grant the sensitive parasites adaptive advantages over the resistant ones when co-infecting the vertebrate host. In such context, the impact of the evolution to an ATN + MF-resistant phenotype undergone by AS-ATNMF-1 was assessed. AS-ATNMF-1 was grown within the same host together with its original progenitor AS-ATN in mixtures containing different initial proportions of the two clones.

It has been observed that changes in parasite fitness can also occur due to repeated sub-inoculations into mice (Mackinnon and Read, 1999; Walliker *et al,* 2005). Since the selection of ATN + MF-resistance was made by consecutive sub-inoculations in mice under drug treatment, the fitness of AS-ATNMF-1 clone was also assessed when in competition with AS-ATN27P, the untreated parasites passaged twenty seven times in parallel with AS-ATNMF-1.

It is important to highlight that accurate estimates of Plasmodium parasites' fitness should take into account the ability of one strain to outcompete a different one throughout each phase of the parasite's life cycle. However, in this work, competition assays will only assess parasite growth in the mammalian host's bloodstream.

III.2.1.1. VALIDATION OF THE COMPETITION ASSAYS

As described in Chapter II, a G to T substitution in position 1155448 on chr 14, in the PCHAS_143160 gene is exclusive to the AS-ATNMF-1 clone. By using proportional sequencing analysis targeting this mutation, it is possible to estimate the relative proportion of AS-ATNMF-1 parasites present in a mixture with parasites displaying the wild-type form of this genotype. However, prior to determining parasite proportions in unknown mixtures, it was crucial to verify whether the accuracy of quantifying parasite proportions using this method was satisfactory for analysing unknown mixtures. For this purpose artificial mixtures with known proportions of AS-ATNMF-1 and AS-ATN27P parasites were prepared and analysed, as described in the Material and Methods, Section 4. The amplified fragments were analysed for each sample in both forward and reverse directions and, subsequently, peak heights were measured in the genetically polymorphic position. These were then used to calculate the relative proportions of each parasite.

Overall, there was a good correlation between the observed and expected values, reflected by a linear pattern in both the forward and reverse directions (Figure 17). However, it was verified that the use of the reverse primer produced slightly more accurate results, as reflected in the observation that the expected and observed values were more correlated to each other than those produced using the forward assay (Figure 17). The assay was subsequently used to estimate parasite proportions in the competition experiments described below.

Artificial Mixtures

expected proportion of sensitive allele (%)	Observed proportions of sensitive allele (%)	
	forward sequence	reverse sequence
100	100	100
99	100	100
90	91.4	91.7
75	78.5	76
50	56.4	52
25	33.6	29.2
10	14.5	15
1	4.5	4.3
0	0	0

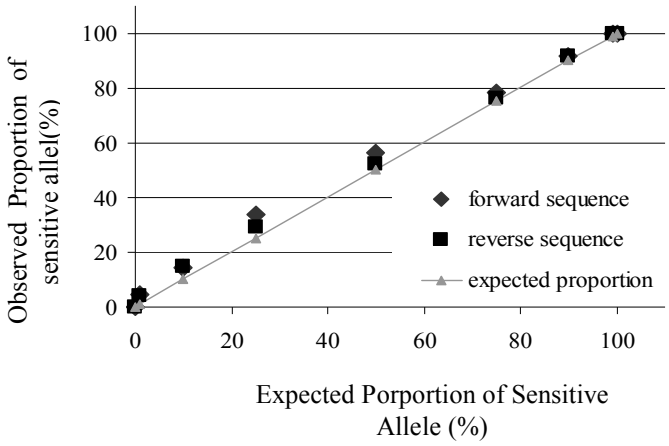


Figure 17 – Validation of proportional sequencing method. DNA present in artificial mixtures of AS-ATNMF-1 and AS-ATN27P parasites was analysed for the detection of a G to T substitution in position 1155448 on chr 14. Analysis of electropherograms resulted in the observed proportion of the sensitive allele calculated for both forward and reverse sequences that are shown in table. The values from both forward (black diamonds) and reverse sequences (black squares) were plotted against the expected values, as depicted in the graph. In addition, the calibration curve is shown (gray triangles and line).

III.2.1.2. WITHIN-HOST COMPETION ASSAYS USING AS-ATN + AS-ATNMF-1

In order to determine if the evolution of resistance to ATN + MF may have caused any impact on the fitness of the AS-ATNMF-1 clone relative to its progenitor AS-ATN, five groups of mice were inoculated with mixtures of the two parasites in different proportions 100:0, 90:10, 50:50, 10:90, and 0:100 (AS-ATN:AS-ATNMF-1). Absolute parasitaemias and proportions of each clone were analysed for each animal along time, as described below.

EVOLUTION OF ABSOLUTE PARASITE GROWTH ALONG TIME

In order to gain an initial insight into the overall parasite growth, absolute percentage parasitaemias were followed over time in mice infected with the above inocula. Mice inoculated with AS-ATN parasites alone (100:0) showed peak parasitaemia of about 38% on Day 10 post-inoculum (pi). In the group of mice inoculated only with AS-ATNMF-1 (0:100), parasites grew faster, and reached higher levels of parasitaemia than AS-ATN (near 63% on Day 8 pi) (Figure 18).

Mice inoculated with unequal proportions of AS-ATN and AS-ATNMF-1 (10:90 and 90:10) showed similar parasite growth as animals inoculated with AS-ATNMF-1 alone (0:100). These mice showed peak parasitaemias of about 50-55% on Day 8 pi. However, after reaching peak parasitaemia, mice inoculated with higher proportion of AS-ATN (90:10) appeared to have a much smoother decrease in parasite numbers when compared to animals inoculated with higher proportion of AS-ATNMF-1 (10:90) or AS-ATNMF-1 alone (0:100) (Figure 18).

A biphasic shape was observed in the curve representing parasite growth of animals inoculated with equal proportions of AS-ATN and AS-ATNMF-1 (50:50). In this group, the first day of detection of parasites in the blood smears differed greatly when comparing the three mice. This indicated a difference in the initial absolute number of parasites inoculated in each animal, most likely due to a technical shortcoming, although inherent biological variations cannot be excluded. Therefore, the biphasic shape of the parasite growth curve does not probably reflect changes in the growth pattern of parasites due to competition within the host.

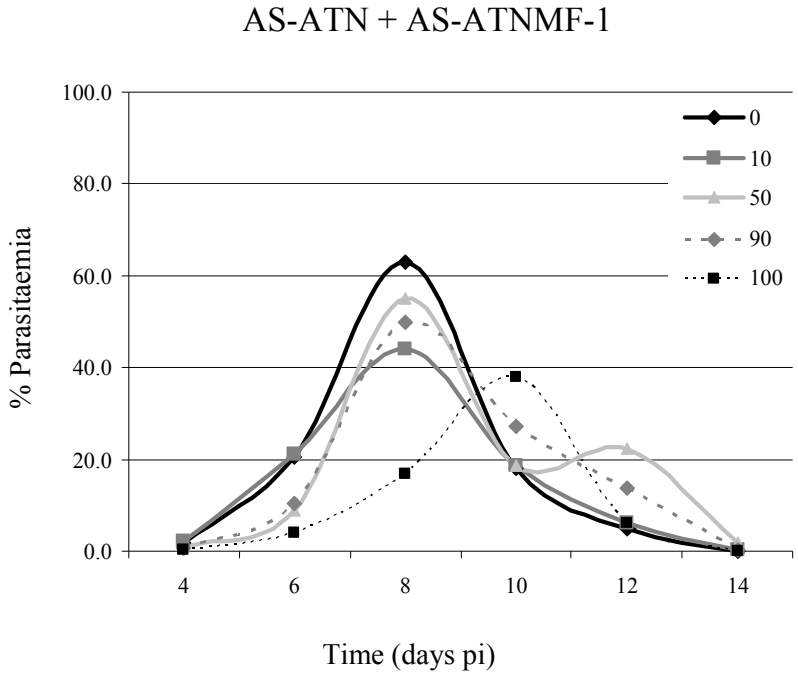


Figure 18 – Percentage (%) parasitaemias were determined along time for animals inoculated with different proportion of the artesunate + mefloquine-resistant AS-ATNMF-1 clone and its progenitor, AS-ATN. Legend indicates initial percentage of AS-ATN clone.

EVOLUTION OF PARASITE PROPORTIONS ALONG TIME

In addition to determination of absolute parasite growth by analysis of percentage parasitaemias along time, the proportion of each clone in the blood of each mouse was measured every two days. This was made by PCR amplification of a fragment of PCHAS_143160 gene containing a G to T mutation in AS-ATNMF-1, and by assessing the peak heights of chromatograms obtained from sequencing each amplified sample.

DNA samples were first obtained from blood mixtures which were later inoculated into mice. Thus, the initial proportions of AS-ATN and AS-ATNMF-1 inoculated in each mouse were determined (Day 0, Figure 20). It was possible to verify that mice inoculated with a higher proportion of AS-ATN (90:10) appeared to have received a smaller proportion of AS-ATN than expected (about 79% of the wild-type allele in the mixture). Conversely, samples obtained from the inoculum where equal proportions of the two clones were injected into mice consisted of a

higher proportion of AS-ATN, as approximately 60:40 was observed instead of 50:50 (AS-ATN:AS-ATNMF-1). Finally, the proportions of each clone in the initial inoculum of the group of mice that received a higher proportion of AS-ATNMF-1 (10:90) seem to be consistent with the expected values (Figure 19).

Regardless of the initial proportion of each parasite in the initial inoculum, when observing the samples obtained from each mouse along time, the presence of the wild-type allele (representing AS-ATN clone) decreased along time. In fact, when observing the animals inoculated with higher proportions of the wild-type AS-ATN clone (90:10) this decrease appeared to be almost linear along time, and from Day 8 AS-ATNMF-1 proportions have increased when compared to the initial proportion inoculated in each mouse in this group (on around 32% on Days 8 and 10, $p<0.005$; around 40% on Day 12 and 61% on Day 14, $p<0.0004$) (Figure 19).

Similarly, in mice initially inoculated with similar proportions of each clone (nominally 50:50, but in reality 60:40), the proportion of the AS-ATN allele decreased from its initial value (60%) to Day 4 (30%, $p<0.0004$), with a further reduction from Day 4 to Day 6 (20%, $p>0.0004$). However, from Day 6 onwards, the proportion remained unchanged up to Day 14, when a further reduction in the wild-type allele was observed (12%, $p>0.0004$) (Figure 19).

Samples obtained from mice inoculated with higher proportions of AS-ATNMF-1 (10:90) also showed a decrease in the proportion of the wild-type allele (AS-ATN), with the reduction of the AS-ATN clone below detection by Day 10 (Figure 19).

The parasite proportions of in blood samples from mice inoculated with only AS-ATN (100:0) or AS-ATNMF-1 (0:100) were also calculated and served as controls. No changes in the initial proportion were found along time in these two groups of mice.

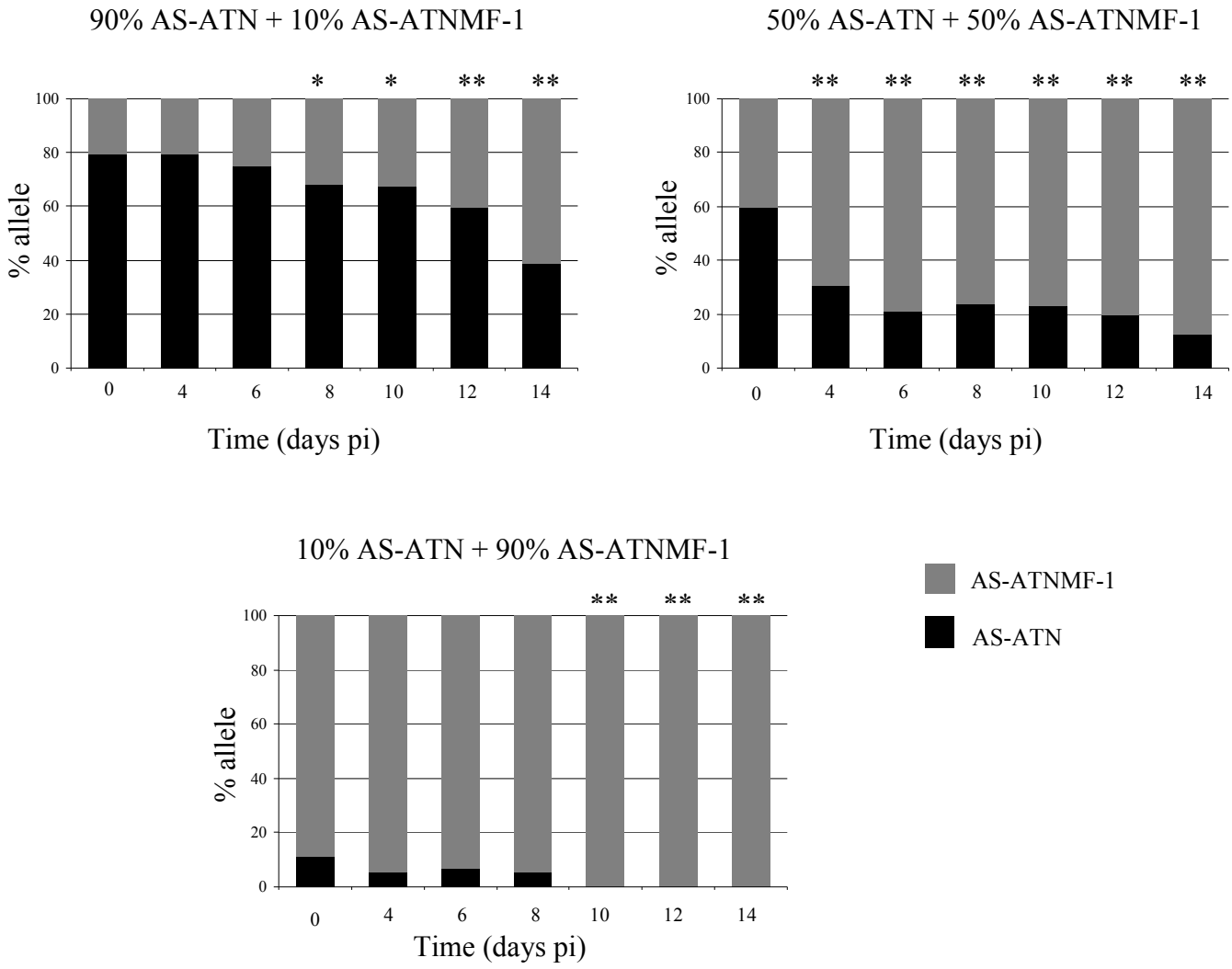


Figure 19 - Competition assays carried out using the artesunate + mefloquine-resistant AS-ATNMF-1 clone and its progenitor, AS-ATN. Bars represent the mean of two animals. Gray bars represent AS-ATNMF-1 (mutant) allele and black bars represent AS-ATN (wild type) allele. Day 0 corresponds to samples obtained from the blood mixture used to inoculate each mouse. From Day 4 onwards, samples were obtained from each animal in each time point. Statistical analysis was made comparing the obtained proportions along time against the proportion inoculated into mice: * $p<0.005$; ** $p<0.0004$.

III.2.1.3. WITHIN-HOST COMPETION ASSAYS USING AS-ATN27P + AS-ATNMF-1

As observed in the previous section, the artesunate + mefloquine (ATN + MF)-resistant AS-ATNMF-1 clone seems to outgrow its sensitive progenitor AS-ATN when in competition within the same host in absence of drug treatment.

In order to determine if consecutive passaging through mice would be responsible for any changes in parasite fitness, the growth of AS-ATNMF-1 was assessed when in competition within the same host with AS-ATN27P, the uncloned parasites passaged twenty seven times in absence of drug treatment. In that context, parasite mixtures were prepared in different proportions 100:0, 90:10, 50:50, 10:90, and 0:100 (AS-ATN27P:AS-ATNMF-1) and injected into mice. Absolute parasitaemias and parasite proportions were determined along time.

EVOLUTION OF ABSOLUTE PARASITE GROWTH ALONG TIME

In a similar fashion as the competition assays performed using AS-ATNMF-1 and AS-ATN clones, the absolute percentage parasitaemia was analysed in mice receiving the different parasite inocula.

Mice inoculated only with AS-ATN27P (100:0) showed similar parasite growth as observed for mice inoculated only with AS-ATNMF-1 (0:100). AS-ATN27P (100:0) showed peak parasitaemias of 79% and AS-ATNMF-1 (0:100) showed peak at 61%, both on Day 8 pi. However, the slope of the AS-ATN27P growth curve was higher than the one observed for AS-ATNMF-1, which may indicate a slight difference in growth of the two parasites, with AS-ATN27P having a slightly higher growth rate (Figure 20).

Differences in slope could also be observed when calculating the parasitaemias of mice inoculated with mixtures of the two parasites. The parasite growth curve shown by mice inoculated with higher proportion of AS-ATN27P (90:10) had a steeper slope when compared with the curve of mice receiving a higher proportion of AS-ATNMF-1 (10:90) (Figure 20).

When examining the animals which received equal proportions of AS-ATNMF-1 and AS-ATN27P (50:50), a considerably smoother parasite growth curve could be observed.

However, parasite growth in the three groups of animals receiving the mixtures of the two parasites in different proportions (90:10, 50:50, and 10:90) seemed to be very similar. All groups of mice showed peak parasitaemias on Day 8, ranging from 50-to-63% (Figure 20).

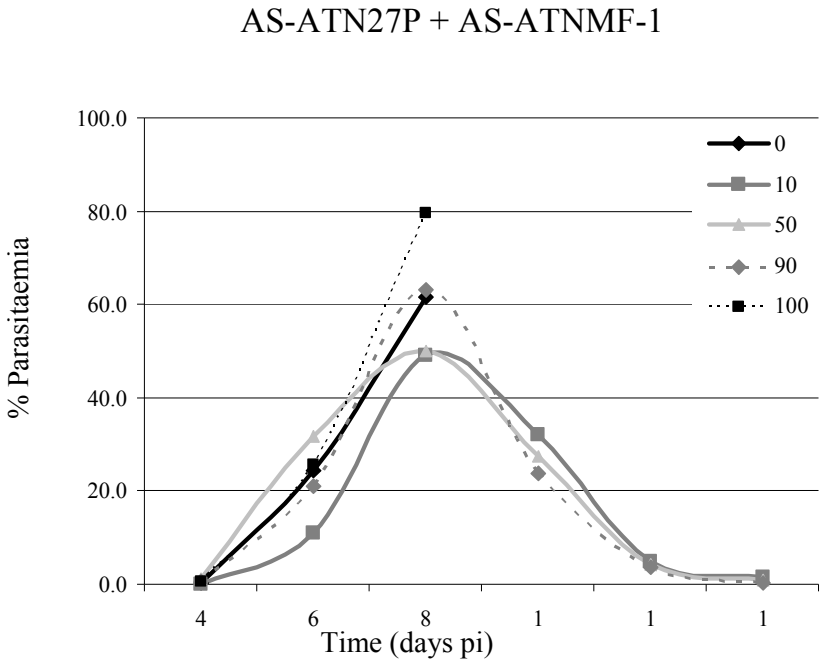


Figure 20 – Percentage (%) parasitaemias shown by mice inoculated with different proportion of the artesunate + mefloquine-resistant AS-ATNMF-1 and the parasites passaged twenty seven times in the absence of drug pressure, AS-ATN27P. Legend indicates initial percentage of AS-ATN27P parasites.

EVOLUTION OF PARASITE PROPORTIONS ALONG TIME

In order to assess the growth of AS-ATN27P and AS-ATNMF-1 in competition within the same host, the parasite proportions in the blood of mice injected with the different mixtures of parasites was analysed in a similar fashion as described above.

Firstly, a sample of the blood mixture used to inoculate each group of mice was analysed, in order to determine the actual proportions initially inoculated in each group of mice (Day 0,

Figure 21). In all cases, the proportion of wild-type and mutant alleles (representing AS-ATN27P and AS-ATNMF-1, respectively) corresponded approximately to the expected values.

After the inoculum, blood samples were extracted from each mouse every two days (from Day 4 onwards) and then analysed.

The group of mice which received an inoculum containing a higher proportion of AS-ATN27P (90:10) showed a slight increase in the proportion of the wild-type allele (representing AS-ATN27P) along time, from 90% on Day 4 to about 93% on Day 10. From Day 12 onwards, however, the proportion of the wild-type allele started to decrease, reaching 89% on day 14 (Figure 21).

The same pattern could be observed in mice which were inoculated with equal proportions of the two parasites (50:50). However, this pattern was more pronounced than in mice inoculated with a higher proportion of AS-ATN27P (90:10). Thus, there was an increase in the proportion of the AS-ATN27P allele from about 53% on Day 4 to almost 70% on Day 8 ($p<0.001$). On Day 10, the proportion began to decrease and reached about 50% on Day 14 (Figure 21).

Mice inoculated with higher proportions of AS-ATNMF-1 (10:90) showed the same pattern of increase of the wild-type allele (AS-ATN27P) along time, however, with some delay. Thus, the proportion of AS-ATN27P appeared stabilise around 10% up to Day 6. On Day 8 it started to increase, reaching about 25% on Day 12 ($p<0.0001$). On Day 14, however, there was a decrease to about half the value measured on Day 12 (about 13%) (Figure 21).

The proportions of each parasite were also calculated in samples extracted from mice inoculated only with AS-ATN27P (100:0) or AS-ATNMF-1 (0:100), and served as control. No differences were found in the initial inoculum and the expected values. In addition, no changes in the percentages of each allele were found along time in these two groups.

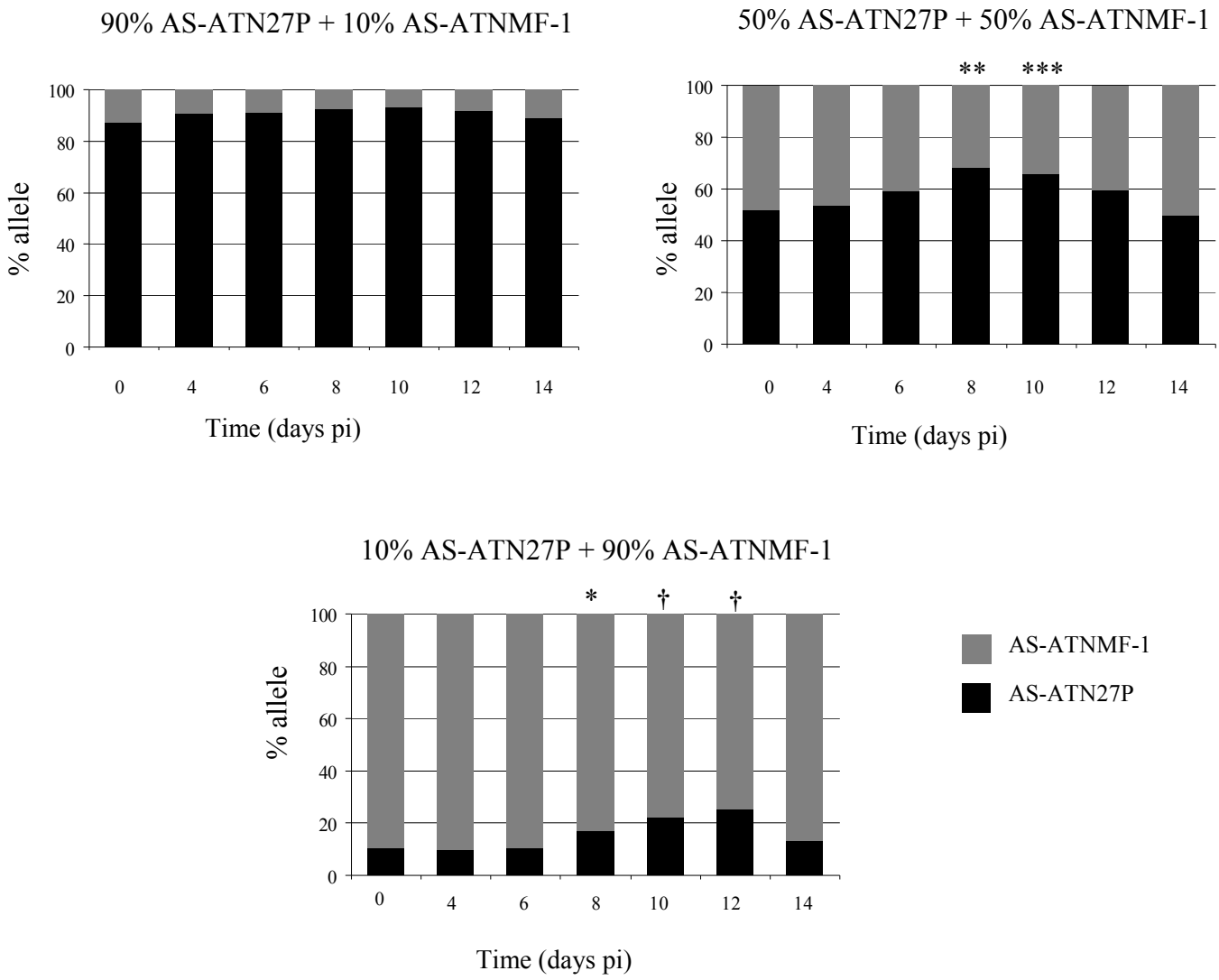


Figure 21 – Competition assays carried out using the artesunate + mefloquine-resistant AS-ATNMF-1 clone and the parasites passaged twenty seven times through mice in absence of drug, AS-ATN27P. Bars represent the mean of two animals. Gray bars represent AS-ATNMF-1 (mutant) allele and black bars represent AS-ATN27P (wild type) allele. Day 0 corresponds to samples obtained from the blood mixture used to inoculate each mouse. From Day 4 onwards, samples were obtained from each animal in each time point. Statistical analysis was made comparing the obtained proportions along time against the proportion inoculated into mice: * $p<0.02$; ** $p<0.002$; *** $p<0.006$; † $p<0.0001$.

CHAPTER SUMMARY

The growth of artesunate + mefloquine (ATN + MF)-resistant AS-ATNMF-1 clone was compared with the progenitor AS-ATN in the absence of drug treatment within the same host. This competition assay provided an indication that the acquisition of resistance to ATN + MF by AS-ATNMF-1 seems to confer no biological disadvantage when in competition with its sensitive progenitor AS-ATN.

In order to assess if consecutive sub-inoculations could be responsible for the changes in fitness observed on the selected AS-ATNMF-1 clone, the growth of this clone was compared to those of AS-ATN27P parasites in absence of drug treatment within the same host.

A slight difference between the ATN + MF-resistant AS-ATNMF-1 clone and the drug-sensitive AS-ATN27P parasites is expressed as an initial increase in AS-ATN27P proportions in relation to AS-ATNMF-1. However, this increase is followed by a reduction in the AS-ATN27P proportions, which tend to return to the initial level. This result seems to indicate that there may be a small fitness cost associated with ATN + MF-resistance in AS-ATNMF-1.

III.3. DISCUSSION

A clone showing moderate level of resistance to artesunate (ATN), AS-ATN was used for the selection of resistance to artesunate + mefloquine (ATN + MF), originating the AS-ATNMF-1 clone. Previous studies have indicated that an increase in drug resistance may cause a decrease in parasite fitness in absence of drug treatment (Rosario *et al,* 1978; Shinondo *et al,* 1994; Peters *et al,* 2002). Sometimes this decrease in fitness is directly related to the mechanism evolved by the parasite to escape the toxic effects exerted by drug treatment (Rosario *et al,* 1978; Walter, 1986).

In order to estimate if ATN + MF resistance could have caused a decrease in fitness of AS-ATNMF-1, this clone was inoculated in the same host together with its progenitor AS-ATN in different initial proportions. In all cases, AS-ATNMF-1 appeared to outgrow its sensitive progenitor, which suggests that AS-ATNMF-1 is fitter than AS-ATN. This result contradicts the above mentioned assumption. In fact, by observing the growth of each parasite alone, AS-ATN seems to have a slower growth rate than AS-ATNMF-1. Thus, through these initial observations, it could be expected that when grown within the same host AS-ATNMF-1 would outcompete AS-ATN.

Other studies have also showed that increased drug-resistance may not always be followed by reduced fitness when compared to the respective sensitive progenitor, as demonstrated in a chloroquine (CQ)-resistant *P. chabaudi* clone (Rosario *et al,* 1978). In addition, there is also evidence that in *P. falciparum* natural populations resistance may not always cause increased fitness cost to parasites, as in some regions CQ withdraw caused a reduction in frequency but not the complete removal of resistant parasites from the population (Borrmann *et al,* 2002; Mwai *et al,* 2009). A possible explanation to such trend may be that in some cases, evolution of resistance is accompanied by acquisition of secondary mutations that compensate for fitness loss induced by the mutation directly responsible for drug resistance (Nair *et al,* 2008; Jiang *et al,* 2008). As discussed in Chapter II, AS-ATNMF-1 has acquired three mutations when

compared to AS-ATN: i) amplification of the *mdr1* gene; ii) a non-synonymous point mutation in the PCHAS_132020 gene; and iii) a non-synonymous point mutation in the PCHAS_143160 gene. Amplification of *mdr1* has been previously described as responsible for increased drug resistance (Price *et al,* 1997; Price *et al,* 1999; Pickard *et al,* 2003; Price *et al,* 2004; Alker *et al,* 2007; Wongsrichanalai and Meshnick, 2008; Lim *et al,* 2009; Rogers *et al,* 2009; Chaijaroenkul *et al,* 2010; Chavchich *et al,* 2010) but, on the other hand, is also associated with reduced fitness of mutant parasites (Preechapornkul *et al,* 2009). As discussed in Chapter II, it is possible that the amplification of *mdr1* gene is the most relevant genetic change for the expression of the ATN + MF resistance phenotype displayed by AS-ATNMF-1. Conversely, the role of the additional two mutations disclosed is unknown. It is possible that the mutation in PCHAS_132020, replacing a STOP codon (present in AS-ATN) for a leucine in position 998, may restore protein function and that the D560Y mutation in PCHAS_143160 may affect its phosphorylation state. Although an extra copy of *mdr1* is very unlikely to contribute for the increased fitness showed by AS-ATNMF-1, there is a chance that the SNPs present in PCHAS_132020 and PCHAS_143160 could be acting as compensatory mutations, and therefore be associated with higher fitness of AS-ATNMF-1 when compared with AS-ATN. However it is important to retain that no functional analyses have been performed, and therefore the attribution of a role for these mutations in parasite fitness is merely speculative.

As such, it would be interesting to analyse the role of each individual mutation described as arising in AS-ATNMF-1 after selection of ATN + MF resistance. This could be done by introducing the mutated allele of each gene (or an extra copy in case of *mdr1*) in AS-ATN separately, generating three mutant clones that could then be compared to the original AS-ATN in within-host competition assays in untreated (and maybe also in treated) mice along time. Another means to assess the contribution of each mutation for fitness cost associated with drug resistance would be by performing genetic crosses between the AS-ATNMF-1 and AS-ATN,

and analyse the clones of the resulting cross-progeny regarding: i) response to drug-treatment and growth rate, and ii) the relation between phenotype and the mutations carried by the various clones generated.

There are reports indicating that in bacteria, compensatory mutations may arise in resistant laboratory strains undergoing repeated culturing or several sub-inoculations in mice (Kugelberg *et al,* 2005; Nilsson *et al,* 2006). In malaria parasites an increase in parasite fitness when compared with the respective progenitor was also observed after subjecting parasites to consecutive sub-inoculations through mice followed by transmission through mosquitoes (Mackinnon and Read, 1999; Walliker *et al,* 2005). We note that AS-ATNMF-1 was generated by repetitive sub-inoculations in presence of drug pressure, and these consecutive rounds of selection could have been responsible for the increase in fitness observed in these parasites when compared to the original AS-ATN clone. In order to test this hypothesis, the AS-ATN clone was passaged through untreated mice twenty seven times, generating the AS-ATN27P parasites. Subsequently, competition assays were performed between the ATN + MF-resistant AS-ATNMF-1 and AS-ATN27P. The pattern observed here is not as clear as the one observed for the competition assays performed using AS-ATNMF-1 and the original AS-ATN clone. AS-ATN27P seems to increase in proportion as parasitaemias grow. However, this tendency seems to disappear at the post-peak phase, and proportions of AS-ATNMF-1 and AS-ATN27P tend to return to initial levels. It could be argued that this may be an indication that AS-ATN27P is slightly fitter than AS-ATNMF-1 and this could be justified by the mutations present in AS-ATNMF-1, which are absent in AS-ATN and AS-ATN27P (such as *mdr1* amplification). On the other hand, the recovery of AS-ATNMF-1 after peak parasitaemia may point towards the opposite, i.e. there is no actual difference between AS-ATNMF-1 and AS-ATN27P. Collectively, these results seem to suggest that there may be a small fitness cost associated with

ATN + MF-resistance in AS-ATNMF-1, and that this fitness cost may be compensated by other factors.

One important consideration worth mentioning is that it is possible that AS-ATN27P does not consist of a clonal population. As such, there is a chance that the many parasites constituting this population show different growth rates and this could be contributing to the contrasting evidence observed when comparing AS-ATNMF-1 and AS-ATN27P proportions along time.

When observing the growth of AS-ATNMF-1 and AS-ATN27P separately, they seem to grow at similar rates. This implies that AS-ATN27P also has a higher growth rate than the original AS-ATN clone. In such context, it is important to highlight that the three mutations mentioned above as appearing in AS-ATNMF-1 after selection for ATN + MF resistance are not present in the AS-ATN27P, there being no difference between AS-ATN27P and the original AS-ATN. However, it is possible that other mutations have emerged after the twenty seven rounds of sub-inoculation that generated AS-ATN27P, and these could be responsible for the increase in growth capacity observed here when compared with AS-ATN.

The results presented here are representative of a single experiment and therefore additional assays would be necessary in order to fully understand the dynamics of within-host competition of the ATN + MF-resistant AS-ATNMF-1 and the ATN + MF-sensitive AS-ATN and AS-ATN27P parasites. Furthermore, the within-host competition assays described here focused solely on the erythrocytic asexual phase of parasite life cycle. However, there are other aspects that were not embraced, such as gametocyte production and transmission efficiency. It has been observed in *P. falciparum* natural populations that increased resistance to CQ and sulphadoxine-pyrimethamine (SP) is associated with lower density of asexual parasite forms in the host bloodstream when compared to what is observed for sensitive strains (Osman *et al,* 2007). On the other hand, in the same study, the drug-resistant parasites produced higher

number of gametocytes. This could result in compensation for the cost of drug-resistance shown by asexual blood forms, as it increases the chance of these parasites being transmitted by mosquitoes. However, the efficiency of gametocytes in generating viable offspring may also limit parasite spread and establishment in the population.

Very few studies focused on the effects of evolution of drug resistance on the transmission potential. This cannot be experimentally assessed in *P. falciparum* natural parasite populations due to ethical constraints. Also, there is a paucity of studies using rodent models for assessing this matter, and in addition results seem confounding. For instance, Shinondo and colleagues (1994) have shown that a PYR-resistant *P. berghei* mutant had similar growth in mice as its sensitive progenitor and, when in mosquitoes, the resistant clone showed delayed production of sporozoites when compared to its sensitive counterpart. On the other hand, in another study mosquitoes were fed with mixed infections of a *P. chabaudi* PYR-resistant clone and its sensitive progenitor. The proportion of the mutated and wild-type allele of *pcdhfr* (where the S106N mutation is responsible for PYR resistance) was assessed in oocysts carried by these mosquitos. The results were not consistent: the wild-type allele increased along time in two experiments, whereas the inverse was observed in the third experiment (Walliker *et al,* 2005).

In the above contexts, it would be interesting to compare the evolution of AS-ATNMF-1 in competition with AS-ATN or AS-ATN27P also taking into account the proportions of gametocytes produced, the sporogonic cycle and also perform a genetic cross between the drug-resistant and one of the drug-sensitive clones and then determining the percentage of the parasites in the resulting cross-progeny which would carry resistant alleles.

Fitness cost of drug-resistance may be an important determinant of spread of drug-resistant parasites, and as a consequence drug treatment useful life. Also, estimating fitness cost of resistance to a given drug may help in better shaping drug policies in order to minimise the factors that would contribute to the maintenance of resistant parasite sub-populations. This

subject was intensively debated in many studies (Walliker *et al,* 2005; Hastings and Donnelly, 2005). Most of them agree that the establishment of drug-resistant mutants in the parasite population is directly dependent on the drug pressure exerted over this population. In addition, considering a region where one drug has been used for long periods, and resistance is already established, it has been postulated that one advantage of the use of combination therapies is that if the cost of mutations conferring resistance to the first drug is up to a certain magnitude in absence of treatment, the administration of this drug as partner in a combination would reduce the effect of the pressure exerted by the first one and thus, result in the gradual removal of resistant strains from the population (Hastings and Donnelly, 2005).

Other factors may play an important role in maintenance and spread of drug resistance. One of them is transmission rate of a given area. In a high transmission setting, the host population usually shows high levels of immunity which implies: i) host immunity eliminates parasites regardless of their drug-resistant or sensitive status; ii) in places where hosts show high levels of immunity the occurrence of asymptomatic infections is frequent. As asymptomatic infections are rarely treated, drug selective pressure is therefore weak. In addition, untreated hosts consist of a reservoir of sensitive strains. In high transmission areas, most malaria cases consist of polyclonal infections (Arnot, 1998), and as such, parasites are subjected to constant competition with other (more sensitive) strains. Finally, in high transmission settings, frequent recombination in mosquitoes may break haplotypes apart, i.e. the reassortment of genetic material may separate mutations determining drug-resistance from the compensatory mutations required for reducing the biological cost of resistance. Together, all these factors seem to indicate that in high transmission settings, spread of drug-resistant parasite strains would be considerably slowed down. In addition, withdrawal of a failing treatment to which resistance is well established, could result in removal of the resistant strains from the parasite pool by the sensitive ones, in a similar fashion as observed for CQ resistance in Malawi

(Kublin *et al,* 2003; Mita *et al,* 2003; Laufer *et al,* 2006). Conversely, in low transmission settings, monoclonal infections are more frequent, and as such, an emerging mutant has higher chance of establishing an infection and consequently has higher chances of being transmitted to other hosts. In addition, most malaria cases are symptomatic, and therefore treated. This increases the advantage of drug-resistant strains over sensitive ones. Finally, due to the low rate of recombination, there is not much genetic diversity, and as consequence, there is smaller chance of advantageous mutations being separated by recombination with more sensitive strains. Resistant parasites which appear in these settings could not be removed from the parasite pool upon drug withdrawal, since there are no sensitive strains to outcompete them.

In this context, our model may be defined as best representing the evolution of drug resistance as described in low transmission areas: AS-ATN parasites were allowed to grow as monoclonal infections, where parasites were subjected to intensive drug pressure (repetitive treatment, even if in sub-therapeutic levels). No genetic recombination with drug-sensitive strains occurred along the course of selection. The parasites obtained using this approach seem to have little or no reduction in fitness when in competition with sensitive counterparts. In case *P. falciparum* natural populations were to follow this trend, it is possible that resistant parasites would persist in the parasite pool even if ATN + MF combination use was abolished. Still, it is important to consider that *P. falciparum* natural populations are highly complex and the factors presented above may consist of a simplification, since they do not fully account for the exposure of parasites to sub-therapeutic levels of drug, the stage of parasite life cycle at which resistance would emerge, etc (White *et al,* 2009).

The results obtained here consist of a preliminary analysis of within host competition between laboratory selected *P. chabaudi* clones. As such they may fail to completely replicate the complexity of *P. falciparum* natural populations. Nonetheless, they are the first to provide

insights on the within-host dynamics of ACT-resistant and sensitive parasite strains, and alert for the importance of rational design of treatment policies in endemic countries.

APPENDIX I

SELECTION OF ARTESUNATE + MEFLOQUINE RESISTANCE BY GENETIC RECOMBINATION

As mentioned in Chapter I, Section I.2, the sexual phase of Plasmodium life cycle takes place into the mosquito's midgut after the parasite's gametocytes are ingested during the blood meal. It is in this stage that the exchange of genetic material between two parasites may occur. This may lead to the emergence of new parasites bearing characteristics deriving from both parentals.

Assuming resistance to artesunate (ATN) and mefloquine (MF) happens separately in two different events (also assuming that resistance to each drug is associated with different genetic determinants), two different parasite populations would exist: one which is resistant to ATN and another one resistant to MF. If the genetic material of these parasites were to recombine in mosquitoes, this could lead to the emergence of parasites bearing genetic traits conferring resistance to both drugs in combination, without ever being exposed to the two drugs simultaneously.

As an attempt to test this hypothesis, a genetic cross between the parasites AS-ATN and AS-15MF was performed. As described in Material and Methods, Section 2.2, mice were inoculated with a mixture in equal proportions of the two clones, and used to feed *Anopheles stephensi* female mosquitoes. As controls, each clone was inoculated separately into two groups of mice and used to feed two other groups of mosquitoes. Due to its ability to be easily transmitted and in order to control the experimental conditions, the AJ clone was injected in a group of mice that were used to feed an additional group of mosquitoes.

On Day 9 after the blood meal ten mosquitoes from each group were dissected and inspected for the presence of oocysts in their midguts. It was possible to find oocysts in all midguts of the mosquitoes that fed on mice infected with the AJ clone. The mosquitoes that fed on mice carrying the AS-ATN clone also had oocysts in their midguts, although in a smaller amount (only 20% of the midguts analysed showed oocysts). No oocysts were found in the mosquitoes that fed on mice infected with the AS-15MF clone. The mosquitoes that fed on

mice carrying the mixture of AS-ATN and AS-15MF, showed the presence of oocysts in 40% of midguts analysed.

Regardless of the detection of oocysts in the midguts analysed, all groups of mosquitoes were allowed to feed on uninfected mice in order to transmit the infection. These mice had their blood inspected for the presence of parasites for up to nine days after transmission.

As expected, AJ was successfully transmitted. On the other hand, in spite of the detection of oocysts, the AS-ATN clone was not. The MF-resistant AS-15MF clone was also not transmitted to new mice. This was not surprising, since no oocysts were detected in the mosquitoes' midguts.

Importantly, the mosquitoes that fed on mice carrying the mixture of AS-ATN and AS-15MF clones were able to transmit the infection to new animals. The parasites borne by those mice (SAMPLE 1, Table 6) could represent the progeny resulting after a recombination event between the two parental clones. In order to verify that, the parasites were investigated for the presence of two genetic markers originating in each of the parental clones used for the genetic cross.

One of these markers is in *pcubp1* gene (PCHAS_020720), and is associated with resistance to ART derivatives. A mutation in this gene (G8089T) was identified as exclusive of the parental AS-ATN (Afonso et al, 2006), whereas the parental AS-15MF carries a different mutation, at a very close position (G8182T).

When analysing the parasite population transmitted through mosquito, the parasites showed the mutation G8089T, indicating these parasites carry the AS-ATN parental allele of this gene.

The other marker analysed was the lysine decarboxylase gene (PCHAS_100330), where a mutation A2467C is exclusive of the MF resistant parental AS-15MF. The parasite progeny

transmitted through mosquito bear the wild type version of this gene, indicating they also carry the AS-ATN parental allele of this gene (SAMPLE 1, Table 6).

No marker belonging to the AS-15MF parental was identified in the progeny resulting from the genetic cross. One explanation could be that the parasites bearing the AS-15MF alleles are underrepresented in the cross-progeny when compared to the ones carrying AS-ATN alleles for the genes investigated. If this was the case, treatment with either ATN, or MF, or both could eliminate the sensitive parasites of the progeny, and could allow the emergency of other parasite populations.

For that purpose, the parasite progeny transmitted through mosquitoes was further inoculated into a group of uninfected mice divided in four groups. Each group received drug treatment as follows: SAMPLE 2) DMSO vehicle; SAMPLE 3) 20 mg/kg/day of ATN, SAMPLE 4) 2 mg/kg/day of MF, and SAMPLE 5) 1 mg/kg/day of MF in combination with 7.5 mg/kg/day of ATN. The parasites were allowed to grow and were analysed for the same markers as the unselected progeny.

Regardless of drug treatment (or the absence of it, as in the DMSO treated group), all the parasites selected here carried the AS-ATN parental allele of both *pcubp1* and lysine decarboxylase genes (Table 6).

Due to the inability to confirm that the parasites generated here are recombinants, the experiments involving the progeny resulting from the AS-ATN x AS-15MF crossing were discontinued.

SAMPLE NAME	ORIGIN	*pcubp1*		lys decarboxylase
		PCHAS_020720 pos. 8089	**PCHAS_020720** pos. 8182	**PCHAS_100330** pos. 2467
REFERENCE		G	G	A
SAMPLE 1	AS-15MF X AS-ATN *	T	G	A
SAMPLE 2	AS-15MF X AS-ATN (NT)	T	G	A
SAMPLE 3	AS-15MF X AS-ATN (20 ATN)	T	G	A
SAMPLE 4	AS-15MF X AS-ATN (2 MEF)	T	G	A
SAMPLE 5	AS-15MF X AS-ATN (1 MEF+ 7.5 ATN)	T	G	A
PARENTAL	AS-ATN	T	G	A
PARENTAL	AS-15MF	G	T	C

Table 6 – Analysis of the progeny transmitted through mosquitoes for mutations present in genes *pcubp1* and lysine decarboxylase, implicated in the expression of resistance to artesunate (ATN) and mefloquine (MF), respectively. Sample 1, parasite progeny after transmission through mosquitoes was passaged into further mice treated as follows: Sample 2, DMSO vehicle; Sample 3, 20 mg/kg/day of ATN; Sample 4, 2 mg/kg/day of MF; and Sample 5, 1 mg/kg/day of MF in combination with 7.5 mg/kg/day of ATN. The genotypes of the parental clones AS-ATN and AS-15MF used for the genetic cross are also indicated.

One possible explanation for the absence of AS-15MF alleles in the parasites resulting from AS-ATN x AS-15MF crossing is that genetic recombination did not take place. In this case, in spite of being fed on mice carrying a mixture of the two clones (AS-ATN and AS-15MF), and showing oocysts in their midguts, the mosquitoes carried oocysts resulting from self-mating of AS-ATN gametes. Indeed, the inability of the AS-15MF clone to be transmitted through mosquitoes has been previously observed (Pedro Cravo, personal communication), indicating a possible reproductive fitness cost associated with the acquisition of MF resistance in this particular clone.

On the other hand, it is possible that genetic recombination between AS-ATN and AS-15Mf did take place. In this case, even after drug treatment, the AS-15MF alleles of the two

genes analysed here may still be underrepresented in the cross-progeny and for this reason, were not detected by PCR analysis.

Alternatively, in case genetic recombination has occurred, it is possible that for these two particular markers no parasites harbouring AS-15MF alleles were present in the cross-progeny. In fact, in order to determine the outcome of the crossing experiment, only two genes were investigated and in both cases, the identified allele represented the ATN-resistant parental clone. Other loci could be investigated in an attempt to confirm whether the ATN-resistant parental was the sole contributor to the production of the cross progeny. It is important to bear in mind that the two clones used here are isogenic, differing only for mutations arising upon each step of selection (see AS lineage). In this work, many mutations were identified in AS-ATN clone which could serve as additional markers for identifying the contribution of each parental. However, by the time the genetic cross was performed, the Solexa whole genome re-sequencing of AS-ATNMF-1 (which than allowed the detection of mutations and their origin in each clone of the AS lineage) was still not but a plan.

As future analyses, it would be interesting to investigate the above mentioned novel markers identified as first appearing in AS-ATN, for which AS-15MF bears the wild-type allele. In addition, it could be interesting to assess if any of the parasites in the cross progeny carries a duplicated copy of *pcmdr1*, another marker representative of the AS-15MF parental clone (in this context).

In case of any AS-15MF markers were identified in the AS-ATN x AS-15MF cross progeny, this would at least confirm the possibility of the biological concept where mating between two parasite clones resistant to ATN and MF, respectively, would be capable of generating viable progeny. Then, further investigation would be required in order to ascertain the level of resistance to treatment with ATN and MF administered alone or in combination shown by the cross progeny (as a whole or each of its component clones).

APPENDIX II

LINKAGE GROUP SELECTION

1. AS-ATNMF-1 x AJ GENETIC CROSS

The genome of the *Plasmodium sp.* is constituted by more than 5,500 genes. This represents a vast ground to be covered when searching for genetic modifications such as random mutations selected by drug pressure, as is the case of the AS-MFATN-5 and AS-ATNMF-1 clones selected here. In addition, genome sequencing and identification of mutations of drug-resistant parasites may provide clues on how drug pressure may affect evolution of the genome of the parasite, however, little can be inferred about the roles of these mutations in determining drug-resistant phenotype. Linkage Group Selection (LGS), on the other hand, provides an overview of the parasite's whole genome, and the influence of each locus in the expression of the phenotype of interest.

Therefore, in order to search the whole genome of the parasite for loci which are relevant for the expression of drug resistance, a genetic cross between the ATN + MF-resistant AS-ATNMF-1 and the genetically unrelated and drug sensitive AJ was performed.

So, as described in Material and Methods, Section 3.8.1, five mice were inoculated with mixed infections containing equal proportions of AS-ATNMF-1 and AJ clones and used to feed female mosquitoes divided into five groups. In addition, two groups of mosquitoes were allowed to feed on mice carrying each parental clone as controls for the experimental conditions. Mosquitoes' midguts were dissected in order to check for the presence of oocysts. Oocysts were found in the group that fed on the mouse carrying AS-ATNMF-1 parental, and in mosquitoes from three out of the five cages fed on mice carrying the AS-ATNMF-1 + AJ mixture. Mosquitoes that fed on AJ infected mouse did not show oocysts in their midguts.

DNA was then extracted from oocysts and analysed for polymorphisms present in gene *pcubp1*. Only the AS allele of this gene was found in oocysts dissected from mosquitoes that fed on the mouse carrying the AS-ATNMF-1 parental. On the other hand, and in spite of the inability of the AJ parental to produce oocysts when transmitted alone, the oocysts resulting

from the mixed infection showed a mixture of alleles from both parentals. This provided evidence that the cross between AS-ATNMF-1 and AJ was successful, i.e. the parasites transmitted through mosquitoes represented the progeny resulting from the genetic cross AS-ATNMF-1 x AJ (Table 7).

	Polymorphism							
	144		149		245		293	
	AS T	AJ G	AS A	AJ G	AS C	AJ T	AS A	AJ G
AS-ATNMF1	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
AS-ATNMF1xAJ	73.40	26.60	73.30	26.70	72.06	27.94	74.62	25.38
AS-ATNMF1xAJ	65.79	34.21	67.04	32.96	62.76	37.24	67.35	32.65
AS-ATNMF1xAJ	89.04	10.96	100.00	0.00	92.81	7.19	92.66	7.34

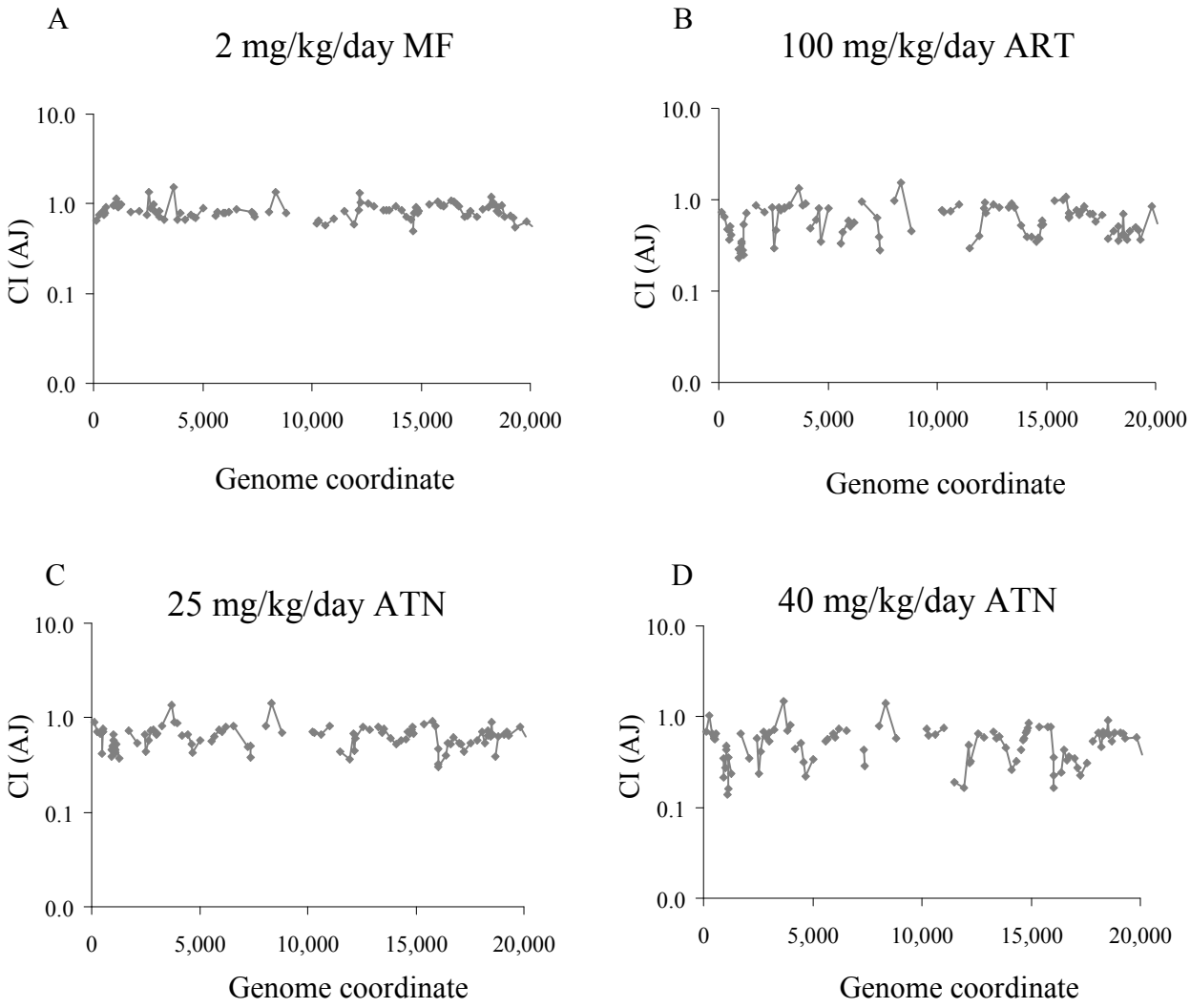
Table 7 – Proportional sequencing analysis of polymorphisms in *pcubp1* gene between AS and AJ parental.

2. SELECTION OF UNCLONED PROGENY

Mosquitoes showing oocysts in their midguts were allowed to feed in uninfected mice in order to transmit the infection. All mice used here showed detectable parasitaemias and on Day 10 after the transmission. The uncloned progeny resulting from AS-ATNMF-1 x AJ cross was propagated and subsequently inoculated into several mice, divided into six groups. Each group received distinct treatment as follows: A) 2 mg/kg/day of MF; B) 100 mg/kg/day of ART; C) 25 mg/kg/day of ATN; D) 40 mg/kg/day of ATN; E) 2 mg/kg/day of MF in combination with 20 mg/kg/day of ATN; and F) DMSO vehicle. The proportion of the sensitive allele in the samples was determined by Pyrosequencing™ for approximately 150 markers distributed along the whole genome of the parasites. A comparative index (CI) between the AS and AJ allele for

each polymorphic position was calculated for each treatment relative to the untreated control and the results were plotted as shown in Figure 22.

The results obtained by this methodology seem to indicate that the drug selection of the progeny was not sufficient to reduce the amount of sensitive alleles present in the mixture, in order to reveal the loci involved in resistance for each drug in AS-ATNMF-1 clone. Therefore, as shown in Figure 22, for every drug treated group, no dominant selection valley could be seen. Instead many small peaks and troughs were observed.



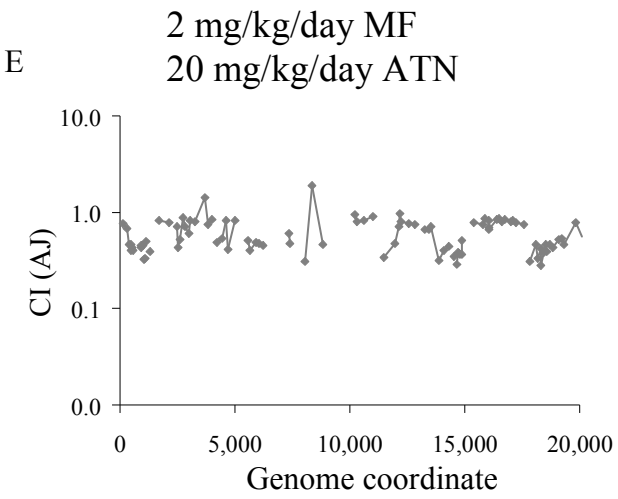


Figure 22 – Linkage Group Selection analysis of the cross-progeny resulting from AS-ATNMF-1 x AJ cross. The parasites were inoculated into six groups of mice, treated with different drugs as follows: A) 2 mg/kg/day of mefloquine (MF); B) 100 mg/kg/day of artemisinin; C) 25 mg/kg/day of artesunate (ATN); D) 40 mg/kg/day of ATN; and E) 2 mg/kg/day of MF in combination with 20 mg/kg/day of ATN. The last group was treated with the DMSO vehicle (non-treated control). The results for each marker along the genome were plotted as the proportion of AJ (sensitive) allele in each treatment, normalised against the non-treated control.

When comparing the drug doses used here and those used for the drug sensitivity test experiments, the absence of selection valleys in the LGS experiment is not at all surprising. All drug doses used for the selection of the cross-progeny obtained after AS-ATNMF-1 x AJ crossing are at least half of the ones used for the drug sensitivity tests, where AS-ATNMF-1 have always shown quick recovery and reached high parasitaemias.

The choice of the drug treatment used for the selection of the progeny resulting from a cross between a drug-resistant and a drug-sensitive parasite must indeed be made with caution. The drug selective pressure here must be low enough in order to maintain parasite sub-populations carrying sensitive alleles, however high enough in order to eliminate the most sensitive parasites from the population. In this sense, competition experiments where mice infected with mixtures of the sensitive and resistant parasites are subjected to treatment with

several drug doses are of great help in the optimisation of the drug dose for treating a progeny resulting from crossing these two clones.

Performing new LGS assays may help in determining the relevance of the mutations identified as appearing in AS-ATNMF-1 in drug resistance, as well as the possible contribution of other mutations already present in the parental AS-ATN which were inherited by AS-ATNMF-1.

				AS-WTSI			AS-SENS					ATNMF-1										
	Chromosome	Type	Analysis	Start (indels only)	End		Base	Read depth	Main Base	Relative coverage	Comparative coverage	Relative coverage	Base	Read Depth	SSAHA/MAQ Quality	Gene ID		Nearest gene	Telomere (x)	Gene abbreviation	Annotation
1	2	SNP	MAQ/SSAHA		217,047	C	C	56	C	1.4	0.2	0.3	A	13	62	PCHAS_020720	**V2697F**			UBP1	deubiquitinating enzyme
2	6	SNP	MAQ/SSAHA		636,862	A	A	19	A	0.5	0.6	0.3	G	14	70			3-PCHAS_061710			seryl-tRNA synthetase
3	7	SNP	MAQ/SSAHA		994,546	G	G	67	G	1.7	0.4	0.7	A	34	99	PCHAS_072830	**S109N**			DHFR	dihydrofolate reductase
4	11	SNP	MAQ/SSAHA		996,332	G	G	56	G	1.4	1.1	1.6	T	80	99	PCHAS_112780	**A173E**			AAT1	aminoacid transporter
5	13	SNP	MAQ/SSAHA		805,658	A	A	31	A	0.8	0.1	0.1	T	3	15	PCHAS_132020	**K998***				conserved plasmodium protein
6	13	SNP	MAQ/SSAHA		805,659	A	A	32	A	0.8	0.1	0.1	T	3	15	PCHAS_132020	**K998L**				conserved plasmodium protein
7	13	SNP	MAQ/SSAHA		1,322,938	G	G	35	G	0.9	1.9	1.7	A	85	99	PCHAS_133430	**E738K**				26S proteasome subunit
8	14	SNP	SSAHA		936,945	T	T	13	T	0.3	0.2	0.1	G	4	20			5-PCHAS_142600			conserved plasmodium protein
9	14	SNP	MAQ/SSAHA		1,155,448	C	C	46	C	1.2	1.4	1.7	A	84	99	PCHAS_143160	**Y560D**				transferase, ARM, WD
10	1	SNP	SSAHA		483,843	T						0.1	G	5	12			3-PCHAS_011300			conserved Plasmodium protein
11	3	SNP	MAQ/SSAHA		82,406	T	T	11	T	0.3	0.7	0.2	G	10	20			PCHAS_030230			Plasmodium exported protein
12	7	SNP	SSAHA		689,803	A						0.1	T	3	15						
13	11	SNP	SSAHA		214,749	A						0.2	C/A	11	17			PCHAS_110620-5			rhomboid protease
14	11	SNP	SSAHA		415,965	A						0.1	C	5	12	PCHAS_111100					myo-inositol 1-phosphate synthase
15	13	SNP	SSAHA		1,792,583	C							T	10	2						
16	bin	SNP	SSAHA		247,620	T						0.1	C	3	6			5.PCHAS_000660			Pc-fam
17	bin	SNP	SSAHA		411,999	T						0.1	C	3	8			PCHAS_001050-3			CIR
18	7	SNP	MAQ/SAAHA		876,917	A	A	54	A	1.4	0.2	0.3	G	16	80			PCHAS_072420-3			conserved malaria protein
19	7	SNP	MAQ/SSAHA		876,919	C	C	54	C	1.4	0.2	0.3	A	17	85			PCHAS_072420-3			conserved malaria protein
20	12	SNP	SSAHA		566,547	A						0.3	G/A	14	27	PCHAS_121630					40S ribosomal protein S3A
21	13	SNP	MAQ		56,840	G	G/A	52	G	1.3	0.1	0.1	A	6	45	PCHAS_130140					HAD hydrolase, putative

Appendix III - All SNPs identified by Solexa in AS-ATNMF-1. Mutations with high confidence of representing true SNPs are highlighted in green, intermediate level, highlighted in orange and low levels of confidence highlighted in red.

				AS-WTSI						AS-50SP				ATNMF-1								
	Chromosome	Type	Analysis	Start (indels only)	End		Base	Read depth	Main Base	Relative coverage	Comparative coverage	Relative coverage	Base	Read Depth	SSAHA/MAQ Quality	Gene ID		Nearest gene	Telomere (x)	Gene abbreviation	Annotation
1	**3**	Deletion	SSAHA	472,273	472,275	3									36/43	PCHAS_031370					conserved Plasmodium protein
2	**13**	Deletion	SSAHA	56	60,867	60,812		25		0.6	0.0	0.0		0		PCHAS_130140					60 Kb deletion
3	**12**	CNV	SSAHA	1,382,067	1,462,751	80,685		43		1.0	2.0	1.9		96		PCHAS_123820					
4	**7**	Deletion	SSAHA	876,894	876,927	34												PCHAS_072420-3'			conserved malaria protein (34 bp deletion)
5	**5**	indel	SSAHA	682,682	685,106	2,425		186		4.2	0.2	0.9		44		PCHAS_051920					S-Antigen
6	**bin**	Deletion	SSAHA	305,928	339,914	33,987		31		0.7	0.0	0.0		0		PCHAS_000890					30 kb deletion involving part of contig 11844
7	**5**	SNP	MAQ		684,139	A	A/G	43	A	1.1	0.7	0.7	G	36	117	PCHAS_051920					S-antigen,_putative
8	**5**	SNP	MAQ/SSAHA		684,988	G	G	37	G	0.9	0.2	0.2	A	10	47	PCHAS_051920					S-antigen,_putative
9	**5**	SNP	MAQ/SSAHA		684,989	A	A	37	A	0.9	0.2	0.2	G	10	44	PCHAS_051920					S-antigen,_putative
10	**1**	Deletion	SSAHA	146,750	146,750	1									3/6			5'-PCHAS_010370			mitochondrial ribosomal protein L41 precursor
11	**1**	indel	SSAHA	342,966	342,993	28						0.2		8		PCHAS_010880					conserved Plasmodium protein
12	**1**	indel	SSAHA	349,058	349,087	30						0.1		6				5'-PCHAS_010900			conserved Plasmodium protein
13	**1**	indel	SSAHA	440,621	440,642	22						0.1		5				5'-PCHAS_011200			conserved Plasmodium protein
14	**1**	indel	SSAHA	474,775	474,825	51		354		8.0	0.2	1.4		72		PCHAS_011280					Myosin-like protein
15	**2**	indel	SSAHA	6,137	6,149	13		22		0.5	0.2	0.1		6		PCHAS_020020					TatD-like deoxyribonuclease
16	**2**	indel	SSAHA	15,580	15,598	19						0.1		6				3'-PCHAS_020080			conserved Plasmodium protein
17	**2**	Deletion	SSAHA	267,432	267,432	1									2/4			5'-PCHAS_020840			conserved Plasmodium protein
18	**2**	indel	SSAHA	274,793	274,834	42		25		0.6	0.2	0.1		7				5'-PCHAS_020860			aspartyl-tRNA synthetase
19	**2**	Deletion	SSAHA	451,164	451,164	1									3/6			5'-PCHAS_021280			transcription factor with AP2 domain
20	**3**	indel	SSAHA	19,730	19,773	44												3'-PCHAS_030070			Pc-fam
21	**3**	indel	SSAHA	53,546	53,574	29										PCHAS_030170					Pc-fam
22	**3**	indel	SSAHA	77,073	77,086	14												3'-PCHAS_030210			CIR
23	**3**	indel	SSAHA	80,256	80,548	293												3'-PCHAS_030230			Plasmodium exported protein
24	**3**	indel	SSAHA	111,510	111,524	15		34		0.8	0.2	0.2		8		PCHAS_030320					conserved Plasmodium protein
25	**3**	Deletion	SSAHA	131,951	131,951	1									3/6			5'-PCHAS_030390			5'-3' exonuclease
26	**3**	indel	SSAHA	137,023	137,033	11		53		1.2	0.2	0.3		15		PCHAS_030400					conserved Plasmodium protein

27	**3** indel	SSAHA	152,213	152,223	11	25	0.6	0.2	0.1	6	PCHAS_030450				aminotrnasferase
28	**3** Deletion	SSAHA	175,685	175,685	1					2/4		5'-PCHAS_030550			conserved Plasmodium protein
29	**3** indel	SSAHA	192,230	192,268	39							5'-PCHAS_030600			iron-sulfur assembly protein
30	**3** indel	SSAHA	211,730	211,752	23							5'-PCHAS_030650			adenylosuccinate lyase
31	**3** indel	SSAHA	347,910	347,936	27							5'-PCHAS_031010			conserved Plasmodium protein
32	**3** Deletion	SSAHA	371,473	371,473	1					2/4		5'-PCHAS_031080			GDP-fructose:GMP antiporter
33	**3** indel	SSAHA	482,384	482,395	12							3'-PCHAS_031400			RING zinc finger protein
34	**3** indel	SSAHA	588,828	588,841	14	34	0.8	0.2	0.2	9	PCHAS_031720				DEAD/DEAH box helicase
35	**3** indel	SSAHA	606,590	606,600	11							5'-PCHAS_031770			40S ribosomal protein S30
36	**3** indel	SSAHA	621,961	622,254	294						PCHAS_031830				Liver Stage Antigen 3
37	**4** indel	SSAHA	227,234	227,247	14							5'-PCHAS_040650			EB1 homolog
38	**4** indel	SSAHA	454,851	454,879	29							5'-PCHAS_041310			conserved Plasmodium protein
39	**4** Deletion	SSAHA	535,524	535,524	1					3/6		5'-PCHAS_041540			HVA22/TB2/DP1 family protein
40	**4** indel	SSAHA	793,798	793,904	107								x		
41	**5** Deletion	SSAHA	404,719	404,719	1					2/4	PCHAS_050980				conserved Plasmodium protein
42	**5** indel	SSAHA	524,702	524,723	22							5'-PCHAS_051400			conserved Plasmodium protein
43	**5** indel	SSAHA	643,158	643,169	12							5'-PCHAS_051790			Sec1 family protein
44	**5** indel	SSAHA	715,298	715,323	26							3'-PCHAS_052020			pyruvate kinase 2
45	**5** indel	SSAHA	796,756	796,766	11							5'-PCHAS_052190			transcription factor with AP2 domain
46	**6** Deletion	SSAHA	288,626	288,626	1					2/4		3'-PCHAS_060750			conserved Plasmodium protein
47	**6** indel	SSAHA	390,005	390,017	13							PCHAS_061000-5'			conserved Plasmodium protein
48	**6** Deletion	SSAHA	593,756	593,756	1					2/3		5'-PCHAS_061570			conserved Plasmodium protein
49	**6** indel	SSAHA	675,837	675,848	12							3'-PCHAS_061760			cysteine repeat modular protein 2
50	**6** Deletion	SSAHA	814,984	814,984	1					3/6		3'-PCHAS_062220			tRNAHis guanylyltransferase
51	**7** indel	SSAHA	72	730	659								x		
52	**7** indel	SSAHA	31,425	31,460	36							PCHAS_070080-5'			Plasmodium exported protein
53	**7** indel	SSAHA	62,990	63,014	25						PCHAS_070150				Pc-fam1
54	**7** Deletion	SSAHA	274,087	274,087	1					3/5		PCHAS_070740-5'			conserved Plasmodium protein
55	**7** Deletion	SSAHA	313,205	313,205	1					4/7		5'-PCHAS_070860			dolichyl-phosphate b-D-mannosyltransferase
56	**7** Deletion	SSAHA	901,865	901,865	1					2/4		3'-PCHAS_072520			conserved Plasmodium protein
57	**7** indel	SSAHA	918,682	918,700	19							5'-PCHAS_072560			RNA binding protein
58	**7** Deletion	SSAHA	975,600	975,601	2					5/9		5'-PCHAS_072770			Prohibitn-like protein
59	**7** indel	SSAHA	1,124,529	1,124,776	248						PCHAS_073120				phospholipase
60	**7** indel	SSAHA	1,146,026	1,146,057	32							5'-PCHAS_073170			Pc-fam1
61	**8** Deletion	SSAHA	40,129	40,130	2					2/4	PCHAS_080100				conserved Plasmodium protein
62	**8** indel	SSAHA	230,789	230,800	12						PCHAS_080470				conserved plasmodium protein

63	**8** Deletion	SSAHA	596,818	596,819	2					2/3		3'-PCHAS_081440			conserved plasmodium protein
64	**8** Deletion	SSAHA	696,273	696,273	1					2/3	PCHAS_081770				conserved plasmodium protein
65	**8** indel	SSAHA	1,140,891	1,140,910	20	42	0.9	0.2	0.2	10	PCHAS_083110				leucine-rich repeat protein 8
66	**8** Deletion	SSAHA	1,277,029	1,277,029	1					2/4	PCHAS_083540				conserved Plasmodium protein
67	**9** indel	SSAHA	16,533	16,564	32							5'-PCHAS_090050			Pc-fam1
68	**9** indel	SSAHA	58,412	58,433	22							3'-PCHAS_090150			syntaxin
69	**9** Deletion	SSAHA	97,123	97,123						2/3		5'-PCHAS_090250			histone H2B
70	**9** indel	SSAHA	154,767	154,784	18							5'-PCHAS_090440			lsm4 homologue
71	**9** Deletion	SSAHA	195,680	195,680						2/4		PCHAS_090520-5'			transcription factor with AP2 domain
72	**9** indel	SSAHA	554,553	554,568	16						PCHAS_091600				conserved plasmodium protein
73	**9** Deletion	SSAHA	1,113,660	1,113,660	1					14/16	PCHAS_093250				conserved Plasmodium protein
74	**9** Insert	SSAHA	1,298,948	1,298,948	1					6/11	rRNA				rRNA
75	**9** Deletion	SSAHA	1,301,785	1,301,785	1					2/2		RRNA-5			
76	**9** indel	SSAHA	1,340,335	1,340,356	22							5'-PCHAS_093820			CIR
77	**9** indel	SSAHA	1,344,932	1,345,061	130								x		
78	**10** indel	SSAHA	113	849	737								x		
79	**10** Deletion	SSAHA	93,825	93,825						2/3		PCHAS_100220-5'			conserved Plasmodium protein
80	**10** indel	SSAHA	140,824	140,837	14							5'-PCHAS_100320			conserved plasmodium protein
81	**10** Deletion	SSAHA	324,986	324,986	1					2/4		5'-PCHAS_100770			40S ribosomal processing protein
82	**10** Deletion	SSAHA	413,152	413,152	1					2/4		5'-PCHAS_101010			conserved Plasmodium protein
83	**10** indel	SSAHA	489,824	489,855	32						PCHAS_101180				protein kinase
84	**10** indel	SSAHA	493,237	493,255	19						PCHAS_101180				protein kinase
85	**10** indel	SSAHA	805,766	805,796	31						PCHAS_102050				conserved plasmodium protein
86	**10** Deletion	SSAHA	845,011	845,011	1					¾		3'-PCHAS_102160			conserved plasmodium protein
87	**10** Deletion	SSAHA	872,167	872,168	2					2/4		5'-PCHAS_102280			40S ribosomal protein S25
88	**10** indel	SSAHA	955,501	956,122	622							3'-PCHAS_102520			calmodulin
89	**10** Deletion	SSAHA	957,793	957,793	1					2/3		3'-PCHAS_102520			calmodulin
90	**10** indel	SSAHA	1,012,866	1,012,887	22						PCHAS_102610				cyclic nucleotide-binding protein
91	**10** Deletion	SSAHA	1,081,207	1,081,207	1					4/8		5'-PCHAS_102780			serine C-palmitoyltransferase
92	**10** Deletion	SSAHA	1,160,559	1,160,559	1					3/6		5'-PCHAS_103000			SufC ATPase
93	**10** Deletion	SSAHA	1,176,268	1,176,268	1					2/4		PCHAS_103050-5'			ubiquitin conjugating enzyme
94	**10** indel	SSAHA	1,303,436	1,303,455	20						PCHAS_103360				conserved Plasmodium protein
95	**10** indel	SSAHA	1,353,510	1,353,520	11							3'-PCHAS_103470			40S ribosomal protein S8e
96	**10** Deletion	SSAHA	1,418,647	1,418,647	1					2/4	PCHAS_103650				conserved Plasmodium protein
97	**10** indel	SSAHA	1,446,774	1,446,787	14						PCHAS_103720				BOP1-like protein
98	**11** indel	SSAHA	61	159	99								x		

99	**11** indel	SSAHA	616,585	616,623	39								5'-PCHAS_111760		N-acetylglucosamine transferase
100	**11** indel	SSAHA	788,148	788,170	23								5'-PCHAS_112230		Ribonuclease HII
101	**11** Deletion	SSAHA	816,282	816,282	1					2/3			3'-PCHAS_112310		N-acetylglucosaminylphosphatidylinositol deacetylase
102	**11** indel	SSAHA	942,002	942,014	13						PCHAS_112630				HECT-domain (ubiquitin-transferase)
103	**11** indel	SSAHA	968,182	968,212	31						PCHAS_112690				conserved Plasmodium protein
104	**11** Deletion	SSAHA	1,032,915	1,032,915	1					2/4			5'-PCHAS_112850		conserved Plasmodium protein
105	**11** indel	SSAHA	1,188,138	1,188,149	12								5'-PCHAS_113300		glutamate - tRNA ligase
106	**12** indel	SSAHA	22	475	454									x	
107	**12** indel	SSAHA	46,671	46,741	71						PCHAS_120130				Plasmodium exported protein
108	**12** Deletion	SSAHA	135,493	135,493	1					3/5			PCHAS_120350-5'		conserved Plasmodium protein
109	**12** indel	SSAHA	288,143	288,154	12								5'-PCHAS_120840		Zinc finger protein
110	**12** indel	SSAHA	343,121	343,143	23								3'-PCHAS_121020		conserved Plasmodium protein
111	**12** Deletion	SSAHA	362,833	362,833	1					2/1			5'-PCHAS_121090		QF122 antigen
112	**12** indel	SSAHA	538,134	538,155	22								PCHAS_121540-5'		conserved Plasmodium protein
113	**12** Deletion	SSAHA	582,127	582,127	1					2/4			PCHAS_121660-5'		conserved Plasmodium protein
114	**12** Deletion	SSAHA	721,489	721,489	1					¾			5'-PCHAS_122020		CRT
115	**12** Deletion	SSAHA	768,299	768,299	1					2/4	PCHAS_122130				conserved Plasmodium protein
116	**12** Deletion	SSAHA	824,806	824,806	1					2/3			5'-PCHAS_122310		pepsinogen
117	**12** Deletion	SSAHA	1,154,153	1,154,153	1					2/3	PCHAS_123130				conserved Plasmodium protein
118	**12** Deletion	SSAHA	1,254,371	1,254,371	1					2/4			3'-PCHAS_123420		conserved Plasmodium protein
119	**13** indel	SSAHA	124,307	124,752	446	98	2.2	0.2	0.4	22	PCHAS_130320				Cyclin-related protein
120	**13** indel	SSAHA	216,839	216,849	11								5'-PCHAS_130520		conserved Plasmodium protein
121	**13** indel	SSAHA	412,367	412,709	343	112	2.5	0.1	0.3	17	PCHAS_130970				TRAP/MIC-2 family member
122	**13** Deletion	SSAHA	511,435	511,436	2					2/4			5'-PCHAS_131190		conserved Plasmodium protein
123	**13** Deletion	SSAHA	631,925	631,926	2					2/4			3'-PCHAS_131550		SEL-1 protein
124	**13** indel	SSAHA	759,029	759,411	383	121	2.7	0.1	0.3	14	PCHAS_131900				rhoptry neck protein 2
125	**13** Deletion	SSAHA	1,624,096	1,624,096	1					2/4			5'-PCHAS_134330		plasmepsin V
126	**13** Deletion	SSAHA	1,681,184	1,681,184	1					3/5			3'-PCHAS_134490		phosphoribosylpyrophosphate synthetase
127	**13** indel	SSAHA	1,763,608	1,763,624	17						PCHAS_134680				conserved Plasmodium protein
128	**13** Deletion	SSAHA	1,871,674	1,871,674	1					2/3			5'-PCHAS_134940		conserved Plasmodium protein
129	**13** Deletion	SSAHA	1,988,346	1,988,346	1					2/3			PCHAS_135370-5'		conserved Plasmodium protein
130	**13** indel	SSAHA	2,002,277	2,002,295	19						PCHAS_135420				conserved Plasmodium protein
131	**13** Deletion	SSAHA	2,043,599	2,043,599	1					4/7	PCHAS_135580				conserved Plasmodium protein
132	**13** indel	SSAHA	2,439,367	2,439,384	18						PCHAS_136610				conserved Plasmodium protein
133	**13** indel	SSAHA	2,589,396	2,589,441	46								3'-PCHAS_137060		phospholipase
134	**14** indel	SSAHA	20	141	122									x	

135	**14**	indel	SSAHA	47,081	47,382	302							3'-PCHAS_140130		CIR
136	**14**	indel	SSAHA	75,415	75,433	19							5'-PCHAS_140190		Pc-fam1
137	**14**	indel	SSAHA	84,847	84,857	11							PCHAS_140200-3'		Pc-fam1
138	**14**	Deletion	SSAHA	217,854	217,854	1					2/2		3'-PCHAS_140520		conserved Plasmodium protein
139	**14**	Deletion	SSAHA	703,779	703,779	1					2/3		5'-PCHAS_141920		conserved Plasmodium protein
140	**14**	indel	SSAHA	876,749	876,775	27	221	5.0	0.2	1.0	51		3'-PCHAS_142390		drug/metabolite transporter
141	**14**	indel	SSAHA	909,600	909,600	1					3/6		5'-PCHAS_142500		ATPase
142	**14**	indel	SSAHA	1,372,295	1,372,311	17							5'-PCHAS_143830		conserved Plasmodium protein
143	**14**	indel	SSAHA	1,415,403	1,415,426	24							5'-PCHAS_143950		transcription factor with AP2 domain(s)
144	**14**	Deletion	SSAHA	1,415,933	1,415,933	1					5/5		5'-PCHAS_143950		transcription factor with AP2 domain(s)
145	**14**	Insert	SSAHA	1,486,111	1,486,111	1							5'-PCHAS_144110		conserved Plasmodium protein
146	**14**	indel	SSAHA	1,546,661	1,546,678	18							PCHAS_144280-5'		conserved Plasmodium protein
147	**14**	indel	SSAHA	1,595,169	1,595,276	108							3'-PCHAS_144430		Cyclin-related protein
148	**14**	indel	SSAHA	1,608,769	1,609,165	397	69	1.6	0.1	0.1	4	PCHAS_144430			Cyclin-related protein
149	**14**	indel	SSAHA	1,922,002	1,922,015	14							5'-PCHAS_145220		RNA binding protein
150	**14**	Deletion	SSAHA	1,958,523	1,958,523	1					2/4		3'-PCHAS_145310		conserved Plasmodium protein
151	**14**	indel	SSAHA	2,320,575	2,320,585	11							5'-PCHAS_146400		conserved Plasmodium protein
152	**14**	Deletion	SSAHA	2,321,521	2,321,521	1					3/5		5'-PCHAS_146400		conserved Plasmodium protein
153	**14**	indel	SSAHA	2,514,727	2,514,817	91								x	
154	**bin**	indel	SSAHA	194	1,494	1,301								x	
155	**bin**	Insert	SSAHA	10,767	10,767	1					2/4		3'-PCHAS_000040		CIR
156	**bin**	indel	SSAHA	17,521	17,618	98							5'-PCHAS_000050		Pc-fam1
157	**bin**	indel	SSAHA	56,257	56,285	29								x	
158	**bin**	indel	SSAHA	116,318	117,043	726								x	
159	**bin**	indel	SSAHA	145,038	145,081	44						PCHAS_000380			none
160	**bin**	indel	SSAHA	208,331	208,874	544						PCHAS_000550			EMP1
161	**bin**	indel	SSAHA	221,643	222,191	549								x	
162	**bin**	Insert	SSAHA	232,938	232,938						2/4		PCHAS_000630-5'		PYST-C2 homologue
163	**bin**	Deletion	SSAHA	260,785	262,250	1,466								x	
164	**bin**	Deletion	SSAHA	352,892	352,892	1					13/13		5'-PCHAS_000050		conserved Plasmodium protein
165	**bin**	indel	SSAHA	412,003	412,076	74							3'-PCHAS_001060		CIR
166	**bin**	indel	SSAHA	417,398	417,520	123								x	
167	**1**	CNV	SSAHA	25,392	27,151	1,760	41	0.9	1.5	1.3	68	PCHAS_010060			conserved Plasmodium protein
168	**1**	CNV	SSAHA	529,707	532,679	2,973				1.2	62	PCHAS_011430			reticulocyte binding protein
169	**1**	CNV	SSAHA	561,780	563,593	1,814				0.0		PCHAS_011490			Pc-fam
170	**1**	CNV	SSAHA	574,110	575,211	1,102				0.1	3	PCHAS_011520			CIR

171	**3** CNV	SSAHA	11,751	12,929	1,179						PCHAS_030040		CIR
172	**3** CNV	SSAHA	40,114	41,716	1,603						PCHAS_030140		Pc-fam
173	**3** CNV	SSAHA	67,747	67,991	245						PCHAS_030190		Pc-fam
174	**4** CNV	SSAHA	748,962	749,599	638						PCHAS_041990		Pc-fam
175	**5** CNV	SSAHA	43,412	45,693	2,282						PCHAS_050100		reticulocyte binding protein
176	**6** CNV	SSAHA	32,175	32,848	674						PCHAS_060090		Pc-fam
177	**8** CNV	SSAHA	1,352,780	1,353,132	353						PCHAS_083760		CIR
178	**9** CNV	SSAHA	889,314	889,544	231						PCHAS_092590		Pc-fam
179	**10** CNV	SSAHA	18,481	18,770	290						PCHAS_100050		Pc-fam1
180	**10** CNV	SSAHA	1,020,416	1,020,650	235						PCHAS_102630		NOT family protein
181	**10** CNV	SSAHA	1,630,200	1,630,574	375						PCHAS_104260		CIR
182	**11** CNV	SSAHA	1,184,288	1,184,534	247	55	1.2	1.7	2.1	105	PCHAS_113280		elongation factor 1 alpha
183	**11** CNV	SSAHA	1,187,665	1,187,896	232	0	0.0	###	0.3	13	PCHAS_113290		elongation factor 1 alpha
184	**11** CNV	SSAHA	1,696,498	1,697,104	607						PCHAS_114700		Pc-fam
185	**13** CNV	SSAHA	2,133,663	2,133,883	221						PCHAS_135850		conserved Plasmodium protein
186	**14** CNV	SSAHA	102,875	103,081	207						PCHAS_140250		CLAG
187	**2** indel	SSAHA	301,741	301,767	27	20	0.5	0.2	0.1	5	PCHAS_020900		ubiquitin carboxyl-terminal hydrolase
188	**3** indel	SSAHA	523,111	523,127	17	21	0.5	0.1	0.1	3	PCHAS_031520		vacuolar protein-sorting protein VPS45
189	**3** indel	SSAHA	524,946	524,964	19	26	0.6	0.2	0.1	5	PCHAS_031520		vacuolar protein-sorting protein VPS45
190	**7** indel	SSAHA	103,522	103,536	15						PCHAS_070250		erythrocyte binding protein 1
191	**8** indel	SSAHA	1,355,069	1,355,081	13							5'-PCHAS_083770	haloacid dehalogenase-like hydrolase
192	**9** Deletion	SSAHA	919,771	919,771	1					2/3		5'-PCHAS_092710	26S proteasome regulatory complex subunit

Appendix IV – Indels and CNVs identified by Solexa. Mutations with high confidence of representing true indels/CNVs are highlighted in green, intermediate level, highlighted in orange and low levels of confidence highlighted in red.

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           1                                                                                                   120
   AS-WTSI  CGTATAAAGG CTGTGACAAC ATTTTAGATA GCCATTATAT ATTTTTCTTT TCAATTTGTA TAATCTACGT TATAAAAGTT CAACCTTTTA AAACAAATGT GGGAATAAAT TACGGTTTGC
   AS-SENS  CGTATAAAGG CTGTGACAAC ATTTTAGATA GCCATTATAT ATTTTTCTTT TCAATTTGTA TAATCTACGT TATAAAAGTT CAACCTTTTA AAACAAATGT GGGAATAAAT TACGGTTTGC
    AS-PYR  CGTATAAAGG CTGTGACAAC ATTTTAGATA GCCATTATAT ATTTTTCTTT TCAATTTGTA TAATCTACGT TATAAAAGTT CAACCTTTTA AAACAAATGT GGGAATAAAT TACGGTTTGC
   AS-50SP  CGTATAAAGG CTGTGACAAC ATTTTAGATA GCCATTATAT ATTTTTCTTT TCAATTTGTA TAATCTACGT TATAAAAGTT CAACCTTTTA AAACAAATGT GGGAATAAAT TACGGTTTGC
 AS-ATNMF-1  CGTATAAAGG CTGTGACAAC ATTTTAGATA GCCATTATAT ATTTTTCTTT TCAATTTGTA TAATCTACGT TATAAAAGTT CAACCTTTTA AAACAAATGT GGGAATAAAT TACGGTTTGC
  Consensus  CGTATAAAGG CTGTGACAAC ATTTTAGATA GCCATTATAT ATTTTTCTTT TCAATTTGTA TAATCTACGT TATAAAAGTT CAACCTTTTA AAACAAATGT GGGAATAAAT TACGGTTTGC

           121                                                                                                 240
   AS-WTSI  CAATCCACAT AATTGTGTTT ATATATGCCA TGCTTGGGGT CCTCCTTATT TCATCCCATA GTAAATAACA AACTGGTTAA TAATCCATCC CATAGTAAAT AACAAACTGT TCAATAGTCC
   AS-SENS  CAATCCACAT AATTGTGTTT ATATATGCCA TGCTTGGGGT CCTCCTTATT TCATCCCATA GTAAATAACA AACTGGTTAA TAATCCATCC CATAGTAAAT AACAAACTGT TCAATAGTCC
    AS-PYR  CAATCCACAT AATTGTGTTT ATATATGCCA TGCTTGGGGT CCTCCTTATT TCATCCCATA GTAAATAACA AACTG----- ---------- ---------- ---------T TCAATAGTCC
   AS-50SP  CAATCCACAT AATTGTGTTT ATATATGCCA TGCTTGGGGT CCTCCTTATT TCATCCCATA GTAAATAACA AACTG----- ---------- ---------- ---------T TCAATAGTCC
 AS-ATNMF-1  CAATCCACAT AATTGTGTTT ATATATGCCA TGCTTGGGGT CCTCCTTATT TCATCCCATA GTAAATAACA AACTG----- ---------- ---------- ---------T TCAATAGTCC
  Consensus  CAATCCACAT AATTGTGTTT ATATATGCCA TGCTTGGGGT CCTCCTTATT TCATCCCATA GTAAATAACA AACTG..... .......... .......... .........T TCAATAGTCC

           241                                                                                                 360
   AS-WTSI  CATCACATTT AATTATTGGC ACATCAATAT TTGCTGGTAG GAAGCAGATA GTATATTTGT AAAGCCTGCA AAAGGAAAAT AGTCACAAAT ACGTTGTATC ATATTAAAGT AGTATGACAT
   AS-SENS  CATCACATTT AATTATTGGC ACATCAATAT TTGCTGGTAG GAAGCAGATA GTATATTTGT AAAGCCTGCA AAAGGAAAAT AGTCACAAAT ACGTTGTATC ATATTAAAGT AGTATGACAT
    AS-PYR  CATCACATTT AATTATTGGC ACATCAATAT TTGCTGGTAG GAAGCAGATA GTATATTTGT AAAGCCTGCA AAAGGAAAAT AGTCACAAAT ACGTTGTATC ATATTAAAGT AGTAT-----
   AS-50SP  CATCACATTT AATTATTGGC ACATCAATAT TTGCTGGTAG GAAGCAGATA GTATATTTGT AAAGCCTGCA AAAGGAAAAT AGTCACAAAT ACGTTGTATC ATATTAAAGT AGTAT-----
 AS-ATNMF-1  CATCACATTT AATTATTGGC ACATCAATAT TTGCTGGTAG GAAGCAGATA GTATATTTGT AAAGCCTGCA AAAGGAAAAT AGTCACAAAT ACGTTGTATC ATATTAAAG
  Consensus  CATCACATTT AATTATTGGC ACATCAATAT TTGCTGGTAG GAAGCAGATA GTATATTTGT AAAGCCTGCA AAAGGAAAAT AGTCACAAAT ACGTTgTATC ATATTAAAGt agtat.....

           361                                       401
   AS-WTSI  ACA CATGGTT ACACTAATAA GGTATGTACA AAACGAACCT G
   AS-SENS  ACA
    AS-PYR  AC
   AS-50SP  ACA CAT
 AS-ATNMF-1
  Consensus  aca....... .......... .......... .......... .
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Appendix V – Confirmation of the 34 bp deletion present in chr 7 in clone AS-ATNMF-1. The clone used as filter for the identification of large indels, AS-50SP also has this mutation, and for that reason this mutation was not identified by Solexa. This deletion first appears in clone AS-PYR in AS lineage. Sequences obtained for the four clones (AS-SENS, AS-PYR, AS-50SP, and AS-ATNMF-1) after di-deoxy sequencing analysis were aligned using MutiAlin software and are indicated above.

pcxxpfyy-zzzz	Gene ID	Forward	Reverse	Sequencing
CHROMOSOME 1				
pc 01 pf 06 - 0142	PFF0100w	TCGTCTAATGCGCTTCTATCTTT	**AAAAACCAAGCATGCCACAT**	TCTATCTTTCAGTGCGTTA
pc 01 pf 06 - 0317	PFF0320c	AGCAATAAAAACGTTGCATAACAG	**TTGCGGATTTGATGTCGTATAA**	ACAGAAATATATATGATGGA
pc 01 pf 06 - 0472	PFF0485c	ACGATGGAGGCATAGGTATATTCA	**CACTGTTCGGCTTGTAATAGATGT**	TGCATAAAAAATCTTCTGT
pc 01 pf 06 - 0510	PFF0590c	GGTGATTATTATAGCGCACTAAAA	**TGATATATGTGCCTTTTGTTTTGA**	TCAAATATACAACTAAATCA
pc 01 pf 06 - 0525	PFF0615c	**CCAACTACACTTGTTGCCAAAAAA**	TCAGCATTTGGTGGTACTCTCAT	GAAAATACATTTACAGCACC
pc 01 pf 06 - 0600	PFF0655c	**TGAATTTAGCATAATCATAATTTG**	TGCTTTACAAAGTTCAGAATGTTT	AGTTCAGAATGTTTATCTAA
pc 01 pf 06 - 0580	PFF0675c	**TGAAATCAAAAGTGATAACCAAGT**	CTTCTGATTTACCTGACCCAGATT	AAGAAATACATAATCACTAT
CHROMOSOME 2				
pc 02 pf 01 - 0406	PFA0515w	AGGCGCGTCAGTCAAATT	**ACTATTTCTTCTGCGGCTCCTA**	ATTGGAGGGTTCTAAAGAG
pc 02 pf 01 - 0399	PFA0510w	AAGGGTAACAAGGTTGAAGATGGT	**GTTGTGATAATCGTCGTTTAATGC**	CAAGGTTGAAGATGGTG
pc 02 pf 01 - 0323	PFA0390w	ACGTCTTTCTATGCAGACACACA	**TTTCTTCCCCTTCATAAAAATCG**	CTATGCAGACACACAAGTAG
pc 02 pf 01 - 0265a	PFA0310c	**ATCATGCTCTGTTGATAAATATGC**	AATGCCACACTGATTTTGAAATAA	CCTATTTCAGTTTTCATACC
pc 02 pf 01 - 0265b	PFA0310c	**TAGCTGTTGCTGCAATTCCTGAA**	TTGACCATTCGTCTGGTTCCTAAA	TCTGGTTCCTAAAGCTAA
pc 02 pf 01 - 0197	PFA0220w	**TTGAACAATTAGGAGGATCAGAA**	CGAGCATTTGTATTTATTGTTTCC	TGCAGGAACATGAAATG
pc 02 pf 01 - 0158	PFA0180w	**ATAATAGCAATCCAAATGGACAAG**	TGTTGCTCTTCAGTGATATATTCA	TTCAAAAAATTTAGAAGCA
pc 02 pf 01 - 0150	PFA0170c	**GAATTCGGATATTTTCGATAGTTG**	TGAAAAGGGCCATAAATCATACAT	GCCATAAATCATACATCCA
pc 02 pf 07 - 1151	PF07_0121	CGGAATCAAATTATAGCGAATTGC	**ATCTCCTGGAAATAAATGTCTTGC**	AGTAAAATTAGAAAAACT
CHROMOSOME 3				
pc 03 pf 02 - 0278	PFB0310c	TGCCGACCCAAAAGTTATCCT	**TTCCGCAAAAACTGTAATTCACTG**	CATGGTACTAATTGTATCGC
pc 03 pf 02 - 0770	PFB0880w	**CACAGTGCTTCGTTTATATCAGCT**	TAGTCGAATCCGAACATAAGGACA	TCAAATTCTTCTTGTATAAT

CHROMOSOME 4				
pc 04 pf 03 - 0231	PFC0225c	AGCTTTGCAAATGATTTATGTATG	**TTTCATTTTTCCGTTGATCATATT**	GCAAATGATTTATGTATGC
pc 04 pf 03 - 0295	PFC0275w	**TCCCTTTGCTACATCAAGATGAA**	TCCATATGATTGCATACAGTAGCT	GCATACAGTAGCTCCGAC
pc 04 pf 03 - 0419	PFC0420w	**CCTTTTAAAATCGAAAAGAAGTGA**	CCGTTGCTTTATATACACAACCA	TTGCTTTATATACACAACCA
pc 04 pf 03 - 0560	PFC0580c	**ATTTTCCTCATCAATCGAAACAT**	GGAATGTGGGGACTAGCAAAA	GTTTAGGCACTAAGACAGAC
pc 04 pf 03 - 0665	PFC0725c	GCCCTTATGGTTAAAGCTGATG	**CGTAATTTCCGATCAGTGTTGG**	GAAGTCTATTTTAACAACCT
pc 04 pf 09 - 0141	PFI0160w	AATATTTGAATCTGCACCATACCA	**TTGCACAAAAGGCTATTGTTCTC**	CACCATACCATGTTTTTC
pc 04 pf 09 - 0133	PFI0155c	AAAAGTTTTGCAATGGTGTAAAGC	**TGGCACTTGTCTCAAAGTATGGTA**	CAATGGTGTAAAGCAAAT
CHROMOSOME 5				
pc 05 pf 10 - 0697	PF10_0169	TCCCTTCTATGTGTCTCAAACAAA	TGGTGCTTCATCATGTTTAATGT	ATATAATTTCAATATGCTGG
pc 05 pf 10 - 1203	PF10_0287	**GAACCGTTTTAAACAACAAGAACT**	GTGATTCATCGGCTAAAAATTGT	TAGGAATTTCAAAAATTTTA
pc 05 pf 10 - 1378	PF10_0337	**ATTAGCAAACAAGCAGGATGTAAA**	CTAGTACCAAAAATTGCCCATTGT	TGATATGGGAAGGCAA
pc 05 pf 04 - 0924	PFD0985wa	TGGACCATCTGGTGATAACAAG	**GACCCATCACTAACATCACTTGTA**	TAAAGGAAATGGAAATTTA
CHROMOSOME 6				
pc 06 pf 12 - 0102	PFL0080c	**ATTAGCAGAAAAGGGAGAAGACAA**	TGGTATATCACAAAAAGGATCAAA	TGATTTATTTCAAGTGGG
pc 06 pf 12 - 0390	PFL0420w	**TGGTGGTTTACTTCTCGTGCTATT**	AATGAAGCGGATTTTTTTGAGA	ATGCGTATGAAAAAATTAA
pc 06 pf 14 - 1522	PF14_0356	**TTCATCCGGTTTTTTATTTCCTAT**	GCAAAAAATGTAGGGAATTTCA	AATTCGGAAAATTGAAA
pc 06 pf 04 - 0540	PFD0590c	TCTGTTCGATTGCTAAGAGTGATA	**TGTTCCCATAATATAAAGCCAGTT**	AGTGATAAACATTTATTACA
pc 06 pf 07 - 0962	PF07_0105	**TGGAAAAATAAGATACCCCCAAAT**	TGTTTGAAAAAACTTCCAAATGAT	TGATTGATGTATTGGGAC
CHROMOSOME 7				
pc 07 pf 11 - 1558	PF11_0407	**TAGTTTTTTGTTGTGGAGCTTCTG**	CCAGAGAAATTTTCATTTTCTTTA	TTTTCATTTTCTTTACAATC
pc 07 pf 11 - 1467	PF11_0385	**TGTCCTAATCCACTAGCGCCTTT**	GAAGGGGGGAAAGAAAGCAAA	GGATATAAACATACACTGGC
pc 07 pf 08 - 0431	MAL8P1.58	TTGGAGGGAATATGATTATTAGTT	**CATAGCGATAAAGAAATGGAAAAA**	TGCCTATATTGTGTAAATGA
pc 07 pf 08 - 0580	MAL8P1.78	CCTCACAATATCCTTGATGACACT	**TGTTTGTCCAGCCATAAAAGC**	TTGTTCATTACTCTTGAATT
pc 07 pf 04 - 0754	PFD0805w	TCCCATTAGCTTCCCATATTCTT	**TATGGTTTATAAGGGGAGAAACAA**	TTAGCTTCCCATATTCTTAC

CHROMOSOME 8					
pc 08 pf 07 - 0175	PF07_0016	AGCATTTTCATTTGATGTGTCTTT	**AACCAATTAACCACCTATGACAAA**	TTATTATCATCTTCATCAGT
pc 08 pf 09 - 0777	PFI0925w	GGATGCTTCCAATGAAAATGTAG	**GATTTATTCCAAATGAGATGACCA**	ATTTAATGAAATATATAAAG
pc 08 pf 09 - 0903	PFI1090w	ATATTTGATCGCAAATTTTATCGG	**ACAAAATTAAAAAACAATGGACCA**	GGATTCCGAAGTAAATAAAA
pc 08 pf 09 - 0944	PFI1140w	**GAAAAGCGAAACAAATCAACATAC**	TCCAGTAAGACTTGTGGCTATAGG	GCTATAGGCTTATTAAATTG
CHROMOSOME 9				
pc 09 pf 11 - 0234	PF11_0065	GGATGGATATATGGTATACTTCGA	**TGATGTCAAAGGTCGATTTGTT**	GTATACTTCGACCATCATG
pc 09 pf 11 - 0533	PF11_0147	**AGCCGATTCAGATTTAAAAAATTA**	AGCCGGTTTTAAATCTCTATGGA	TCTTCTGTCAAAAATATTGG
pc 09 pf 11 - 1100	PF11_0294	AGCAGGAATAGCTGTCGAAAATA	**GGTACAATGAGCTGCTCCAATTT**	AATTATGTAACATCCGTTAG
CHROMOSOME 10				
pc 10 pf 14 - 1029	PF14_0244	**TTTAGATCAGTAGGTTTACCTTGG**	CATGCAAAAAATGTCCAGATTC	TTACATCAACCTAGTAGTCA
pc 10 pf 14 - 0920	PF14_0218	AGGAAGATCAAATTCGCTAGTTAT	**TACATCCAAAATAGCTTCCTCTCT**	TGGAGGTGATTATTTTGA
pc 10 pf 14 - 0562	PF14_0139	TCGCCCTATATGACGGACTTTGA	**CAGGTGATCGTTTTGTACGCAATA**	TCAGTTTGTAAGCTAAAAAT
pc 10 pf 14 - 0102	PF14_0029	ATGGAAACCTTGCAATCATATTAA	**CAGGACAAAAATAAAAATCATTAA**	AAACGTATTTTACCTTTTT
CHROMOSOME 11				
pc 11 pf 05 - 0177	PEF0225w	AACTATTGTCACATGGGGTTCATT	**CAGATTTTTGCACCGTTTCAATAT**	AAAATGAAAAATGCAGC
pc 11 pf 06 - 0782	PFF0830w	ACTGGTTGAAAATTTATATGAAGA	**GTTTCATATTTTTGGAAAATCAAT**	AATATGTTTAATGCTTGTTC
pc 11 pf 06 - 1001	PFF1135w	TGCTTAGGCATATTTGCTTCAA	**TTGAAGGTTTTGTAGCTCCTTCGA**	CCATAAAATTATATTCTGG
pc 11 pf 06 - 1044	PFF1185w	TCGTTTTACCTAACCCCATTTCA	**GAAAGGAGGAAAAAATGATTCTGG**	AAAAATGATTTTAATAACCA
pc 11 pf 13 - 2207	MAL13P1.284	**ATATGCTCTCCAGGAGGTATTACA**	AACCCCATGCTCACAAAGAT	TTTTTTTCAAGGGTATATAA
pc 11 pf 13 - 2508	PF13_0345	TGTGCCATTTTTAATTTGATCTGA	**GAAAAGTATACCCCAATTGAAGCT**	ATATTAGCACCGTTAAAATT

CHROMOSOME 12								
pc	12	pf	10	- 0141	PF10_0034	**ACATGCGTTGTATTTTCGTATGT**	TGGATGAAGAGGAATGCTTTACA	TGAATTTGTTAAATTCACTT
pc	12	pf	10	- 0317	PF10_0077	**AAATTCATTAGATGCACCAACTCA**	TGGACATGATTTTTAAATCGTTCA	TTTAACTGCTTCTAAACCTA
pc	12	pf	10	- 0435	PF10_0108	CTCATGGTAATTATAAGCTCATCA	**GGCAATTTCCTCAAACATTCTT**	ACAACAATTATGGGCC
pc	12	pf	03	- 0868	MAL3P7.12	GTCGCAAACACATCGAAATC	**CATCGGCAAGCACCATTT**	TCGTGTTTTTCATGATTT
pc	12	pf	08	- 1006	MAL8P1.134	**CGACCAAAACAAACAGTAACAGA**	TCCCCTATATGGATTAACTTGAAA	TCTACTTTGCCTGGACA
pc	12	pf	06	- 0623	PFF0730c	GTTATTTTTGGTGTATGGCCACCT	**CAGCATCAAATGGCAAAACATC**	CCAAACGAATGGAAATA
pc	12	pf	05	- 0885	PFE1090w	GCAGAAAACGAAAATGGAATAAA	**GGATTTAATAAGCATAGCATCAGA**	AAATTCATAAGGATTT
pc	12	pf	05	- 0991	PFE1185w	ATGACCCCAATAAATTTTCAAGAA	**AACAATTTTCCTTTCGGTATTCTG**	CCCAATAAATTTTCAAGAAG
pc	12	pf	05	- 1057	PFE1265w	GCAAATCAGTCGATTTATTGTG	**ACCCCTATATTCCCTTCTAAAAA**	AAATCAGTCGATTTATTGTG
pc	12	pf	05	- 1136	PFE1360c	**TTCTGACCATCCCTACATATTGA**	GGAACAACAGGGATAGGTGATATT	TCAGAAAAACACAAAAAATT
pc	12	pf	05	- 1178	PFE1430ca	GAAAAAACAAAATCATTGGAAGAA	**ACATGTTTTGGGGCTATGGTA**	TTATAAAATGAAAGGCCA
pc	12	pf	05	- 1260	PFE1545c	AAGAACCCGTTTTAAAATCGTCA	**TGTTTCACTTTCATCCTCCATAA**	TCAAAAGAAAAAAAAAATAC
pc	12	pf	05	- 1286	PFE1560c	**AAAATTTTATGCATTTTTAATGGG**	CCCGTTTTAAAACAAAATATTTCC	TTTTAACAATTGTATGTTGG
CHROMOSOME 13								
pc	13	pf	14	- 1658	PF14_0386	**GTTTGCGCTCAATTCATCTTTAT**	GGTATATCGAAAATGTTGTAGGTA	AATATCAGCAAACATATCAT
pc	13	pf	14	- 2118	PF14_0489	CCGATGAAAATTACGTAAAGTTTG	**GGTTTTGGTTGGTAAGGCTAAAT**	AAAAAAAAGTTGTAGAGC
pc	13	pf	14	- 2213	PF14_0512	**TTAAGTGCTGCCTCAGTTTTATGC**	CGCGTTTTCCATTATTCCCCTTAT	ACATAAGCCCGTGCC
pc	13	pf	14	- 2445	PF14_0571	TAACGATACCCAAACATCATCAAA	**TGCACATGTTTCAAATTTTATCTC**	AATATTATTCATTTTCACTT
pc	13	pf	14	- 2708	PF14_0632	AGGCAACGAAGAAGCTGTTAATA	**TGGCATTTCGTCCACATATATCTA**	TGCCACTTTTAACTGATAC
pc	13	pf	14	- 2860	PF14_0664	**ACGAATATACATGAAAAAAAGTGA**	CTGTTTTTTCCAGGAGGAACTTT	AAAATTCGGTATCATCAAC
pc	13	pf	13	- 0906	MAL13P1.120	**TCAAAAGGTTTTGGGTTCATACA**	TCAAAAGGTTTTGGGTTCATACA	CTTCTGAAGCTTTATGAAA

pc	13	pf	13	- 1050	PF13_0143	**AGATTTGCAGATGGAGAAGTATCA**	TTTTCTATCTTGACGTGCATAACC	GATTTTCATTAACTGGTGG
pc	13	pf	13	- 1356	MAL13P1.172	TTGAAACTGCTTCAGATTTGTCTT	**CATTACCCAACTTATGCCATTACT**	ATCATCTTTACATATGCTTG
pc	13	pf	13	- 1507	PF13_0211	ATTTGGATGATCCATTTGTTTC	**AAAAAACAGGTCAAGCAAAAGC**	TGATCCATTTGTTTCATAA
pc	13	pf	13	- 1652	PF13_0233	GTTACAATTGCTGGTGGAAATAGA	**CGTCTTTTGGCTCAATTCGACTA**	GGTGGAAATAGAATTGAAG
pc	13	pf	13	- 1956	MAL13P1.256	TCGCTTGTTTCTTTCCAATCAG	**TTTGAGCCAGAAAACAGGAAGAG**	TCCAATCAGATTGAAAAT
CHROMOSOME 14								
pc	14	pf	13	- 0185	MAL13P1.23	**CACAAATTAATGGTGGCAGAGAT**	TTGGAGGTAAATGTTTTATAAGGG	ATAAGGGAATATTTATCTAC
pc	14	pf	13	- 0448	PF13_0063	TGATATTGGTGGATGTAAAGAACA	**CGAGCTGTTAATGTTTTTCCTGTA**	CCCAAGGGTGTTTTACT
pc	14	pf	13	- 0609	MAL13P1.74	AGTTTGGAAGCTCGTACTTAAACA	**ATATGCTCCAAATCCACTTAAAGG**	TGTTGATTATTTAAGATCAT
pc	14	pf	13	- 0737	MAL13P1.95	ACCCCGAATATCTTCTCTTATTGA	**AATCGAATATGCGCAAGGCTTA**	TGTTGTTCATGAGAAAAA
pc	14	pf	07	- 0524	MAL7P1.66	**TGACGGGAAAAAAATATGTCTG**	GGCCAATGGCTAGTGCAATA	CAATGGCTAGTGCAATAAT
pc	14	pf	07	- 0595	PF07_0062	ATTGTTGATACACCTGGGCATTC	**TGGTTTGATTTTTTGGTCCTTCTA**	TGATACACCTGGGCA
pc	14	pf	08	- 0650	MAL8P1.92	**CAAGTCTCAAAGCGCACAAAT**	TGGTTTTTTGATCACCCTTTCAAT	CTATCGAGGCAGTAGTAAA
pc	14	pf	08	- 0819	PF08_0098	GAAAGCTATCCGCAACCTATTAGA	**TTCGACTGATGATTTTACAAGTCT**	TTATGTCTAATTGATTTTGG
pc	14	pf	12	- 0627	PFL0735w	GGTGGACCATCTTCTTCTAAACTT	**TGCTTTATATAATCAAGGCCCTCA**	CCATACCTTTCAAAACAGT
pc	14	pf	12	- 0934	PFL1120c	ATGCTGCTGCTATGAGATATACA	**AACATCAACAAGGTTATGACTTGG**	GAAAAAGAACCTAAAGTG
pc	14	pf	12	- 1086	PFL1295w	CGTCACCATCATCATCCATATT	**TTGAACCTAAAAATCCAAATCAA**	TCTGACTTGTTCATACTATA
pc	14	pf	12	- 1160	PFL1385c	AGCACTCGAATTAGGAGAAGAAGA	**TGGTTCAGGGACAATTCCTTTAT**	AGAAGTACCAATAGTTAGCG
pc	14	pf	12	- 1830	PFL2100w	**ATCTGCACTTTCCTGACTACTAGC**	AAGCAGAAGCCTTTATACACTGGT	TATGACAGTGGAAATCCT
pc	14	pf	12	- 2125	PFL2505c	GAATGAAGGTGCTAATTTTGAAA	**TCGTTGCATTCACCTGATTT**	ATTAGAAAAGTTTTGGC

Appendix VI – List of primers forward, reverse and sequencing used for pyrosequencing™ analysis of the progeny resulting from the AS-ATNMF-1 x AJ cross. Biotinilated primers are highlighted in bold. Pcxxpfyy-zzzz is the marker designation, xx and yy refers to *Plasmodium chabaudi* (pc) and *P. falciparum* (pf) chromosomes, respectively.

MATERIAL AND METHODS

1. GENERAL PROCEDURES

1.1. SUBSTANCES AND SOLUTIONS

Citrate saline solution
0.9% (w/v) NaCl
1.5% (w/v) Sodium Citrate
pH 7.2

Deep Freeze solution
28% (v/v) Glicerol
3.0% (v/v) Sorbitol
0.65% (v/v) NaCl
(sterilised by filtration)

Erythropoietin (10 µg/ml stock solution)
10 µg Mouse Erythropoietin (Sigma)
1 ml PBS (Sigma) + 0.1% Bovin Serum Albumin (Sigma)
(sterilised by filtration)

Hypotonic Amonium Chloride Solution
50 mM NH_4Cl
2 mM EDTA
5 mM Hepes-Na, pH 7.4
1% protease inhibittor cocktail (Sigma)
(sterilised by filtration)

Loading Buffer
250 mM Tris-HCl, pH 6.6
3% SDS
20 % Glicerol

Parasite Lysis Buffer
150 mM NaCl
25 mM EDTA
0.25% SDS

TAE Buffer (50x stock solution)
242 g Tris
57.1 ml Glacial Acetic Acid
37.2 g $Na_2EDTA.2H_2O$
(final volume 1 litre)

TE buffer
10 mM Tris-HCl
0.1 mM EDTA

Xanthurenic Acid (20 mM stock solution)
41 mg Xanthurenic Acid (Aldrich)
10 ml PBS (Sigma)
(adjust pH 7.4)

1.2. PARASITES AND HOSTS

Malaria parasites belonging to the rodent infecting species *P.chabaudi chabaudi* were used. These parasites were originally isolated from the blood of a shiny thicket rat (*Thamnomys rutilans*) from the Central African Republic (Landau and Chabaud, 1965). For this work, two lines were used, AS and AJ.

The AS lineage (as described in Chapter II, Section II.1.2.1) consists of an isogenic line of clones obtained along time after many steps of selection with different anti-malarials. All these clones share a common precursor, the drug-sensitive clone, AS-SENS (Table 8).

PARENTAL	CLONE NAME	RESPONSE TO DRUG TREATMENT
Natural host	AS-SENS	sensitive
AS-SENS	AS-PYR	resistant to PYR
AS-PYR	AS-50SP	resistant to sulphadoxine
AS-PYR	AS-3CQ	resistant to low doses of CQ
AS-3CQ	AS-15CQ	resistant to intermediate doses of CQ
AS-15CQ	AS-15MF	resistant to and MF
AS-15CQ	AS-ATN	resistant to and ATN
AS-15CQ	AS-30CQ	resistant to high doses of CQ
AS-30CQ	AS-ART	resistant to ART

Table 8 – *P.chabaudi* AS lineage. Nine parasite clones used in this work, their respective parentals and response to drugs are represented.

The other parasite line used here is the drug-sensitive AJ clone. This clone is genetically non-related to AS, differing in a great number of known genetic markers throughout the genome.

The parasites were maintained in different strains of mice (*Mus musculus)*. Six-to-eight weeks old female CBA and C57BL/6 mice were used mainly for the genetic cross between the ACT-resistant clone selected here and AJ. Six-to-eight weeks old male CD1 mice were used for the remaining procedures.

Mice were kept in polypropylene cages, and sawdust was used as bedding. 41B mouse maintenance diet (Harlan-Teklad, UK) and 0.05% p-Aminobenzoic Acid (Sigma) supplemented water were available *ad libitum*. The room temperature ranged from 14ºC to 26ºC with light periods of 12 hours per day.

1.3. MOSQUITO MAINTENANCE CONDITIONS.

Anopheles stephensi mosquitoes were kept in groups of up to 200 mosquitoes (both male and female). Inside of the cages, 10% Glucose solution supplemented with 0.05% p-Aminobenzoic Acid was available to the mosquitoes *ad libitum*. The room was maintained at 24-to-26ºC, the air humidity was kept above 60%, and had light-dark cycles of 12-12 hours.

1.4. PARASITAEMIA COUNT

A drop of blood was extracted by a small puncture on the mouse tail vein and used to make a thin smear on a glass slide. The blood was fixated with pure methanol and subsequently stained using a 20%

Giemsa solution during approximately 20 minutes. The slides were washed with water and read under optical microscopy. The number of parasitized Red Blood Cells (pRBC) and total RBC present in a particular microscopic field were counted for at least 5 different fields. These values were entered into an equation to calculate the percentage Parasitaemia (%P) as follows:

$$\%P = (pRBC / total\ RBC) \times 100$$

1.5. PARASITE PASSAGING, PREPARATION AND STORAGE OF PARASITE STOCK VIALS

Frozen parasite-infected blood was thawed on ice after which uninfected mice were injected intraperitoneally with 0.1ml of infected blood using a 1.0 ml seringe with a 27G x 1/2" needle. Percentage parasitaemias in individual mice were then followed from day 4 post-inoculum (pi) onwards. After reaching the desired parasitaemia values (at least above 10%), 5 µl of blood collected from the above donor mice, in a glass capillary, was diluted into 195 µl of Citrate Saline Solution and RBC density was determined using a Abacus Junior haemocytometer (Diatron, Hungary). Subsequently, parasite density was determined by multiplying the RBC density by the parasitaemia and the dilution factor (40x). Consequently, blood was diluted such that 0.1 ml would contain the desired amount of pRBC to be inoculated into each acceptor mouse.

The donor mice were then anaesthetised by an intra-muscular (im) injection of a mixture of 1:2 xylasine:ketamine and then exsanguinated under general anaesthesia by a puncture on the brachial artery. Blood was collected using a micropipette and mixed with ice cold Citrate Saline Solution, according to the calculations of parasite density. The blood would then be inoculated into uninfected mice, starting a new infection.

Alternatively, in order to store and preserve the parasites, the mixture of blood and Citrate Saline Solution was immediately centrifuged at 3000 rpm for 5 minutes at room temperature and the supernatant was discarded. The volume of the pellet was estimated and mixed gently with 2,5x volume of Deep Freeze Solution, which prevents the formation of crystals and the consequent disruption of the RBCs. 300 µl aliquots were made and kept in liquid nitrogen. In order to start a new infection, these aliquots were removed from liquid nitrogen and upon thawing on ice 0.1 ml was injected intraperitoneally into donor mice, as described in the beginning of this section.

1.6. ANTI-MALARIAL DRUGS: DILUTION AND ADMINISTRATION

All antimalarials used in this work were available as powder and were diluted into pure DMSO (Merk). Artesunate (ATN) and artemisinin (ART) were donated by Daphra Farma. Mefloquine (MF) hydrochloride powder was purchased from Roche Farmaceutics. The concentrations were adjusted according to body weight so the volume given to each animal would range from 0.06 ml to 0.12 ml. The drug was then administered orally by gavage.

The diluting vehicle DMSO was administered to mice used as untreated controls, in order to certifify that neither DMSO alone nor the stress caused by the procedure would interfere with the observed parasite growth.

2. SELECTION OF PARASITES RESISTANT TO ARTESUNATE AND MEFLOQUINE IN COMBINATION

2.1. SELECTION OF RESISTANT PARASITES BY DRUG PRESSURE

Our objective was to generate resistance to the artesunate (ATN) + mefloquine (MF) version of ACT. The strategy to achieve resistant parasites was based on the prolonged exposure of parasites to consecutive small increments in drug doses in treated mice over many generations. To achieve this goal, two parasite clones were used as the starting biological material, as follows: i) parasites resistant to a low level of artesunate (*P. chabaudi* AS-ATN) which were exposed to a combination of ATN and MF and ii) mefloquine-resistant parasites (*P. chabaudi* AS-15MF) which were exposed to ATN. Thus, blood containing each of parental clones above was thawed and inoculated intraperitoneally into two CD1 mice. When these mice reached peak parasiteamia, donor blood was extracted to inoculate 10^7 pRBC, diluted in citrate saline, into two groups of two mice each. One group was left untreated and parasites were sub-inoculated every seven days into two uninfected mice in the absence of treatment and in parallel with the treated group. The remaining two mice were treated with drugs dissolved on DMSO on days 3, 4 and 5 post-inoculum by gavage. Infection progression in individual mice was monitored from day 3 onwards by counting the percentage parasitaemia in Giemsa-stained thin blood smears. The treated mouse containing the highest parasitaemia on day 7 post-inoculum, was then exsanguinated by section of the brachial artery under general anesthesia and served as donor for the next round of drug selection. The starting drug-selecting dose was of 1 mg/kg/day of MF administered together with 5 mg/kg/day of ATN for AS-ATN and 10 mg/kg/day of MF for AS-15MF, and it was increased whenever parasitaemias reached above 2% on day 7, under drug treatment. On the other hand, when the parasites were not able to grow beyond this value, the dose was maintained. The dose was reduced only as an extreme measure, to avoid losing the parasites, in cases when the parasitaemias were nearly undetectable. After a number of rounds of drug selection, when a satisfactory degree of resistance was achieved, parasites under drug pressure were cloned by limiting dilution. Over the selection course, aliquots of parasites were frozen down in order to prevent the loss of the parasites under selection, in case the next round of treatment would eradicate the parasites from the host mice. This would also provide a useful tool for the analysis of the gradual acquisition of tolerance by these parasites, during the course of reaching high levels of resistance.

2.2. CLONING

At the end of the last round of selection, the resistant parasites population selected as described above was cloned by limiting dilution (Rosário, 1981). Thus, mice harbouring the resistant parasite populations were exsanguinated by puncture of the brachial artery and blood was diluted in Citrate Saline Solution. Serial dilutions were prepared and inoculated into uninfected mice as follows: forty CD1 mice were sub-divided into two groups of twenty animals each. The first group was inoculated

with the equivalent of 1 pRBC and other twenty animals received the equivalent of 0.5 pRBC. Since the parasite inoculum consists of a suspension of cells, the probability of each mouse receiving the exact amount of parasites as calculated follows a Poisson distribution. In this case, this means that, for instance, of all mice inoculated with the equivalent of one parasite, the mice showing detectable parasitaemias are assumed as carrying a clonal population if at least 37% of the mice show no detectable parasitaemias.

Additionally in order to control parasite growth, ten mice were subdivided into three groups of three animals and were inoculated with the equivalent of 10^3 pRBCs, 10^2 pRBCs, and 10 pRBCs. The last animal was inoculated with the equivalent of 10^6 pRBC. Parasitaemias were followed for at least fifteen days.

The animals showing detectable parasitaemias were exsanguinated and the blood of each animal was processed separately. The samples were subsequently stored into liquid nitrogen.

In this work, the clones with the fastest growth rate were used for further analysis.

2.3. DRUG SENSITIVITY TEST

Two different artesunate + mefloquine (ATN + MF)-resistant parasite populations resulted after selection as described in Section 2.1. These populations were cloned (Section 2.2), and the clone displaying the fastest growth rate from each of population was further inspected in the course of this work. Therefore, two clones were analysed, one of which was named AS-MFATN-5 and was derived from the mefloquine (MF)-resistant clone, AS-15MF. The second was named AS-ATNMF-1 and was obtained using the artesunate (ATN)-resistant AS-ATN clone as parental.

In order to assess the degree of resistance obtained by the clones, different drug sensitivity tests were performed. For the first one, the clones used were: i) the AS-MFATN-5 clone, ii) its MF-resistant parental AS-15MF, iii) AS-ATN clone, which shows some degree of resistance to ATN, iv) AS-3CQ, which shows low levels of resistance to CQ, and is sensitive to ATN and MF. Thus, CD1 mice were divided into four groups of nineteen animals and were inoculated with 10^7 pRBC of each of the clones mentioned above. Each group was sub-divided into three groups of five mice that were treated with the following anti-malarials: ATN, MF or ATN in combination with MF. The remaining two mice received only the DMSO vehicle (non-treated control). The treatment consisted of three daily doses of anti-malarial to be administered on Days 1, 2, and 3 pi (Figure 23).

In parallel, the AS-15MF population passaged along the course of selection in the absence of drug treatment (referred to as AS-15MF36P herein) was also tested. For that purpose, CD1 mice were divided into two groups of nineteen animals. One group received 10^7 pRBC of the population AS-15MF36P and the other group was inoculated with 10^7pRBC of the original AS-15MF clone. In a similar fashion as described above, each of these groups were subdivided into three groups of five animals that were treated with ATN, MF or ATN in combination with MF. The remaining four animals received the DMSO vehicle (Figure 23).

Similarly, the level of resistance acquired by the AS-ATNMF-1 clone was also assessed. For that purpose, CD1 mice were divided in five groups of seventeen animals and were inoculated with 10^7 pRBC of the following parasites: i) AS-ATNMF-1 selected here, ii) its parental, the ATN-resistant AS-

ATN clone, iii) the AS-ATN parasite population left untreated and passaged in parallel with the treated group during the selection course, referred herein as AS-ATN27P, iv) the MF-resistant AS-15MF clone, and v) the CQ-resistant, ATN and MF-sensitive AS-3CQ clone. Each group of mice was further divided into four groups of animals. Three of which were treated with 3 daily doses (Days 1, 2, and 3 pi) of ATN, MF or ATN in combination with MF. The fourth group was left untreated and received only the DMSO vehicle (Figure 23).

In all cases, the parasitaemias were assessed daily from Day 4 onwards and the results are expressed as daily average % parasitaemia of all mice in each group.

In order to prevent suffering, the animals were sacrificed through cervical dislocation whenever reaching parasitaemias above 40% and displaying symptoms of disease.

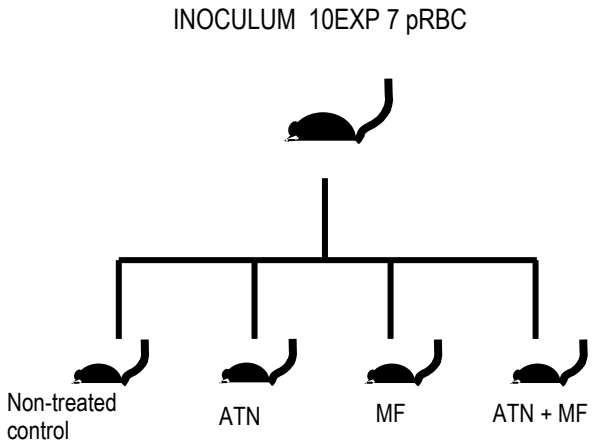

Figure 23 – Schematic representation of drug sensitivity test carried out to assess the level of resistance exhibited by AS-MFATN-5 selected from the AS-15MF clone after several rounds of treatment with stepwise increasing doses of ATN. A similar protocol was used for determining the level of resistance of AS-ATNMF-1, selected by exposing AS-ATN clone to several rounds of treatment with increasing doses of the combination ATN + MF. The parental clones as well as AS-3CQ were used as controls. In case of the AS-ATNMF-1 clone, the parasite population passaged in absence of drug response to each drug was also assessed. For determining the response of AS-15MF36P parasite population to different drug treatments, only the original clone AS-15MF was used as control.

2.4. SELECTION OF RESISTANT PARASITES THROUGH GENETIC CROSSING

2.4.1. PARASITE CROSSING

In order to generate artesunate + mefloquine (ATN + MF)-resistant parasites through genetic recombination between artesunate (ATN)- and mefloquine (MF)-resistant parasites, a genetic cross between AS-ATN and AS-15MF was performed. Thus, one aliquot of blood of each clone was thawed and inoculated into two donor CD1 mice per clone. In addition, one aliquot of blood containing AJ was also used as control, due to its ability to be easily transmitted (Figure 24A). Parasites were then allowed

to grow in these mice for at least five days after which animals were all bled out. Collected blood was diluted so that 0.1 ml would contain 10^5 pRBCs. Concurrently, acceptor mice were treated with the erythropoiesis-stimulating agent Erythropoietin (EPO) (Sigma) prior to (and after) being injected with blood from the donor mice. Thus, eight CD1 mice received a daily intraperitoneal injection of 0.1 μg of EPO, during five consecutive days. On the third day of EPO treatment, mice were subdivided into four groups, and each group received an intraperitoneal injection containing infected blood extracted from the donor mice as mentioned above. Thus, the first group was inoculated with 10^5 pRBCs of the AJ clone, the second group received 10^5 pRBCs of AS-15MF and the third group received 10^5 pRBCs of AS-ATN. Blood infected with AS-15MF parasites was mixed in equal proportions with blood containing AS-ATN parasites, and this mixture was inoculated into the last group of two mice (Figure 24B).

Simultaneously, one-day-old female *Anopheles stephensi* mosquitoes were divided into four cages containing about two hundred female-reared mosquitoes. Mosquito drinking water was further supplemented with 200 μM Xanthurenic Acid (XA) (Aldrich) diluted freshly from 20 mM stock solution (Section 1.1) and was replaced every two days.

In order to assure that the majority of the mosquitoes would feed on blood, therefore increasing the chances of successful transmission, on Day 5 pi, drinking water was removed two hours prior to blood meal to promote starvation. Acceptor mice were anaesthetised and used to feed the mosquitoes for 20 minutes. After removing the mice, drinking water was again supplemented with 200 μM XA and made available for 24h (Figure 24C). After that, XA supplement was removed and mosquitoes remained with standard 10% Glucose, 0.05% p-Aminobenzoic Acid supplemented water from now on.

On the fourth day after the blood meal, the drinking water was once more removed and after two hours, eight uninfected mice were anaesthetised and two animals per cage were used to boost-feed the mosquitoes for 20 minutes. Similarly to the procedure described above, the drinking water was restored after the end of the blood meal.

On the tenth day after the first blood meal – using the infected acceptor mice – ten mosquitoes were taken from each cage. Their midguts were dissected and observed at light microscopy for the presence of oocysts.

On the fourteenth day after the first blood meal in order to transmit the infection drinking water was removed two hours prior to the blood meal and eight uninfected CD1 mice were anaesthetized. Two animals were placed on each mosquito cage and the mosquitoes were allowed to feed for 20 minutes (Figure 24D). After recovering from the anaesthesia the mice were kept under standard conditions. A schematic representation of the genetic cross procedure is presented in Figure 24.

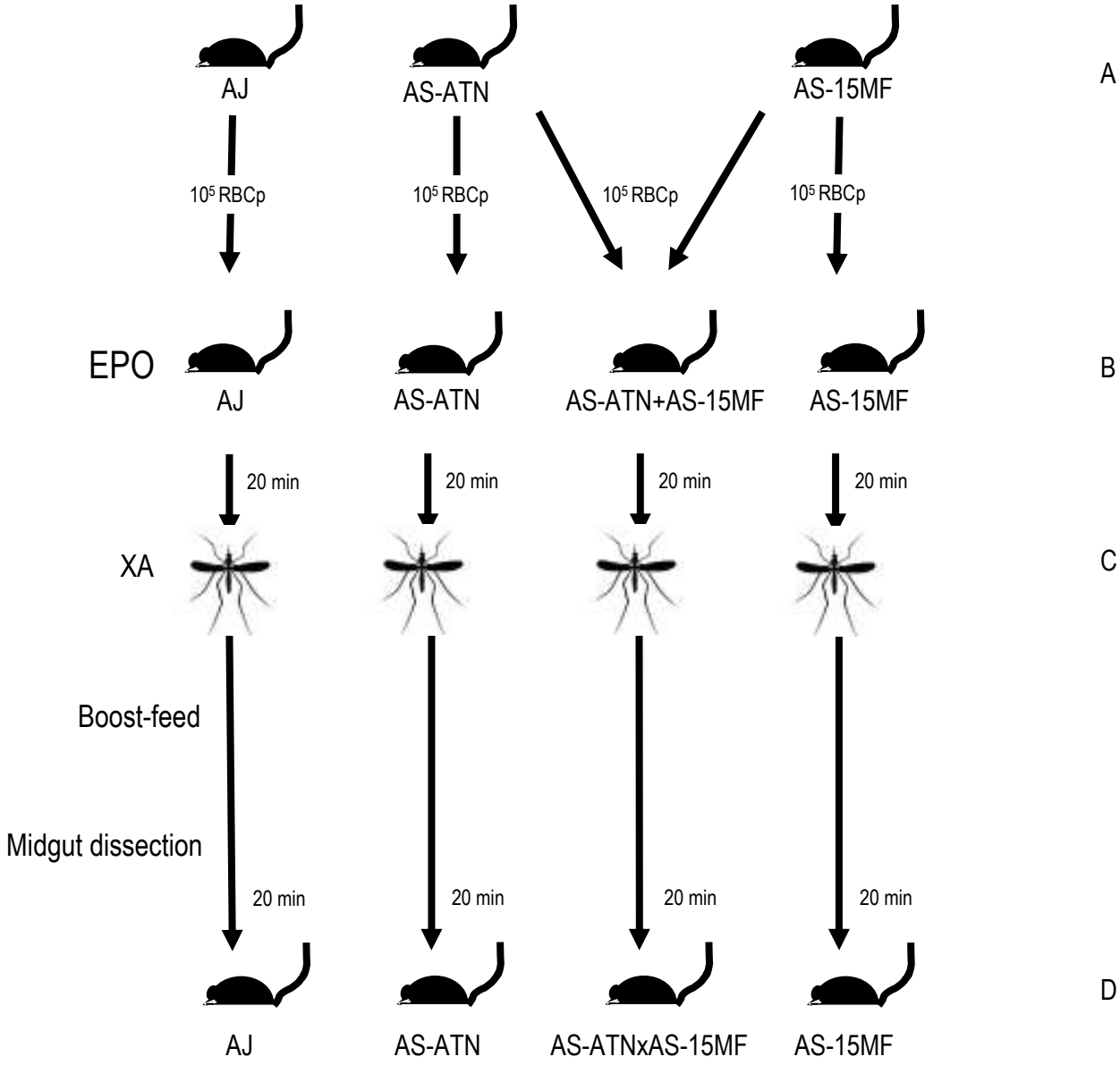

Figure 24 – Schematic representation of the genetic cross performed using AS-ATN and AS-15MF clones as parentals. The AJ clone was used as a control, due to its ability to be easily transmitted. (A) CD1 mice were inoculated with each clone and served as donors. (B) EPO treated acceptor mice were injected with 10^5 pRBC of each parasite or a mixture in equal proportions of AS-ATN and AS-15MF (AS-ATN + AS-15MF). (C) These animals were used to feed XA treated *Anopheles stephensi* mosquitoes. Mosquitoes' midguts were inspected for the presence of oocysts and were allowed to feed on uninfected mice in order to transmit the infections. (D) Mice parasitaemias were followed. The parasites cross-progeny (AS-ATN x AS-15MF) was further analysed.

In order to verify the effectiveness of the transmission procedure, the animals' parasitaemias were followed for the following ten days. The animals showing detectable parasitaemias on Day 9 were exsanguinated. The progeny resulting from the genetic cross between AS-ATN and AS-15MF (AS-ATN x AS-15MF) was further analysed. Thus, the animals carrying these parasites were exsanguinated and a drop of blood was collected into Whatman Nº4 filter paper for DNA extraction (as will be described in

detail in Section 3.1) and will be referred to as Sample 1. The remaining blood was used for inoculating eight uninfected CD1 mice with 10 exp6 pRBC (Figure 25).

The next step was to select the parasite progeny with different drugs in order to eliminate the sensitive parasites from the population. To this extent, the animals were divided into three groups treated as follows: i) DMSO vehicle; ii) 20 mg/kg/day of ATN; iii) 2 mg/kg/day of MEF; and iv) 1 mg/kg/day of MF in combination with 7.5 mg/kg/day of ATN (Figure 25). The parasitaemias of the eight animals were followed and on Day 7 pi the animals were exsanguinated. The blood of the two animals in each group was pooled together and a drop was dried into Whatman Nº4 filter paper for DNA extraction (as described below in Section 3.1) and will be refered to as Samples 2 – to – 5, as shown in Figure 25. The remaining blood was processed (Section 1.4) and stored into liquid nitrogen.

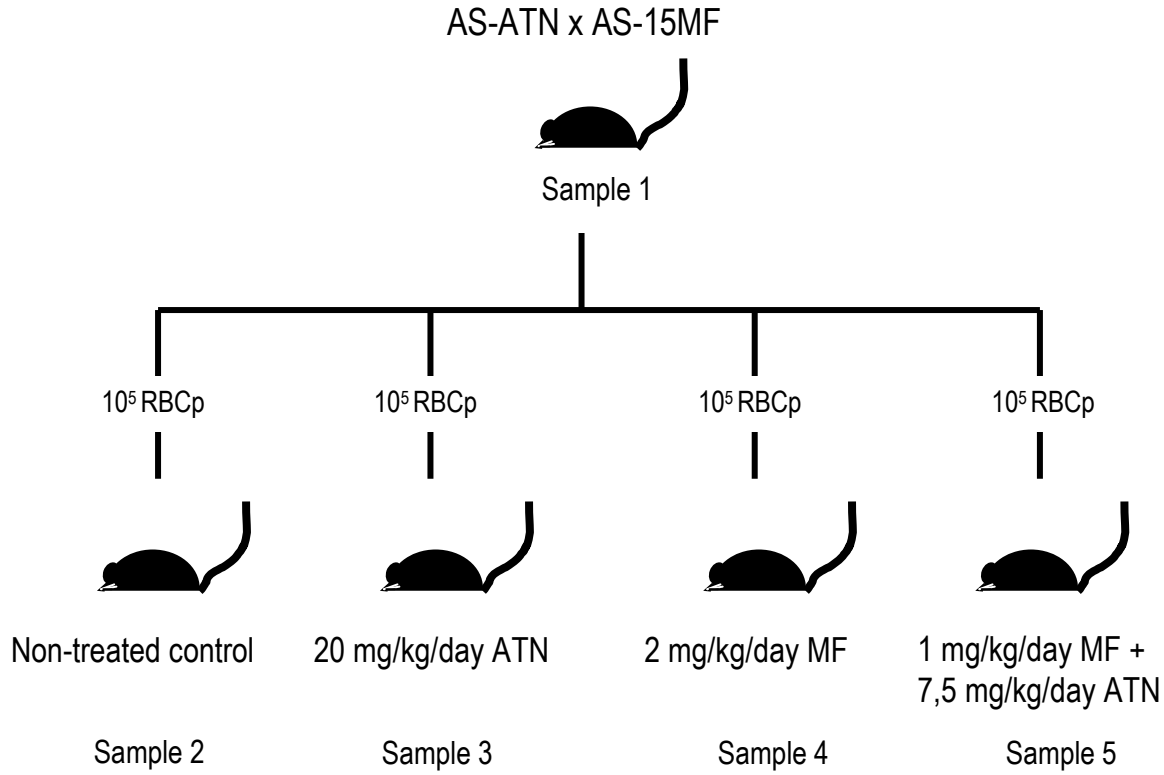

Figure 25 – Parasite progeny resulting from the genetic cross was selected with different drug treatments. Mice carrying the cross-progeny AS-ATN x AS-15MF had their blood preserved in filter paper for DNA extraction (Sample 1). Then, the mice were exsanguinated and their blood was inoculated into eight mice divided in four groups. Each group was treated as follows: DMSO vehicle (non-treated control), 20 mg/kg/day of ATN, 2 mg/kg/day of MF or 1 mg/kg/day of MF in combination with 7,5 mg/kg/day of ATN. Parasitaemias were followed. Mice were exsanguinated and blood preserved for DNA extraction (Samples 2 to 5, respectively).

2.4.2. ANALYSIS OF PARASITE CROSS

In order to attest that the parasite progeny obtained as described above was a product of the genetic cross between AS-ATN and AS-15MF clones, the genetic traits belonging to both parents was investigated.

For that purpose, each of the five DNA samples obtained above were analysed by PCR for two genetic markers. One of them is the *pcubp1* (PCHAS_020720) where the V2467F mutation is exclusive of the AS-ATN parental clone, and results from a G to T substitution in position 8089 of the coding sequence of this gene (according to the lattest Sanger annotation) (Hunt *et al,* 2005). AS-15MF does not carry the wild type allele of this gene. Instead it carries a SNP placed downstream to the one present in AS-ATN, a G8182T. This mutation codes for another V to F substitution, this time in aminoacid 2728. The presence of either of the two mutations seems to be in linkage with ATN or ART resistance, respectively (Hunt *et al,* 2005).

The second marker analysed was the PCHAS_100330 gene, which codes for a lysine decarboxylase (*P.falciparum* PFD0285c), where the SNP A2467C causes de non-synonimous mutation T823P (Borges, 2009). This mutation is exclusive of the AS-15MF parental clone, while AS-ATN carries the wild type allele. The presence of this mutation seems to be in linkage with resistance to MF, but its presence is not mandatory for the expression of the resistat phenotype.

PCR conditions, oligonucleotide primers and further analysis will be described in detail in Section 3.2.

3. GENOTYPING

3.1. DNA EXTRACTION

DNA was extracted from mouse blood by two different methods, according to the size and preservation of the blood sample. The methods are described in detail bellow.

DNA form dried blood spots preserved in Whatman nº4 filter paper was extracted using the Chelex-100 method. Briefly, 1 cm^2 of filter paper was incubated overnight in 1 ml PBS (Sigma) + 0.5% saponin (Sigma) at 4ºC. The solution was removed and the filter paper was incubated with 1 ml PBS at 4ºC for 30 minutes. After the removal of the PBS, the filter paper was incubated at 100ºC with pre-heated 200 µl PBS + 5% Chelex-100 (Bio-Rad) for 10 minutes and vortexed vigorously every 5 minutes. The tubes were centrifuged at 14000 rpm for 2 minutes and the supernatant was placed into a fresh tube. The supernatant (containing the DNA) was centrifuged again in order to insure the complete removal of the Chelex-100 matrix and stored at -20ºC.

For large amounts of blood obtained from of one mouse or many mice, DNA was extracted by Phenol:chlorophorm. Concisely, mice were exsanguinated by puncture in the brachial artery and blood was mixed with ice cold Citrate Saline Solution. The mixture was passed twice through cellulose (CF11, Sigma) 5 ml column and in addition, it was also filtrated using two Plasmodipur™ filters (Euro-Diagnostica), to ensure the complete removal of the leucocytes. The filtrates were centrifuged at 3000 rpm for 5 minutes at room temperature and the supernatant was discarded. The volume of the erythrocyte pellet was estimated and resupended into twice the volume of PBS + 0.15% saponin, in order to disrupt the erythrocytes' membranes, releasing the parasites. The mixture was mixed gently until its colour turned from red to burgundy. The lysis was stopped by the addition of an excess of PBS, followed by centrifugation at 4000 rpm for 5 minutes. The supernatant was discarded and parasite pellet obtained was resuspended into PBS and centrifugation and wash repeated. The supernatant was discarded and the parasite pellet was kept in -70ºC for further use.

Upon thawing on ice, the parasite pellets were resuspended into 5 volumes of pre-warmed Parasite Lysis Buffer (Section 1.1) containing 0.125 mg/ml Proteinase K. The mixture was maintained at 37ºC overnight. Phenol (Ambion) pH 7.9 (equilibrated with TE Buffer) was added in an equal volume and mixed thoroughly for 2 minutes. The mixture was then centrifuged at 5000 rpm for 3 minutes. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of phenol:chlorophorm (1:1) (Ambion) pH 7.9 and centrifuged as before. The aqueous phase was carefully removed (in order to avoid contamination with proteins present in the interface) and transferred to a new tube where it was mixed with an equal volume of chlorophorm. After mixing and repeating the centrifugation, the aqueous phase was transferred to a fresh tube and 1 µl of RNAse H 1.5 U/µl (Promega) was added per 100 µl of aqueous phase. The mixture was incubated at 37ºC for 10 minutes.

The DNA precipitation was made by the addition of 1/9 volume of 3M sodium acetate (pH 5.2). Two volumes of ice-cold absolute ethanol were added to this mixture and it was incubated on ice for 30 minutes, followed by centrifugation at maximum speed for other 30 minutes. The ethanol was removed and the DNA pellet was washed twice in ice cold 70% ethanol. Finally, the mixture was centrifuged at 12000 rpm for 1 minute and the supernatant was discarded. The pellet was let air dry and re-suspended into 50 to 100 µl of TE buffer and incubated at 37ºC for 10 minutes.

3.2. PCR

Along the course of this work, different genes were analysed with different purposes. For instance, the two clones selected by drug pressure (Section 1.2) were investigated for the presence of novel mutations in specific candidate genes. Thus, an overlapping fragment DNA sequencing strategy was applied in order to cover the whole coding sequence of each gene under investigation. The sequences of the *P.chabaudi* orthologs of the *P.faciparum pfatp6* (PfA0310c) and *pfubp-1* (PfA0220w) genes were first retrieved from PlasmoDB. These sequences correspond to *P. chabaudi* orthologues PCHAS_020540 and PCHAS_020720, respectively, and were used as template for designing PCR oligonucleotides to amplify and sequence the two genes. However, by the time this analysis was performed, the lattest assembly of the *P.chabaudi* genome was not available yet. An updated version was recently released (available the Welcome Trust Sanger Institute website dating of September of 2009) and the coding sequence of the gene *pcubp1* is now annotated as composed by 8766 nucleotides. So, only the last 3500bp segment of the currently annotated sequence of this gene was investigated here. On the other hand, no differences were found when comparing the coding sequence previously available of the *pcatpase6* gene and its latest annotated version.

Oligonucleotide primer sequences as well as the cycling conditions for the *pcatpase6* (PCHAS_020540) gene were described previously by Afonso et al, 2006 whereas the primer sequences and cycling conditions for the gene *pcubp1* (PCHAS_020720) were described previously by Hunt et al, 2007. The only exceptions are the primers surrounding the mutation exclusive of AS-ATN (Hunt et al, 2007), the G8089T substitution (nucleotide position according to the new Sanger annotation). The forward sequence used here was 5'-GTTACCAATTGATACGACTG-3' and the reverse sequence 5-CAGAATTAGTATGAGGTGGC-3'. The cycling conditions were: denaturation at 94ºC for 3 minutes, 15 cycles of 94ºC for 15 seconds, 40ºC for 45 seconds and 68ºC for 4 minutes, followed by 30 cycles of

94ºC for 1 minute, 45ºC for 45 seconds and 72ºC for 3 minutes. The final elongation was run for 10 minutes at 72ºC.

The progeny resulting from the genetic cross between the parasites AS-ATN and AS-15MF was also investigated for mutations. In this case, the aim was not the identification of novel mutations appearing in the coding sequence of genes of interest. The intent here was to seek for known mutations previously described as being present in the parental clones used for the cross, serving as genetic markers. For that purpose, two genes were inspected. The gene *pcubp1*, described previously in this Section, was analysed regarding the presence of the G8089T mutation, which is exclusive of AS-ATN clone. Thus, the primers and cycling conditions applied were the same as described above. In addition, another SNP could be identified in the same segment flanked by the two primers used for the analysis of this gene. This is the G8182T substitution, and can be found in AS-15MF.

Another gene used as marker of the parental clones used for the genetic cross was *P.chabaudi*'s PCHAS_100330 (*P.falciparum* PFD0285c). This gene codes for a lysine decarboxylase and the mutation A2457C is present in AS-15MF clone, as decribed in Chapter II, Section II.1.2.1. The sequences of the primers used for amplifying the region around this mutation were forward 5'-TGGTTTGCTTATGCTTGTTTC-3' and reverse 5'-CCATTTCTCCTATTTGTATTTC-3'. The cycling conditions were 95ºC for 3 minutes, followed by 48ºC for 1 minute, 65ºC for 1 minute, and then by 35 cycles of 94ºC for 1 minute, 50ºC for 1 minute and 65ºC for 1 minute. The final elongation step was made at 65ºC for 10 minutes.

For all reactions performed here, 1 µl of DNA extracted by Chelex-100 method (Section 3.1) was used as template in 50 µl reactions. The other reagents were added to 10x Green GoTaq® Flexi Buffer (Promega) to a final concentration of 1,5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (each), 0.2 pmol/µl forward and reverse primers, and 1,25 U of Go Taq Flexi DNA Polymerase (Promega).

The products resulting after PCR were analysed by electrophoresis in TAE Buffer on an ethidium bromide stained 1% agarose gel. When a single band was observed, the products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). After purification, the products were sent for being analysed by di-deoxy sequencing using the commercial services provided by STABVIDA laboratories. The sequences obtained here were aligned using Multi-Alin (Corpet, 1988), a multiple sequence alignment software available online (http://bioinfo.genotoul.fr/multalin/multalin.html). The chromatograms were also investigated in some cases, using Chromas 2.33 software (Technelysium) obtained online (http://www.technelysium.com.au).

3.3. RNA EXTRACTION

Total RNA was extracted from blood stage parasites in order serve as template for cDNA synthesis.

For that purpose, CD1 mice were inoculated with infected blood. When reaching peak parasitaemia, the animals were bled out and the mixture of blood and Citrate Saline Solution was filtered in a cellulose (CF11, Sigma) 5 ml column. Subsequently, it was centrifuged at 3000 rpm for 5 minutes at room temperature and the supernatant was discarded. The volume of the erythrocyte pellet was

estimated and an equal volume of Citrate Saline Solution was added to it and mixed gently. The mixture was divided into vials containing at least 300 µl each. The vials were kept at -70ºC prior to use.

For RNA extraction, the blood on the vials was defrost on ice, and the Aquapure RNA Blood Kit 100 (BioRad) was used. The protocol was followed as the manufacture's instructions, except for minor changes described bellow. Briefly, 300 µl of blood was mixed by inversion with 900 µl of RBC Lysis Solution, provided with the kit. The mixture was incubated at room temperature for 10 minutes and then centrifuged at 14000 rpm for 10 minutes at 4ºC. The supernatant was discarded leaving approximately 20 µl of residual liquid that was thoroughly mixed with the pellet. Subsequently, 300 µlof the kit's RNA Lysis Solution was added and mixed by pipetting repeatedly. 100 µlof the Protein-DNA Precipitation Solution was added and the mixture was incubated on ice for 10 minutes. The samples were centrifuged at 14000 rpm for 5 minutes at 4ºC and the pellet was discarded. 300 µl of 100% Isopropanol was added to the supernatant and centrifuged at 14000 rpm for 5 minutes at 4ºC. The supernatant was poured out and the tube was briefly dried using an absorbent paper. The pellet was washed in 70% Ethanol. After being centrifuged again at the same conditions as before, the supernatant was rejected and the tube was allowed to air dry for 15 minutes. 50 µl of RNA Hydration Solution was added to the dry pellet and the tube was kept on ice for 30 minutes.

The RNA sample obtained as described above was either used immediately for cDNA synthesis or kept at -70ºC.

3.4. cDNA SYNTHESIS

cDNA synthesis was carried out using the RNA samples extracted from mouse blood infected with different clones, as described in the previous section (Section 3.3).

Before starting the cDNA synthesis, the samples were treated with DNAse to ensure the removal of any contaminant DNA. Thus, 4 µl of the RNA sample was mixed with 1,0 µl of 10x DNAse Buffer (FERMENTAS^{TM}), 1,0 µl of 1U/µl DNAse (FERMENTAS) and 5,0 µl of DEPC treated water and incubated for 15 minutes at room temperature. 1 µl of 25mM EDTA was added to the reaction mixture and incubated at 65ºC for 5 minutes.

The reverse transcription of the DNAse treated RNA began by the addition of 2 µl of 10x concentrated Hexanucleotide Mix (Roche). The tube was placed in a dry bath at 70ºC for 5 minutes and later cooled down on ice. After that, a mixture consisting of 4 µl of 10x M.Mulv Buffer (FERMENTAS), 2 µl of deoxynucleoside triphosphate mix (10 mM each), 3 µl of 100mM DTT, 0.5 µl of 400U/µl RNA inhibitor Ribolock™ (FERMENTAS), and 7,5 µl of DEPC treated water was prepared. 17 µl was added to the mixture of DNAse treated RNA and Hexanucleotides and it was incubated at 25ºC for 5 minutes. 1 µl of 200 U/µl M-MuLV Reverse Transcriptase (FERMENTAS) was added and the reverse transcription was performed by one cycle of 25ºC for 10 minutes, 42ºC for 1 hour, and 70ºC for 10 minutes. The samples were allowed to cool down in ice and kept at -20ºC prior to use.

3.5. REAL-TIME PCR ANALYSIS

Real-Time PCR TaqMan assays were performed in order to assess the copy number and RNA expression profile of *mdr1* gene in the clones selected in this work. The values were obtained by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001), using the *pcα-tubulin ii* as housekeeping gene.

For that purpose, the sequences of the *P.chabaudi* homologous of the *P.falciparum pfmdr1* gene (PFE1150W) and alpha-tubulin ii gene (PFD1050w) were obtained from Plasmo DB. These genes are annotated as PCHAS_123820 and PCHAS_052240, respectively. Their sequences were used as template for the design of oligonucleotide primers and probes.

Both assays were devised to be performed simultaneously (in the same plate and under the same conditions) using the commercial services provided by TIB MOLBIOL. The sequences of the primers and probes are depicted on Table 9. Probes were labelled with 6-FAM (6-carboxyfluorescein, succinimidyl ester) at the 5' end, and the dye's fluorescence was quenched by the presence of Black Berry™ Quencher 650 on the 3' end of the probe.

	PRIMER SEQUENCE
mdr1 foward	5'-CAC ACA ATT TGA AAG ACG TTG ACT-3'
mdr1 reverse	5'-ATT TAA TGA AGA ATC GCT ACT TCC G-3'
α-tubulin ii foward	5'-TGC TCC AAT TAT TAG TGC TGA AAA AG-3'
α-tubulin ii reverse	5'-ACA GCA AGC CAT ATA TTT TCC ATG TC-3'

	PROBE SEQUENCE
mdr1	5'-6FAM-TCA TGG TGA ATC CAT TTT CCA TTG CTT CT--BBQ-3'
α-tubulin ii	5'-6FAM-CCT GCA TCT ATG ATG GCA AAA TGT GAT CCT--BBQ-3'

Table 9 – Primers and probe sequences for mdr1 (PCHAS_123820) and α-tubulin ii (PCHAS_052240) genes used for quantification of copy number (genomic DNA) and RNA expression (cDNA) of the *mdr1* gene by Real-Time PCR.

The reaction mix was prepared by adding primers and probes to Faststart Universal Probe Master Mix with ROX (Roche), to a final concentration of 900 μM and 250 μM respectively.

In order to assess the number of copies of *mdr1* gene shown by the clones selected here, 2 µl of DNA extracted from dried blood spots as described in Section 3.1 was added to the reaction mix, in triplicates, to a final volume of 20 ul. AS-SENS, AS-ATN and AS-15MF clones were used as controls, since the latter harbours a duplication of the *mdr1* gene (Cravo *et al,* 2003) whereas the former two carry a single copy.

For the RNA expression analysis, 2 µl of cDNA synthesised from RNA extracted as described in the previous sections was added to the reaction mix, also in triplicates, to a final volume of 20 µl. AS-3CQ clone, which has a single copy of this gene, was used as control instead of AS-SENS. Similarly to the copy number determination, AS-ATN and AS-15MF clones were also used as controls.

The cycling conditions for copy number analysis were 95ºC for 10 minutes followed by 50 cycles of denaturation at 95ºC for 15 seconds and annealing/extension at 57ºC for 1 minute, carried out in a Gene Amp 5700 Light Cycler (Applied Biosystems).

The cycling conditions for the RNA expression analysis were the same used for the copy number assays. However the experiments were carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems).

The assays were repeated at least 5 times, and the mean values and standard errors calculated. A Student's T-Test was used to verify statistic significance of the results found here.

3.6. WESTERN BLOT ANALYSIS

In order to determine expression of gene *mdr1*'s product P-gh1 in the ATN + MF resistant parasites AS-ATNMF-1, protein extracts were prepared from erythrocyte stage parasites. Thus, mice were injected with infected blood and upon reaching peak parasitaemia were exsanguinated. The blood was diluted in Citrate Saline Solution and then filtrated once using a cellulose (CF11, Sigma) 5 ml column and centrifuged at 3000 rpm for 5 minutes. The pellet was washed twice in ice-cold PBS and re-suspended into two volumes of PBS. The mixture was divided into 500 µl aliquots. RBC lysis was performed by adding 1 ml of Hypotonic Ammonium Chloride Solution and incubating at 4ºC for 3 minutes. The samples were centrifuged at 10000 rpm for 7 minutes at 4 ºC and supernatant was discarded. The pellet was washed twice in PBS and finally centrifuged at 10000 rpm at 4ºC for 7 minutes. The volume of the pellet was estimated and re-suspended into twice the volume of Loading Buffer. Samples were kept at -20ºC and sent to analysis by Western Blot performed by Cecilia Sanchez as part of a collaboration maintained with Heidelberg University.

Thus, upon arrival, samples were prepared as follows: protein amounts were determined by Bradford assay (BioRad, Germany). Samples were then run on NuPAGE Novex Tris-Acetate gels (Invitrogen, Germany) and transferred onto a 0.2 µm PDVF membrane (BioRad, Germany). Membranes were blocked overnight at 4°C using 5% milk in PBS. Primary antibodies (α-Pgh-1 and α-tubulin, diluted 1:1,000 and 1:2,000, respectively) were incubated for 1 h at room temperature in 1% BSA + PBS. Membranes were washed three times using PBS + 0.1% Tween for 10 min at room temperature and then blocked again in 5% milk in PBS for 1 h. Secondary antibodies (Alexa Fluor 680 goat anti-rabbit IgG, or Alexa Fluor goat anti-mouse IgG, both diluted 1:10,000) were added to 1% BSA + PBS for 30 min at room temperature. After washing four times in PBS + 0.1% Tween for 5 min, signals were

read using an Odyssey-Li-cor infrared imaging system (Li-cor Biosciences). Fluorescence intensities for Pgh-1 were normalized using fluorescence intensities measured for tubulin. The resulting values were then expressed as relative to AS-3CQ and AS-ATN.

The results were then expressed as means of five experiments and a Student's T Test was used to determine statistical significance of results obtained.

3.7. GENOME-WIDE IDENTIFICATION OF MUTATIONS

3.7.1. SOLEXA GENOME RESEQUENCING

In order to perform a complete analysis covering all the genome of AS-ATNMF-1 clone, DNA obtained as described in Section 3.7 was processed using standard methods according to the manufacturer's recommendation (http://www.illumina.com/technology/sequencing_technology.ilmn). Samples were run at the Genepool facilities at the University of Edinburgh, UK, using the Illumina Solexa high-throughput sequencing technology. Sequencing was performed with 50 bp reads, using a paired-end read approach. Two software packages were used to align sequences against a reference genome (the isogenic AS-WTSI genome, curated by the Wellcome Trust Sanger Institute) and identify genome wide mutations, namely MAQ (Mapping and Assembly with Quality) (Li *et al,* 2008) and SSAHA2 (Sequence Search and Alignment by Hashing Algorithm) (Ning *et al,* 2001). The SSAHA2 analysis was done in collaboration with Thomas Otto at the Wellcome Trust Sanger Centre in Cambridge, while MAQ analysis was done in collaboration with Urmi Trivedi at the Genepool in Edinburgh.

SNP detection was performed by both MAQ and SSAHA2 softwares, using in-built algorithms, with a read depth of 3 set as the minimum threshold for SNP detection and no upper threshold selected. In order to filter out SNPs arising in the sensitive progenitor AS-SENS, the lists of SNPs obtained for AS-ATNMF1 was compared against a list previously obtained for AS-SENS (Hunt *et al*, 2010). The two filtered lists (for MAQ and SSAHA2) were then combined.

Small indels (< 3bp) were detected using SSAHA2's internal algorithm only. The list of small indels was filtered against a similarly obtained list for AS-SENS (Hunt *et al*, 2010). Larger indels (≥ 3bp) and copy number variation (CNV) detection was performed using SSAHA2 as previously described (Hunt *et al*, 2010). Briefly, the approach is based upon comparing coverage between the mutant clone and the sensitive progenitor in order to detect differences that indicate potential mutations. Coverage was normalised for each clone to take into account differences in average coverage (e.g. because some clones were sequenced with a greater number of Solexa lanes, thus yielding greater read depth). So, the coverage in each position was divided by the mean coverage of the whole genome in order to obtain a so-called "relative coverage" for each clone. Then, the "relative coverage" for each nucleotide in the resistant clone was compared to the relative coverage of the sensitive clone, obtaining the "comparative coverage". The comparative coverage along a given chromosome can be visualised by using Artemis Software (Rutherford *et al,* 2000).

Two simple algorithms were used to indicate areas of significantly decreased or increased coverage (Hunt *et al*, 2010). Since AS-ATNMF-1 was sequenced using paired-end reads but AS-SENS was originally sequenced using single end reads, this approach could not be adopted using this clone, due to

the excessive variation introduced by the different re-sequencing strategies adopted. Instead, a clone selected for sulphadoxine resistance (AS-50SP) and re-sequenced using paired-end reads (Martinelli unpublished results) was selected to act as a "filter", with the caveat that false positives and negatives are likely to appear. Due to a complete sequencing and analysis of other clones in the lineage (Hunt *et al*, 2010), we felt confident that this limitation could be overcome by resorting to the data previously obtained to resolve any uncertainties and discover false negatives.

The positions of all the mutations described here are in accordance with the latest version of the *P.chabaudi* genome available on Welcome Trust Sanger Institute website (ftp://ftp.sanger.ac.uk/pub/pathogens/P_chabaudi/).

3.7.2. VALIDATION OF MUTATIONS BY DI-DEOXY SEQUENCING

Some SNPs and indels identified as described in the previous section were chosen for confirmation through traditional di-deoxy sequencing. For that purpose, a region of about a 1000 bp around each mutation was used for designing oligonucleotide primers and the fragments were amplified by PCR. DNA samples were obtained from dried blood spots for all clones in AS lineage, as described in Section 3.1. DNA and PCR reagents were added as described in Section 3.2. There was no variation in cycling conditions used for all reactions, except for annealing temperatures, which varied according to the primers. Cycling conditions were 93ºC for 3 minute, followed by 35 repeats of denaturation at 93ºC for 30 seconds, annealing for 45 seconds, and elongation at 72ºC for 1 minute. A final elongation step was performed at 72ºC for 10 minutes, and samples were kept at 4ºC until processing. The sequences of oligonucleotide primers and their respective annealing temperatures are listed in Table 10.

The products thus obtained were resolved into an agarose 1% gel, as previously described in Section 3.2, and when showing a single band, were purified and sequenced using the commercial services of STABVIDA Laboratories. The chromatograms were inspected using the Chromas 2.33 software (Technelysium).

GENE (position)		SEQUENCE	ANNEALING TEMPERATURE
PCAS_020900	sense	GGT ATG ATC CAT TAT CTT GTT CG	51 ºC
	anti-sense	TGT ATC AGG ATC TAG TAG TTC G	
PCAS_031370	sense	GCT TGC TTA TGC ATA CTT CG	53 ºC
	anti-sense	TCG CGA AAT CGA ACT TAA AAT AC	
PCAS_031520	sense	GAG GGT TTA TAG TGT TTG TAT C	54 ºC
pos. 523111 to 523127	anti-sense	CAT TCA AAC TCA TCA GCC TTA G	
PCAS_031520	sense	GAA CAG ACA TTT CTT GAT TTT GC	54 ºC
pos. 524946 to 524964	anti-sense	CTA TTC AGG CAA TGG TAA CC	
3'-PCAS_061710	sense	CAT TCA GCT TGT AAC TTG GG	56 ºC
	anti-sense	GAG AAG GTA TTA TGG TTC CTG	
PCAS_070250	sense	CCC GCC CCA GCA AAA TAA AC	54ºC
	anti-sense	GCT GAA AAG AAA GCG GAA GAG	
PCAS_072830	sense	TAA ATC TTC ATT GAT ATC TGG	51ºC
	anti-sense	CAT TCC CAA GTC CCT TAA AA	
PCAS_072420-3'	sense	CGT ATA AAG GCT GTG ACA AC	52 ºC
34 bp deletion chr 7	anti-sense	CAG GTT CGT TTT GTA CAT ACC	
5'-PCAS_083770	sense	GAA AGT GAA GCA CCA TTC GG	56 ºC
	anti-sense	CAC TGT GTC ATA TTC AAG ATT TG	
5'-PCAS_092710	sense	GCA TGC ATG TAG ATC ATA ACA C	54 ºC
	anti-sense	GTT TAC ATT CTT CGC TGC TAT C	
PCAS_121630	sense	CCC TTT ATC ACC CGA ATA TTT C	55 ºC
	anti-sense	CAT ATT TTT TAA GGG GTA AGT GC	
PCAS_130140	sense	CTC TGG GTC CGA AAT CAA TG	51 ºC
	anti-sense	GTC ACC ATT AGG CCC ATA AG	
PCAS_133430	sense	GGT TGT TGT TTA GGA TTA GGT C	49 ºC
	anti-sense	CAT ACC ACC ATA TCT GAT TAT TG	
PCAS_132020	sense	CCA GAT ATT AAG CAA TAC AGC AC	56 ºC
	anti-sense	CGG CCA CAC ATA TAT TAA GGA	
PCAS_143160	sense	GAG GAC GCG AAA AGG AAA G	53 ºC

Table 10 – List of primers and their respective annealing temperatures. Results obtained by Solexa were analysed by di-deoxy sequencing using the primers listed above.

3.7.3. BIOINFORMATICSANALYSIS OF MUTATIONS

The role of any mutation located within the coding sequence of a predicted protein was further analysed through the use of widely available bioinformatics tools. The wild type sequence was retrieved from the PlasmoDB database (www.plasmodb.org). The predicted sequence of the mutated protein was obtained by the translation of the manually mutated nucleotide sequence by the Sequence Manipulation Suite Software available online (Stothard, 2000). Subsequently, mutated and wild-type nucleotide and amino-acid sequences were aligned against each other using the alignment software Multi-align (Corpet, 1988).

Any potential role of the predicted protein was retrieved from the PlasmoDB database. Additionally, the predicted protein sequence was used for performing a BLAST search (non-redundant protein sequences (nr) database using Blastp (protein-protein BLAST) algorythm) (Altschul *et al,* 1997; Altschul *et al,* 2005) in order to identify the presence of regions showing similarities to known conserved domains in other proteins and organisms. Protein sequences were also inspected for conserved domains and functional sites using PROSITE (Hulo et al, 2007) and Pfam (Welcome Trust Sanger Institute). Although this information can sometimes be found using BLAST, localisation of predicted transmembrane domains in the protein sequences was also made by the use of HMMTOP (Tusnady and Simon, 1998).

3.8. LINKAGE GROUP SELECTION

3.8.1. GENETIC CROSS AND DRUG TREATMENT

In order to perform a genetic cross between the ATN + MF- resistant AS-ATNMF-1 selected as described previously (Section 2.1) and the drug-sensitive genetically unrelated AJ, the protocol was very similar to the one used in Section 2.2. However, it contains a few modifications. Briefly, four CBA donor mice were divided into two groups. The first inoculated with the AS-ATNMF-1 clone and the other one inoculated with AJ. After reaching parasitaemias above 10%, blood was extracted by the tail vein and used to prepare the mixed infections. Seven C57BL/6 acceptor mice (here the acceptor mice were not treated with EPO) were divided into three groups: i) one mouse was inoculated with 10^5 pRBC of AS-ATNMF-1; ii) one mouse received 10^5 pRBC of AJ; iii) five animals received a mixture of equal proportions of the two clones, containing 10^5 pRBC. On Day 8 pi, when reaching parasitaemias above 5%, the seven mice were anaesthetised and used for feeding around two hundred *Anopheles stephensi* mosquitoes (both male and female) for 30 minutes. The mosquitoes here were not previously treated with XA. The mosquitoes' drinking water (10% Glucose + 0.05% PABA solution) had been removed 12 hours prior to feeding, and was replaced immediately after the end of the blood meal.

Similarly to Section 2.2, four days after the blood meal, uninfected mice were used to boost-feed the mosquitoes, and on the tenth day ten mosquitoes per cage had their midguts dissected and inspected for the presence of oocysts. The oocysts were preserved in PBS and DNA was extracted using the High Pure PCR Template Preparation Kit (Roche), following the manufacturer's instructions. Later, nested PCR products were obtained for the *pcubp1* gene (PCHAS_020720) using the primers shown in Table 11. DNA and PCR reagents were added as described in Section 3.2. Cycling conditions were: 95ºC for 1 minute, followed by 30 cycles of 95ºC for 1 minute, 52ºC for 1 minute and 65ºC for 1 minute. Final

extension was made at 65ºC for 10 minutes. Samples were then resolved in 1% agarose gels, and further processed as described in detail in Section 3.2.

	PRIMER SEQUENCE
Outer Forward	5'-ATG CAA ACT TAC TTT CAA AAC G-3'
Outer Reverse	5'-TTG TTG CAT TTC GAG CAT TTG-3'
Inner Forward	5´-CAA ATA AAA AAT ATG TTT CAC CAG-3'
Inner Reverse	5'-CGA GCA TTT GTA TTT ATT GTT TCC-3'

Table 11 – Primer sequences used for nested PCR amplification of *pcubp1* gene using DNA extracted from oocysts dissected from mosquitoes' midguts.

The amplified fragment contains four polymorphisms when comparing the sequences obtained for AS and AJ parentals. It is then possible to determine the proportions of the alleles from each parental, by observing the electropherogram peak heights at each polymorphic position. This method is known as Proportional Sequencing (Hunt *et al,* 2005) and will be described in detail below (Section 4).

On the fourteenth day, mosquitoes were allowed to feed on two CBA uninfected mice per cage in order to transmit the infection. For that purpose, mosquitoes' drinking water was removed at least 12 hours prior to the blood meal and anaesthetised uninfected mice were made available to mosquitoes for 30 minutes. After recovering from anaesthesia, the mice were kept on standard conditions and parasitaemias followed for 10 days.

On Day 10 after the transmission, all the mice carrying the uncloned progeny resulting from AS-ATNMF-1 x AJ cross were exsanguinated and their blood was mixed. The parasites were propagated into new mice in a two-step process: i) 10^6 pRBC from the mixed blood was inoculated into five CBA female mice. On Day 7 the mice were exsanguinated and their blood pooled together. ii) 10^7 parasites from this second pool were inculated into new five CBA female mice. On Day 7, when reaching parasitaemias above 20%, blood was extracted from the five mice and mixed.

After propagation, the selection of the uncloned progeny was made as follows: thirty CBA female mice were divided into six groups of five mice, and each one received a 10^7 pRBC inoculum. The mice were then treated with three daily doses of different anti-malarial drugs on Days 1, 2 and 3 pi as follows: i) 25 mg/kg/day of ATN; ii) 40 mg/kg/day of ATN; iii) 2 mg/kg/day of MF; iv) 2 mg/kg/day of MF in combination with 20 mg/kg/day of ATN; v) 100 mg/kg/day of ART; and vi) DMSO vehicle. The parasitaemias were assessed daily from Day 6 onwards.

3.8.2. SAMPLE PREPARATION

Upon reaching peak parasitaemia the mice carrying the drug treated uncloned parasite progeny resulting from AS-ATNMF-1 x AJ cross were exsanguinated and the blood used for DNA extraction by phenol-chloroform, as described at Section 3.1.

The DNA concentration in each sample was determined using a QUIBIT™ fluorometer using Quant-iT™ dsDNA assay kit (Invitrogen). Dilutions were prepared so each reaction would contain between 1 pg/ml and 300 pg/ml.

DNA samples were analysed by quantitative Pyrosequencing™ (Cheesman *et al,* 2007) using a genome-wide library of approximately 150 Pyrosequencing™ SNP assays, produced by the Pyrosequencing™ Assay Design Software version 1.0.6 (Biotage AB) to distinguish between AS/AJ alleles. These assays consist in two oligonucleotide primers (one of which is biotinylated) and a sequencing primer that anneals to the sequence close to the SNP. Primer sequences are listed in Appendix VI.

The DNA samples were amplified by PCR using each pair of oligonucleotide primers, under the same conditions described in Section 3.2. The cycling conditions used in this case were: 96ºC for 1 minute, followed by 30 cycles of 94ºC for 1 minute, 51ºC for 1 minute and 72ºC for 2 minutes. The final elongation step was run at 65ºC for 10 minutes. The samples were analysed in 1% agarose gel, stained with Safe View Nucleic Acid Stain.

3.8.3. PYROSEQUENCING

The Pyrosequencing™ analysis of the samples was carried out by adding 10 µl of PCR product obtained as described in the previous section to 38 µl Binding Buffer (Biotage), 30 µl of high purity Milli-Q water (Millipore) and 2 µl of Streptavidin Sepharose™ High Performance beads (GE Healthcare), on a 96-well PCR plate. The plate was placed on a shaker until further processing.

The content of the wells was then removed by suction and the biotinylated PCR product attached to the beads was trapped to the probes of a Pyrosequencing™ Vacuum Prep Tool (Biotage). The probes were washed in a 70% ethanol solution for 5 seconds, following a wash in denaturation solution containing 0.2 M Sodium Hydroxide for 5 seconds, and a third wash, in Wash Buffer (Biotage) for 5 seconds. The probes were allowed to vacuum dry the products for other 5 seconds.

A second plate was prepared by adding 0.5 of sequencing primer to 11.5 µl of Annealing Buffer (Biotage) in a Pyrosequencing instrument-specific 96-well plate (Biotage). This plate was placed under the Pyrosequencing™ Vacuum Prep Tool and the vaccum released, allowing the beads to be mixed with the wells' content. The plate was heated at 80ºC for 2 minutes and let cool down to room temperature. The plate was subsequently analysed on the PSQ™ HS-96A instrument using the Pyro Gold reagents (Biotage).

The Comparative Index (CI) was calculated as the proportion of the sensitive allele (AJ) in the treated population relative to the untreated populations (Culleton *et al,* 2005).

4. FITNESS COST ANALYSIS

In order to assess the biological cost the parasites might incur as consequence of the acquisition of resistance to ATN + MF, the fitness of AS-ATNMF-1 clone was estimated in comparison with its progenitor, AS-ATN.

In addition, it is also known that consecutive passaging through mice might lead to an increase in parasite fitness (Mackinnon and Read, 1999; Walliker *et al,* 2005). To attest that, AS-ATNMF-1 growth was compared with the parasite population passaged in absence of drug treatment, AS-ATN27P.

For that purpose, two clones were mixed in different proportions and grown together in the same host. Changes in proportions of parasites were followed along time using a method known as Proportional Sequencing (Hunt *et al,* 2005). By this method, the proportion of different alleles present in a mixture is estimated by measuring the peak heights shown by the DNA sequence gel electropherogram in a polymorphic position. As exemplified in Figure 26, the proportion of the allele A is determined by the height of peak A as a percentage of the sum of the two peaks (A and B) in that position, so:

$$\%A_{allele} = A_{height} \times 100 / (A_{height} + B_{height})$$

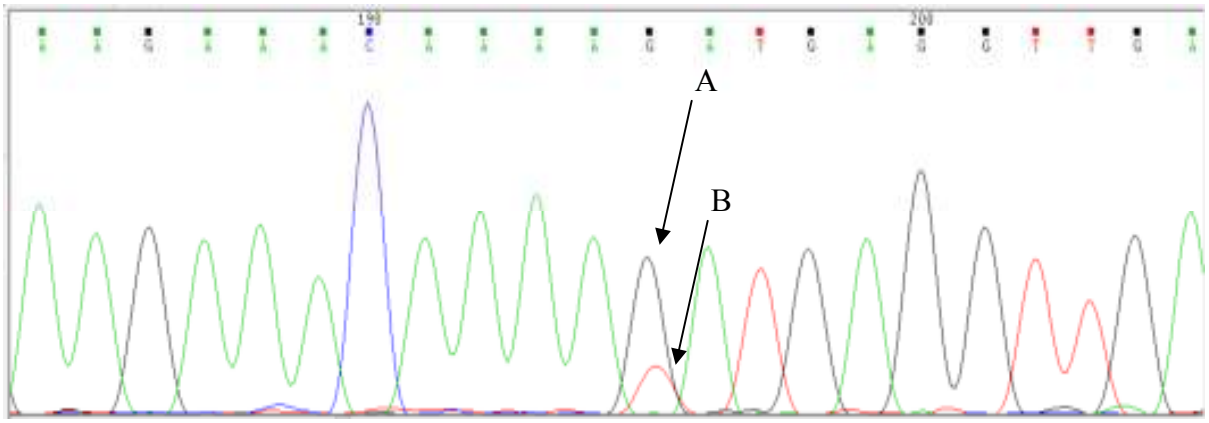

Figure 26 – Example of an electropherogram visualised using Chromas 2.33. A G to T polymorphism can be observed (black arrows). The heights of peaks A and B are measured and percentage of the each allele was calculated as the percentage of the sum of the two peaks.

4.1. VALIDATION

Although this method provides the determination of proportions of different alleles with high accuracy, limitations intrinsic of DNA amplification and sequencing make necessary validation with a calibration curve.

To that extent, four mice were divided into two groups. Mice bearing the AS-ATNMF-1 clone or the parasites passaged in absence of drug, AS-ATN27P (Section 2.1), were exsanguinated upon reaching peak parasitaemia. Their blood was diluted into Citrate Saline Solution so that 0.1 ml had the equivalent

of 10^6 pRBC. The blood was subsequently mixed in the following proportions (AS-ATNMF-1:AS-ATN27P): 100:0, 90:10, 75:25, 50:50, 25:75, 10:90, and 0:100.

A drop of blood from each mixture was dried in Whatman Nº4 filter paper and DNA was extracted by Chelex-100 method (Section 3.1).

DNA samples were subsequently amplified by PCR, using oligonucleotide primers specific for the region flaking a G to T substitution in position 1155448 on Chr 14 found by Solexa, and confirmed to be exclusive of the artesunate + mefloquine-resistant clone, AS-ATNMF-1 (Chapter II, Section II.2.2.3). The amplified fragments were sequenced by di-deoxy sequencing using the commercial services of STABVIDA laboratories. The chromatograms were visualised using Chromas 2.33 software and the heights of the overlapping peaks corresponding to the mutation were measured.

The percentages were calculated for both forward and reverse sequences and the proportions were plotted on a graph against the expected values.

4.2. COMPETITION ASSAYS

In order to estimate AS-ATNMF-1 parasites' fitness, CD1 mice were divided into two groups, inoculated with AS-ATNMF-1 or AS-ATN27P. Upon reaching peak parasitaemias, the animals were exsanguinated and their blood was diluted so 0.1 ml contained the equivalent of 10^6 pRBC. The blood carrying each parasite was mixtured in the following proportions: 100:0, 90:10, 50:50, 10:90, 0:100.

A group of fifteen CD1 mice were divided into five groups of three animals inoculated intraperitoneally with 0.1 ml of one of the mixtures of parasites. Mice's parasitaemias were assessed every two days, from Day 4 pi onwards.

A drop of blood was collected from the tail of each mouse and dried in filter paper at the same days the parasitaemias were checked. DNA was extracted (Section 3.1), fragment surrounding position 1155448 on Chr 14 was amplified by PCR, and sequenced. The proportions of AS-ATN27P (1155448G) and AS-ATNMF-1 (1155448T) alleles in each sample were determined by proportional sequencing analysis using the reverse sequence only. The results are expressed as mean percentage of two mice for both alleles, at each time point.

Additionally, the assay was repeated using the original parental AS-ATN in mixtures with AS-ATNMF-1 derived from it, and the proportions were assessed along time.

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