Studies on the genetics of artemisinin resistance in malaria

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Professor Doutor Pedro Vítor Lemos Cravo

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I declare that unless stated otherwise, the work presented here is my own.
A number of figures and tables have been kindly made available by other scientists or developed in partnership with other scientists.

Ana Júlia Pinto Fonseca Sieuve Afonso Rodrigues

Ítaca

Se um dia partires rumo a Ítaca,
reza para que o caminho seja longo,
cheio de aventura e de conhecimento.
Não temas monstros como os Ciclopes ou o zangado Poseidon:
Nunca os encontrarás no teu caminho
enquanto mantiveres o teu espírito elevado,
enquanto uma rara excitação agitar o teu espírito e o teu corpo.
Nunca encontrarás os Ciclopes ou outros monstros
a não ser que os tragas contigo dentro da tua alma,
a não ser que a tua alma os crie em frente a ti.
Deseja que o caminho seja bem longo
para que haja muitas manhãs de Verão em que,
com quanto prazer, com tanta alegria,
entres em portos que vês pela primeira vez;
Para que possas parar em postos de comércio fenícios
aí comprar coisas finas, madrepérola, coral e âmbar,
e perfumes sensuais de todos os tipos
tantos quantos puderes encontrar;
e para que possas visitar muitas cidades egípcias
e aprender e continuar sempre a aprender com os seus escolares.
Tem sempre Ítaca na tua mente.
Chegar lá é o teu destino.
Mas não te apresses na viagem.
Será melhor que ela dure muitos anos
para que sejas velho quando chegares à ilha,
rico com tudo o que encontraste no caminho,
sem esperares que Ítaca te traga riquezas.
Ítaca deu-te a tua bela viagem.
Sem ela não terias sequer partido.
Não tem mais nada a dar-te.
E, sábio como te terás tornado,
tão cheio de sabedoria e experiência,
já terás percebido, à chegada, o que significa uma Ítaca.

Konstantinos Kaváfis (1863-1933)

.

Ithaca

When you set out on your journey to Ithaca,
pray that the road is long,
full of adventure, full of knowledge.
The Lestrygonians and the Cyclops,
the angry Poseidon -- do not fear them:
You will never find such as these on your path,
if your thoughts remain lofty, if a fine
emotion touches your spirit and your body.
The Lestrygonians and the Cyclops,
the fierce Poseidon you will never encounter,
if you do not carry them within your soul,
if your soul does not set them up before you.
Pray that the road is long.
That the summer mornings are many, when,
with such pleasure, with such joy
you will enter ports seen for the first time;
stop at Phoenician markets,
and purchase fine merchandise,
mother-of-pearl and coral, amber and ebony,
and sensual perfumes of all kinds,
as many sensual perfumes as you can;
visit many Egyptian cities,
to learn and learn from scholars.
Always keep Ithaca in your mind.
To arrive there is your ultimate goal.
But do not hurry the voyage at all.
It is better to let it last for many years;
and to anchor at the island when you are old,
rich with all you have gained on the way,
not expecting that Ithaca will offer you riches.
Ithaca has given you the beautiful voyage.
Without her you would have never set out on the road.
She has nothing more to give you.
And if you find her poor, Ithaca has not deceived you.
Wise as you have become, with so much experience,
you must already have understood what Ithacas mean.

Konstantinos Kaváfis (1863-1933)

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3D7 *P. falciparum* culture, sensitive
6-PGD - 6-Phosphogluconate dehydrogenase
A - Adenine (in DNA context)
A Alanine (Ala) (in protein context)
a.a. - Amino acid
ACT - Artemisinin combination therapy
AFLP - Amplified fragment length polymorphism
ART - Artemisinin
AJ - *Plasmodium chabaudi* clone, totally drug sensitive. Genetically different from AS line.
AS-ART - *Plasmodium chabaudi* clone, pyrimethamine-, high chloroquine- resistant, obtained from AS-30CQ by artemisinin drug pressure (clone obtained during this project)
AS-ART* - *Plasmodium chabaudi* parasite line, pyrimethamine-, high chloroquine- resistant, obtained from AS-30CQ by artemisinin drug pressure (parasite line obtained during this project), this parasite line was then cloned by limiting dilution
AS-ATN - *Plasmodium chabaudi* clone, pyrimethamine-, intermediate chloroquine- resistant, obtained from AS-15CQ by artesunate drug pressure (clone obtained during this project)
AS-ATN* - *Plasmodium chabaudi* parasite line, pyrimethamine-, intermediate chloroquine- resistant, obtained from AS-15CQ by artesunate drug pressure (parasite line obtained during this project), this parasite line was then cloned by limiting dilution
AS-SENS - *Plasmodium chabaudi* clone, totally drug sensitive. Genetically different from AJ.
AS-PYR - *Plasmodium chabaudi* clone, pyrimethamine-resistant obtained from AS-SENS by pyrimethamine drug pressure
AS-3CQ - *Plasmodium chabaudi* clone, pyrimethamine and low chloroquine resistant obtained from AS-PYR by chloroquine drug pressure
AS-15CQ - *Plasmodium chabaudi* parasite isolate, pyrimethamine and intermediate chloroquine resistant, obtained from the AS-3CQ by cloroquine drug pressure
AS-15MEF - *Plasmodium chabaudi* parasite clone, pyrimethamine, intermediate chloroquine resistant and mefloquine resistant, obtained from the AS-15CQ by mefloquine drug pressure
AS-30CQ - *Plasmodium chabaudi* parasite clone, pyrimethamine and high chloroquine resistant, obtained from the AS-15CQ by cloroquine drug pressure
ARMD - Accelerated resistant to multiple drugs phenotype
ama-1 - Apical membrane antigen-1
ATN - Artesunate
atp6 - Encoding the Sarcoplasmic and Endoplasmic Reticulum Ca^{2+} atpase gene
C - Cysteine (Cys)
CDC - National Center for Disease Control
cDNA - Complementary DNA
cg10 - Gene coding for a putative protein transporter, *P. chabaudi* orthologue of the *P. falciparum crt.*
CI - Comparative intensity
CQ - Chloroquine
crt - Chloroquine resistance transporter gene
CSP - Circumsporozoite protein
D - Aspartic acid (Asp)
Dd2 *P. falciparum* culture, from Indochina, pyrimethamine, mefloquine and chloroquine resistant
DDT - Dichloro-diphenyl-trichloroethane
DHA - Dihydroartemisinin
DHFR Dihydrofolate-reductase enzyme
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
F - Phenylalanine (Phe)
gDNA - Genomic DNA
HB3 - *P. falciparum* culture, from Honduras, pyrimethamine, mefloquine and chloroquine resistant
IC_{50} - The drug dose necessary to eliminate 50% of the parasites
II Intensity index
i. p. - Intraperitoneally
iRBC - Infected red blood cells (erythrocytes)
K_i Biochemistry catalytic constant

L	Leucine (Leu)
LDH -	Lactate dehydrogenase
LGS -	Linkage group selection
Mb -	Mega base
MCD -	Minimum curative dose
***mdr1* -**	Multi drug resistance 1 gene
msp-1	Merozoite surface protein-1
msp-2 -	Merozoite surface protein-2
N -	Asparagine (Asn)
NADH -	Hydrogen nicotinamide adenine dinucleotide
NCBI/NIH -	National Institute of Health
nM -	Nanomolar
PABA -	Paraminobenzoic acid
***pcdhps* -**	Gene of *Plasmodium chabaudi* codifying for enzyme dihydropteroate synthetase
PCR -	Polymerase chain reaction
Phe -	Phenylalanine
PYR -	Pyrimethamine
RBC -	Red blood cells (erythrocytes)
RIIs -	Relative intensity indices
RMP -	Rodent malaria parasite
RNA -	Ribonucleic acid
rRNA -	Ribosomal RNA
RTQ-PCR -	Real time quantitative – PCR
S -	Serine (Ser)
s. c. -	Subcutaneous
SDS -	Sodium dodecyl sulfate
sRNA -	Small RNA
SP -	Sulfadoxine-pyrimethamine
T -	Tymine
***tctp* -**	Translationally controlled tumour protein gene
tRNA -	Transfer RNA
Tyr -	Tyrosine
ubp-1 -	De-ubiquitinating enzyme, ubiquitin carboxyl-terminal hydrolase, putative, 1
ubp-1	De-ubiquitinating enzyme, ubiquitin carboxyl-terminal hydrolase, putative, 1 gene
V -	Valine (Val)
WHO -	World Heath Organization
Y -	Tyrosine (Tyr)
6-PGD -	6-Phosphogluconate dehydrogenase

ABSTRACT

Resistance of *Plasmodium falciparum* to multiple drugs including chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) is a major problem in malaria control. New drugs, such as artemisinin (ART) derivatives, particularly in combination with other drugs, are thus increasingly used to treat malaria. Although stable resistance to ART has yet to be reported from laboratory or field studies, its emergence would be disastrous because of the lack of alternative treatments.

The work presented in this thesis describes the selection of parasites with stable resistance to ART and artesunate (ATN), and their genetic analysis. This work was carried out using the rodent malaria parasite *Plasmodium chabaudi chabaudi* (*Plasmodium chabaudi*).

Two different rodent malaria parasite lines AS-15CQ and AS-30CQ were continually passaged in the presence of increasing concentrations of ATN or ART, respectively. After selection, these lines, named AS-ATN and AS-ART, showed 6-fold and 15-fold increased resistance to ATN and ART respectively. Resistance remained stable after cloning, freeze/thawing, blood passage in the absence of drug pressure and transmission through mosquitoes. The nucleotide sequences and the gene copy number of the possible genetic modulators of ART resistance *mdr1*, *cg10*, *tctp* and *atp6*; were compared between sensitive and resistant parasites. No mutations or changes in the gene copy number of these genes were found.

Linkage Group Selection (LGS) was used to investigate the genetic basis of ART resistance. Genetic crosses between AS-ART or AS-ATN and the ART-sensitive clone AJ were analysed before and after drug treatment. Using quantitative markers, a genetic locus on chromosome 2 was found to be under strong selection. Loci on chromosomes 1, 8 and 14 of *P. chabaudi* also appear to be under selection. On chromosome 2, two different mutations V739F and V770F in a de-ubiquitinating enzyme (ubp-1) were identified in AS-ATN and AS-ART respectively, relative to their sensitive progenitors. The implications of these results are discussed.

RESUMO

A existência de estirpes do parasita, *Plasmodium falciparum* resistentes a multiplos fármacos tais como; cloroquina (CQ) e sulfadoxina-pirimetamina (SP) é um dos problemas mais graves no controlo da malária.

Novos fármacos, como a artemisinina (ART) e seus derivados, particularmente em combinação com outros fármacos, são cada vez mais utilizados no tratamento da malaria. Embora até ao momento a fármaco-resistência estável à ART quer *in vitro* quer *in vivo* não tenha sido registada, o seu surgimento seria desastroso devido á falta de alternativas.

O trabalho apresentado nesta tese descreve a selecção de resistência estável a ART e ao artesunato (ATN). Este trabalho foi realizado usando o modelo roedor de malária *Plasmodium chabaudi chabaudi* (*Plasmodium chabaudi*).

Duas linhas parasitáricas diferentes, AS-15CQ e AS-30CQ, foram feitas crescer na presença de concentrações crescentes de ATN e ART, e que no final apresentavam uma resistência de 6 e 15 vezes superior ao ATN e à ART, respectivamente (estas novas linhas obtidas foram nomeadas AS-ATN e AS-ART).

A resistência é estável mesmo após clonagem, congelamento/descongelamento, passagem sanguínea na ausência de pressão de fármaco e transmissão através do mosquito vector.

A sequência nucleotídica e o número de cópias dos genes descritos como moduladores putativos de resistência à ART: *mdr1, cg10, tctp* e *atp6*; foi comparada entre parasitas resistentes e sensíveis. Não tendo sido encontradas alterações na sequência ou no número de cópias destes genes.

Numa tentativa de identificar os genes encolvidos na resistância à ART e ao ATN a técnica de Linkage Group Selection (LGS) foi utilizada e dois cruzamentos genéticos entre os clones fármaco-resistentes; AS-ART e AS-ATN e o clone geneticamente distinto dos anteriores e sensível aos fármacos em estudos; AJ; foram realizados. Foram encontrados sobre selecção em ambos os cruzamentos genéticos quatro loci; cromossomas de *P. chabaudi* 1, 2, 6 e 8. Atendendo a que, a selecção no cromossoma 2 era mais forte, este locus foi submetido a análises genéticas subsequentes. Tendo sido encontradas duas mutações diferentes (V739F e V770F) num gene que codifica para um enzima de desubiquitinação (ubp-1). As implicações destes resultados serão discutidas.

CHAPTER I

INTRODUCTION

1.1 Malaria: general features

Malaria parasites are micro-organisms that belong to the genus *Plasmodium*. There are more than 100 species of *Plasmodium*, which can infect many animal species such as reptiles, birds, and various mammals. Only four species of *Plasmodium* infect humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* [Reviewed by: Aikawa M 1971; Collins WE *et al.* 2005; Cowman *et al.* 2006*;* Gauthier C *et al.* 2005; Mackinnon MJ *et al* 2004].

Although the symptoms of malaria were known since ancient times, the discovery of the causative agent of the disease had to wait until the end of the nineteenth century. Charles Louis Alphonse Laveran, a French army surgeon stationed in Constantine, Algeria, was the first to notice parasites in the blood of a patient suffering from malaria (see Figure 1). For his discovery, Laveran was awarded the Nobel Prize in 1907 [Anderson WK *et al.* 1927; Celli A 1933; Hoeppli R 1959, Hippocrates - English translation by W. H. S. Jones 1923; Jones WHS 1909, Lehrer S 1979, Schmidt GD *et al.* 2004].

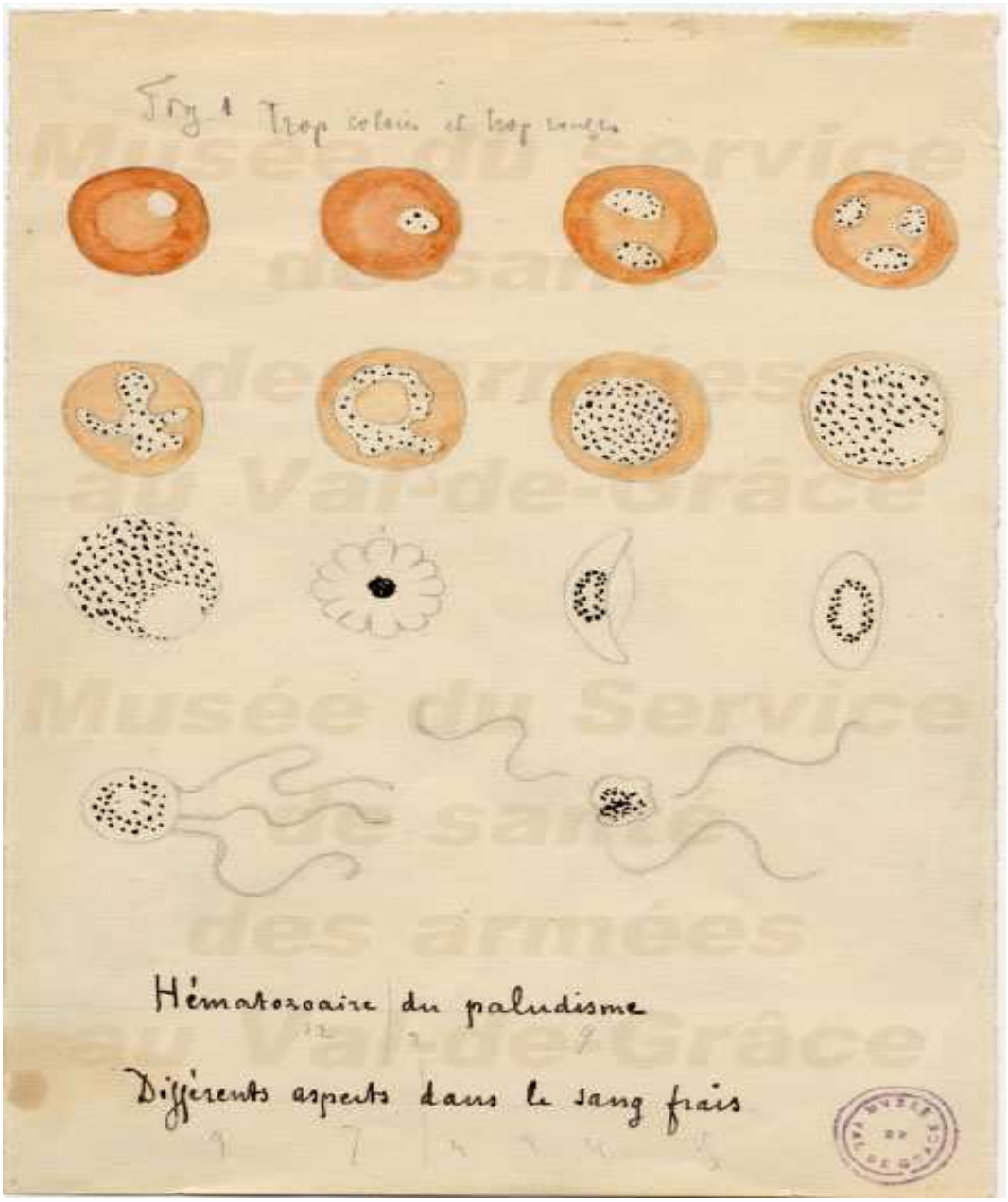


Figure 1 - Illustration drawn by Laveran of various stages of malaria parasites as seen on fresh blood. Dark pigment granules are present in most stages. The bottom row shows an exflagellating male gametocyte.
Source: National Center for Disease Control (CDC).with kind permission of CDC

1.2 Malaria today

Although more than 100 years have gone by since the pioneering malariologists uncovered the causes of the disease, malaria is today the world's most important parasitic infection, ranking among the major health and developmental challenges for the poor countries of the world [Sachs J *et al.* 2002]. Although four parasite species of the genus *Plasmodium* infect human beings nearly all malaria deaths and the larger proportion of morbidity are caused by *Plasmodium falciparum.*

More than a third of the world's population (about 2 billion people) live in malaria endemic areas and 1 billion people are estimated to carry parasites at any one time (See Figure 2). In Africa alone, there are an estimated 200-450 million cases of fever in children infected with malaria each year [Breman JG *et al.* 2001]. Estimates for annual malaria mortality range from 0.5 to 3 million people [Marsh K 1998], although malaria related mortality is particularly difficult to measure because the symptoms of the disease are non-specific and most deaths occur at home. Although the use of ineffective antimalarials will inevitably result in an increase in mortality [Trape JF 2001], the real effects of antimalarial drug resistance on malaria morbidity and mortality tend to be under-estimated [White NJ 1999].

After World War II, the widespread use of DDT coupled with the covering and draining of breeding grounds resulted in a substantial reduction in mosquito populations. This, together with effective treatment, eradicated malaria in Southern Europe, Russia and some parts of Asia. Substantial successes were achieved in subtropical regions but control of malaria in the tropics proved far more challenging. The effectiveness of the control effort was undermined through a combination of difficulties with access to health facilities, the lack of health infrastructures, and the gradual development of insecticide resistance. As a consequence, plans for eradication of malaria through mosquito vector control had to be abandoned in the late 1960s.

Nowadays, prompt and effective drug treatment is probably the most cost-effective element of malaria control [Goodman CA *et al*. 1999]. The majority of antimalarial therapy worldwide is oral drugs for uncomplicated *P. falciparum* malaria. Oral treatment prevents progression to severe disease and complications, and if the drugs are efficacious and applied effectively they reduce overall malaria morbidity and mortality. However, most people living in endemic areas have little or no access to correct diagnosis and treatment. Malaria treatment is commonly inadequate: drugs are of poor quality, effective drugs are not available or if available they are not taken correctly due to incorrect prescription or poor adherence.

P. falciparum has become resistant to almost all drug classes except the artemisinin derivatives. Nowadays chloroquine-resistant *P. falciparum* occurs across all malaria endemic areas. The effectiveness of sulfadoxine-pyrimethamine has rapidly declined in all regions where it has been introduced due to resistance, and multidrug resistance is now established in Southeast Asia, South America and Africa (See Figure 3) [Collins WJ *et al.* 2006; Green MD 2006; Kshirsagar NA 2006; Linares GE *et al.* 2007].

Drug resistance is most likely to emerge when background immunity is weak, parasite numbers in an individual are high, transmission is low and drug pressure is intense or very intense [Hastings IM *et al.* 2000].

With an increase in insecticide and antimalarial-drug resistance, the development of a malaria vaccine and above all new drugs or new drug combinations, using drugs already in use, carries huge expectations [Chatterjee S *et al.* 2006; Girard MP *et al.* 2007; Greenwood B *et al.* 2007; Matuschewski K 2006, Shanks GD 2006; Stepniewska K *et al.* 2006].

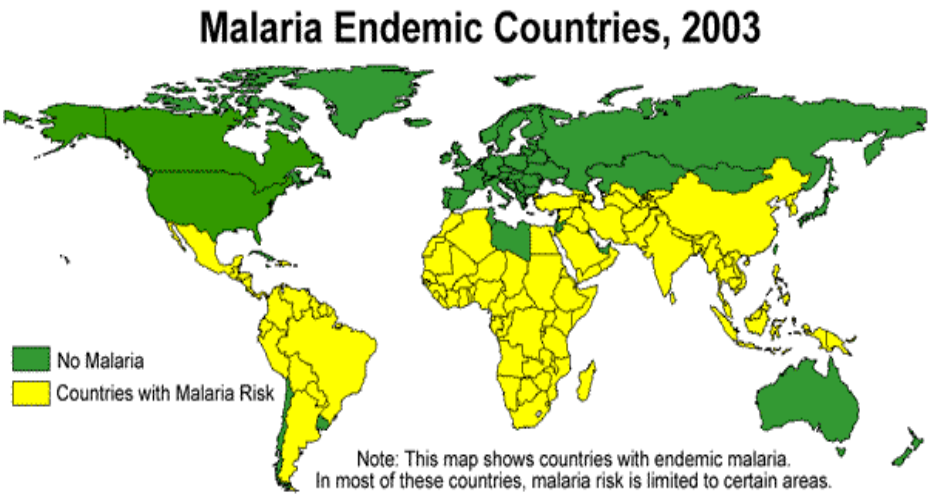


Figure 2 – World geographic distribution of malaria, data from 2003.

Source: National Center for Disease Control (CDC) with kind permission of CDC.

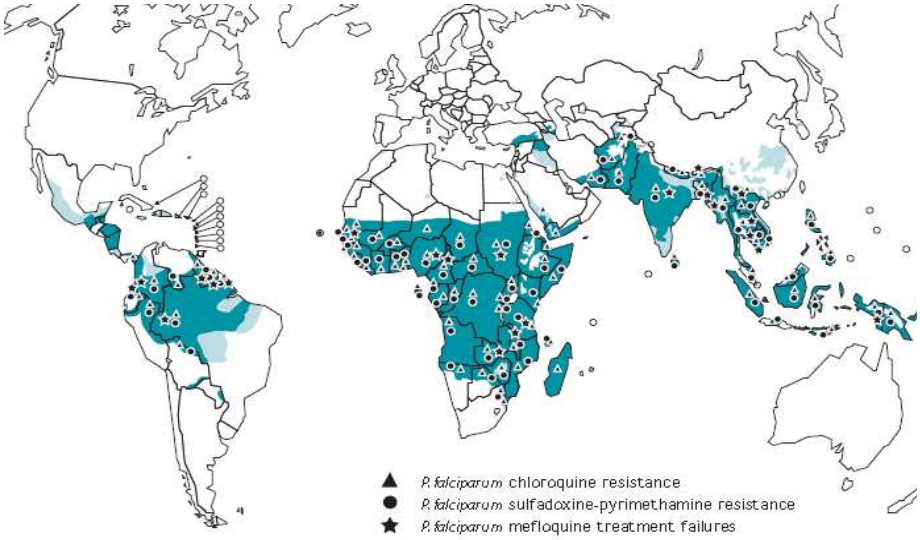


Figure 3 – Malaria transmission areas and *P. falciparum* drug resistance distribution data from World Heath Organization data from 2004.

Source: World Heath Organization (WHO) with kind permission of WHO.

1.3 The parasite and its life-cycle

In nature, malaria parasites spread by infecting successively two types of hosts: humans and female *Anopheles* mosquitoes.

Malaria is transmitted among humans by female mosquitoes of the genus *Anopheles*. Female mosquitoes require blood meals in order to carry out egg production, and such blood meals are the link between the human and the mosquito hosts in the parasite life cycle. Of the approximately 430 known species of *Anopheles*, only 30-50 transmit malaria in nature ("vectors"). The successful development of the malaria parasite in the mosquito (from the "gametocyte" stage to the "sporozoite" stage – See Figure 4) depends on several factors. The most important is ambient temperature and humidity and whether the *Anopheles* survives long enough to allow the parasite to complete its cycle in the mosquito host ("sporogonic" or "extrinsic" cycle, duration 10 to 18 days). Malaria's life cycle is comprised of both the sexual and asexual forms (See Figure 4). The sexual cycle occurs mainly in the mosquito; while the asexual cycle takes place in the human host after the parasites have entered the host's blood stream when the mosquito bites for a blood meal. During a blood meal, a malaria infected female *Anopheles* mosquito inoculates sporozoites into the human host. Though the salivary glands of an infected mosquito contain thousands of sporozoites, less than 100 of these are transmitted in any one bite [Rosenberg R *et al.* 1990]. Within 30-45 minutes of the parasite's sporozoites entering the bloodstream, they enter parenchymal cells of the liver; this is achieved by the binding of the thrombospondin domains of the circumsporozoite and thrombospondin-related adhesive proteins (csp and trap respectively) to the heparin sulphate proteoglygan on the hepatocytes [Frevert U *et al.* 1993]. This phase is called the pre-erythrocytic stage lasting 5-15 days in which the parasite undergoes asexual reproduction (schizogony): the end products of this are the merozoites. In *P. vivax* and *P. ovale* a dormant stage called hypnozoites, can persist in the liver and can cause relapses by invading erythrocytes weeks or years later [Durante Mangoni E *et al.* 2003].

Hepatocytes rupture to release merozoites that enter red blood cells. Invasion of erythrocytes by merozoites involve an initial low affinity interaction between proteins on the surface coat of the merozoite (merozoite surface protein-1 (msp-1), and apical membrane antigen-1 (ama-1)) and the surface of the erythrocyte. Once inside the erythrocyte the merozoite initiates the feeding process forming the intracellular parasite, the trophozoite (erythrocytic schizogony stage). Mitotic divisions (asexual reproductive stage) occur in the cells giving rise to schizonts, which contain up to about thirty haploid merozoites. This red cell ruptures, releasing more mature merozoites, which invade more red blood cells, hence maintaining this

“asexual cycle”. Some parasites undergo gametocytogenesis within the erythrocyte, producing male or female micro and macrogametocytes respectively. These remain in the blood circulation where they are available for ingestion by a feeding mosquito. The asexual reproductive stage, occurring in the blood of the vertebrate host (human, primate or rodent), is the target of most antimalarial drugs, including artemisinin and its derivatives. Inside the mosquito mid-gut, female and male gametocytes undergo gametogenesis, in which the female macrogametocyte escapes from the erythrocyte membrane and the male microgametocyte undergoes the process of exflagellation which produces 8 motile microgametes. The micro and macro-gametes fuse to form a zygote that in turn becomes an ookinete, (the only diploid stage of the parasite). The ookinete crosses the gut wall and encysts on the outer wall of the gut beneath the basal lamella forming an oocyst or sporocyst. Division and multiplication of the sporocyst takes place to produce many haploid sporozoites. When the sporocyst bursts the sporozoites are released and then migrate to the salivary gland, waiting to re-infect again once the mosquito takes another blood-meal [Barnwell JW *et al.* 1998; Beier JC *et al.* 1998; Sinden RE 1997].

See Figure 4 for details on the parasite life cycle.

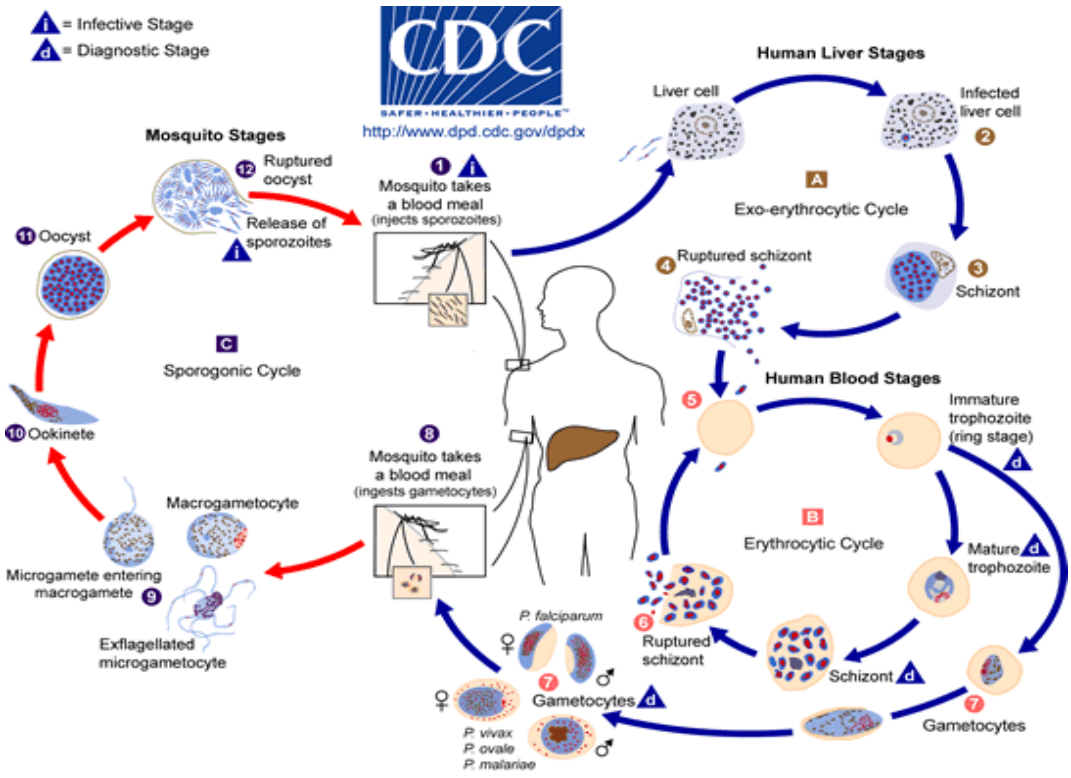


Figure 4 - Life cycle of malaria parasite.

Most of the biological work presented here occurs in the erythrocytic cycle of the parasite (presented in the Figure as B). The drug resistance selection process occurs on this part of the parasite life cycle.

Briefly: the malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host[1]. Sporozoites infect liver cells[2] and mature into schizonts[3], which rupture and release merozoites[4]. After this initial replication in the liver (exo-erythrocytic schizogony[A]), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony [B]). Merozoites infect red blood cells[5]. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites[6]. Some parasites differentiate into sexual erythrocytic stages (gametocytes)[7]. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal[8]. The parasites' multiplication in the mosquito is known as the sporogonic cycle[C]. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes[9]. The zygotes in turn become motile and elongated (ookinetes)[10] which invade the midgut wall of the mosquito where they develop into oocysts[11]. The oocysts grow, rupture, and release sporozoites[12], which make their way to the mosquito's salivary glands. Inoculation of the sporozoites [1] into a new human host perpetuates the malaria life cycle.

Source: National Centre for Disease Control (CDC) with kind permission of CDC.

1.4 The genetics of malaria parasites

Malaria parasites, as all members of the phylum Apicomplexa, are haploid for almost their entire life cycle (exo-erythrocytic and erythrocytic blood stages, sporogony and microgametogenesis), and in the haploid phase of the parasite life cycle they multiply by mitosis.

The only phase of the parasite life cycle where the parasite genome is diploid is the zygote stage (ookinetes), prior to the meiotic division that results in the production of sporozoites.

Malaria parasites have three individual genomes; an extra-chromosomal mitochondrial genome, a 35kb circular genome and a large nuclear genome.

The mitochondrial genome, also known as the 6kb element contains genes encoding two truncated ribosomal sRNA and three proteins components involved in the electron transport system; cytochrome c oxidase subunits I and II and cytochrome b [Funes S *et al* 2004]. The inheritance of the 6 kb element appears to follow the same pattern as other mitochondrial genomes in eukaryotes meaning it is inherited from the female parent only [Creasey AM *et al.* 1993].

The 35kb circular genome associated with the apicoplast encodes 30 proteins, mainly rRNA, tRNA, which are primarily involved in gene expression [Funes S *et al.* 2004, Gardner MJ *et al.* 2002]. The exact role of the apicoplast remains unclear, but it is known to be involved in the anabolic synthesis of fatty acids, isoprenoids and haem [Gardner MJ *et al.* 2002].

The haploid nuclear genome of *P. falciparum* is where most parasite genes reside. It consists of 14 chromosomes and encodes approximately 5,300 genes with a total genome size of 22.8 Mb. The parasite chromosomes have a central domain that contain conserved coding regions and chromosome ends that consist of telomeric repeat sequences and subtelomeric repeat regions, containing polymorphic gene families (for example *pfemp1*, *stevors* and *rifins*) [Lanzer M *et al.* 1994].

The *P. falciparum* nuclear genome is very (A+T)-rich, with an overall (A+T) content of 81%, rising to 90% in intronic and intergenic regions [Gardner MJ *et al.* 2002]. There is considerable chromosomal size polymorphism between strains of parasites [Corcoran LM *et al.* 1986], which could be due to unequal crossing-over of homologous chromosomes during meiosis, or non-meiotic chromosome breaking and healing events [Babiker HA *et al.* 1994, Gardner MJ *et al.* 2002, Hernandez-Rivas R *et al.* 1996, Scherf A *et al.* 1992].

Various genetic polymorphisms can be observed when comparing different strains even within the same *Plasmodium* species. This diversity observed to the genotype level has its origin on either spontaneous genetic mutation, occurring at any stage of the parasite

development and also through genetic recombination occurring in the mosquito vector stage. Genetic recombination which the parasites undergo in the mosquito midgut can then result in independent assortment of genes on different chromosomes (See Figure 5) [Walliker D *et al.* 1998].

When a mosquito feeds on an infected host containing two genetically distinct parasites, gametocytes from the two parasites are taken up, and may fertilise, recombining into heterozygous zygotes, by meiosis, producing four genetically distinct haploid daughter cells, which are called the recombinant progeny (See Figure 5). In the case where there are equal numbers of male and female gametes present from each parental strain in the mosquito, then selfing will occur 50% of the time, resulting in 25% of the zygotes being genetically identical to one parental strain, and 25% identical to the other. The remaining 50% will be hybrid between the two parentals.What is meant is that if the recombinant progeny was undergoing a normal Mendelian segregation pattern meaning without any kind of selfing/crossing bias, thus in the presence of a random segregation and if there are equal numbers of male and female gametes present from each parental strain in the mosquito, then selfing (recombination within the same strain) will occur 50% of the time, resulting in 25% of the zygotes being genetically identical to one parental strain, and 25% identical to the other. The remaining 50% will be hybrid between the two parentals.

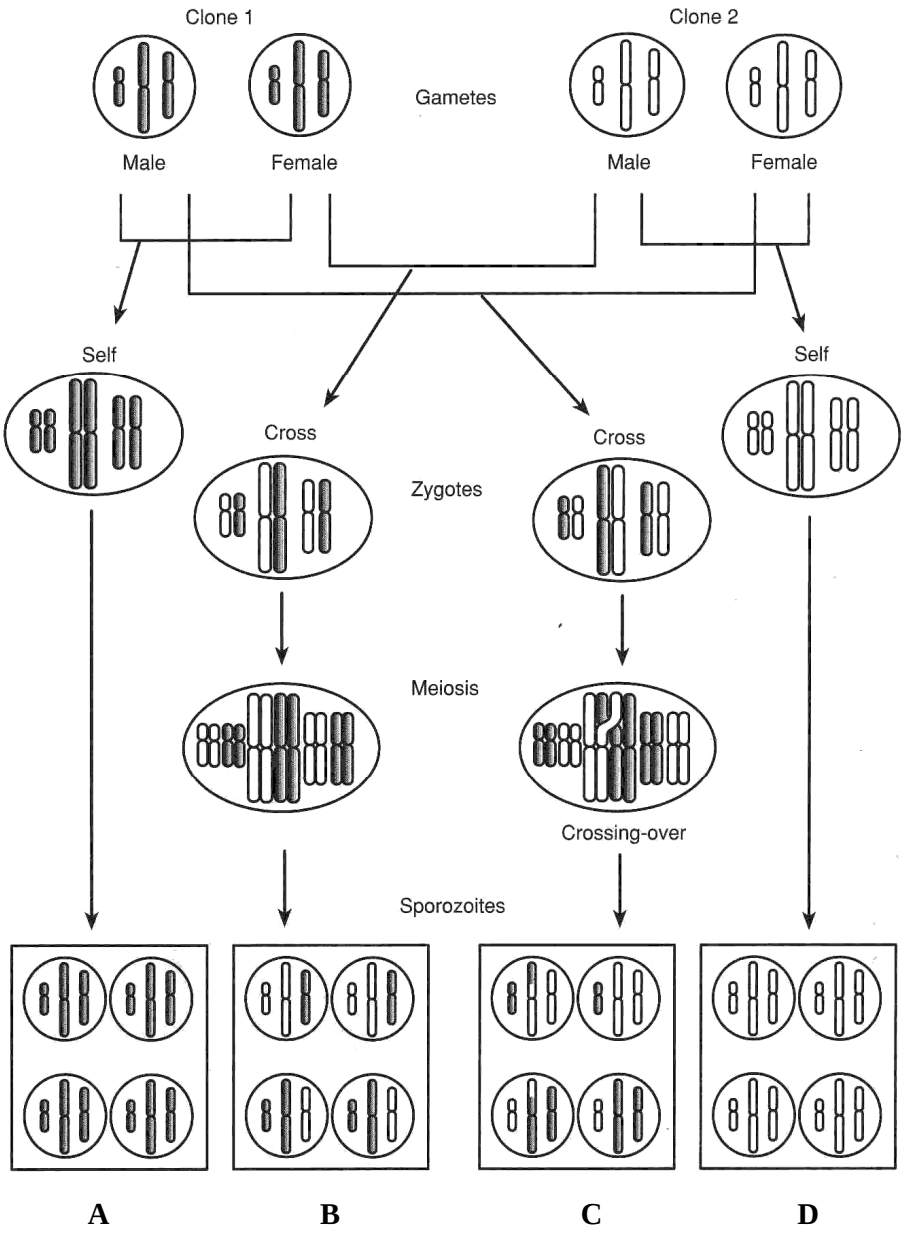


Figure 5 – Crossing and chromosomal events in *Plasmodium.*

Clone 1 and clone 2 are gametes (haploid stage) from two genetically distinct parasites, after the blood meal zygotes are formed. The formation of these zygotes can result from selfing (equal to the progenitor gametes) or from crossing (cross between different clones and are therefore heterozygous). Through meiosis four genetically distinct haploid daughter cells are produced, which are called the recombinant progeny. A and D are the result of selfing on the other hand B and C are the product of recombination.

From: Walliker D 2000, with kind permission of Professor David Walliker.

Recombination between malaria parasites was first proven with genetic crossing experiments done with the rodent parasites *Plasmodium yoelii* [Walliker D *et al.* 1971], and *Plasmodium chabaudi* [Walliker D *et al.* 1975]. In the *P. chabaudi* experiments, two cloned parasites

which differed in their response to the anti-malarial drug pyrimethamine and in the electrophoretic patterns of two enzymes (6-phosphogluconate dehydrogenase (6-PGD) and lactate dehydrogenase (LDH)) were mixed in mosquitoes and the resulting progeny were cloned and characterized for their enzyme type and their phenotypic response to pyrimethamine. It was found that not only had the two enzyme isoforms recombined, but that pyrimethamine susceptibility segregated independently [Walliker D *et al.* 1975], which showed that recombination between the parental characters had occurred.

In *P. falciparum* the production of heterozygotes (in the oocyst) between two heterologous malaria parasites has also been demonstrated experimentally, by dissecting individual oocysts from mosquitoes that had fed on a mixed *P. falciparum* blood infection of clones 3D7 and HB3. After performing genetic typing of alleles of *msp-1* and *msp-2* genes, it was found that some oocysts contained alleles exclusively from HB3, some contained alleles only from 3D7, and the remainder of the oocysts contained alleles from both parents, and were therefore hybrids, meaning, the products of fertilization between the two different parental strains. The proportion of the homozygous and heterozygous forms was consistent with random fertilization between parents [Ranford-Cartwright L *et al.* 1993].

With the objective of sequencing the genome of the human malaria parasite *Plasmodium falciparum* (clone 3D7), an International Malaria Genome Sequencing Consortium was formed in 1996. The genome was sequenced by three groups: The Institute for Genomic Research and the Malaria Program of the Naval Medical Research Center (chromosomes 2, 10, 11 and 14), The Wellcome Trust Sanger Institute (chromosomes 1, 3-9, 13) and Stanford University (chromosome 12).

In 2002, the complete genome of *Plasmodium falciparum* was published, triggering the “post-genomic” age of malariology [Gardner MJ *et al.* 2002].

1.5 Antimalarial drugs and targets

Antimalarial drugs are one of the most important measures to control the disease. The drug of choice depends on the parasite species and local conditions, drug resistance prevalence and specificity. Traditionally, antimalarial agents are classified as blood schizontocides, tissue schizonticides, gametocides and sporontocides, depending on the stages of the malaria life cycle which are targeted by the drug [Tracey J *et al.* 1996]. For details see Table 1.

Blood schizontocides are drugs acting on asexual intraerythrocytic stages of malarial parasites. They suppress the proliferation of plasmodia in the erythrocytes.

Tissue schizontocides prevent the development of hepatic schizonts. They are causally prophylactic because they affect the early developmental stages of the protozoa and prevent the invasion of the erythrocytes.

A hypnozoiticide acts on persistent intrahepatic stages of *P. vivax* and *P. ovale* in the liver.

Gametocides destroy the intraerythrocytic sexual forms (gametes) of the protozoa and the prevent transmission from human to another mosquito. Antimalarials are rarely used clinically just for their gametocidal action

Table 1 – Classification of antimalarial agents according to their stage of action.

Stage of Action	**Antimalarial**
Tissue schizontocides	Primaquine, pyrimethamine, sulfonamides (and other 8-aminoquinolines and other folate inhibitors)
Hypnozoiticides	Primaquine, tafenoquine
Blood schizontocides	*Type 1, quick onset*: Chloroquine, mefloquine, quinine, halofantrine, artemisinin *Type 2, slow onset*: Pyrimethamine, sulfonamides, sulfones, other antibiotics, atovaquone
Gametocides	Primaquine for *P. falciparum* Quinine for *P. vivax, P. malariae* and *P. ovale*
Sporontocides	Primaquine, chloroquine

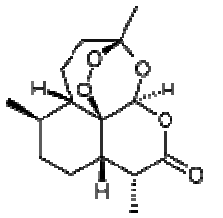
1.6 Artemisinin and its derivatives

Artemisinin, known in Chinese as Quinghaosu, is the active principle extract of the medicinal herb, know in Chinese as Qinghao (*Artemisia annua* L. also know as Sweet Wormwood, Annual Wormwood, Sweet Annie or Chinese Wormwood), and has been used in traditional medicine in China for about 2000 years [Antimalaria studies on Qinghaosu 1979; Klayman DL 1985].

Figure 6- Schematic representation of *Artemisia annua* L.
Source: www.hort.purdue.edu/hort/

The effective antimalarial crystal was isolated in 1979. Qinghaosu high resolution mass spectrum and elemental analysis have led to the molecular formula of $C_{15}H_{22}O_5$ .Its structure is shown in Table 2 based on the data of spectral analysis chemical reactions and X-ray diffraction [Antimalaria studies on Qinghaosu 1979].

Table 2 – Qinghaosu chemical information.

	
Chemical name	(3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one
Chemical formula	$C_{15}H_{22}O_5$
Molecular mass	282.332 g/mol

Chemically, artemisinin is a sesquiterpene trioxane lactone containing a peroxide bridge (C-O-O-C), unique among antimalarial drugs, which is essential for its activity [Antimalaria studies on Qinghaosu 1979]. This peroxide bridge corresponds to a very unusual chemical property that may contribute to the molecule's unique bioactivities, and it is more stable in general than other peroxides, for example it is poorly soluble in water and in oil and it shows remarkable thermal stability [Balint GA 2001, Klayman DL 1985]. The lactone that constitutes artemisinin can easily be reduced (with sodium borohydride), resulting in the formation of dihydroartemisinin (reduced lactol derivative of artemisinin), which has even more antimalarial activity *in vitro* than artemisinin itself [van Agtmael MA *et al.* 1999]. See Figure 7 for details in the molecule structure.

Since artemisinin is poorly soluble in water or oil, water-soluble derivatives (artesunate and artelinate) and oil-soluble derivatives (artemether and arteether) have been synthesized and newer semi synthetic and synthetic derivatives are also being developed.

Artesunate, the most widely used of the derivatives, is available in oral, parenteral and suppository formulations. Artemether, a methyl ether derivative of artemisinin, is available in ampoules for intramuscular injection or as capsules for oral administration. Arteether, another lipophilic ester is available for intramuscular injection. As artemisinin itself, both the lipophilic and hydrophilic derivatives are converted to dihydroartemisinin, the active metabolite. Dihydroartemisinin itself is available in an oral preparation [Lee IS *et al.* 1990; Li

WH *et al.* 1982; Meshnick SR *et al.* 1996; Woodrow CJ *et al.* 2005]. See Table 3 for details in the pharmacokinetic of artemisinin and some of its derivatives.

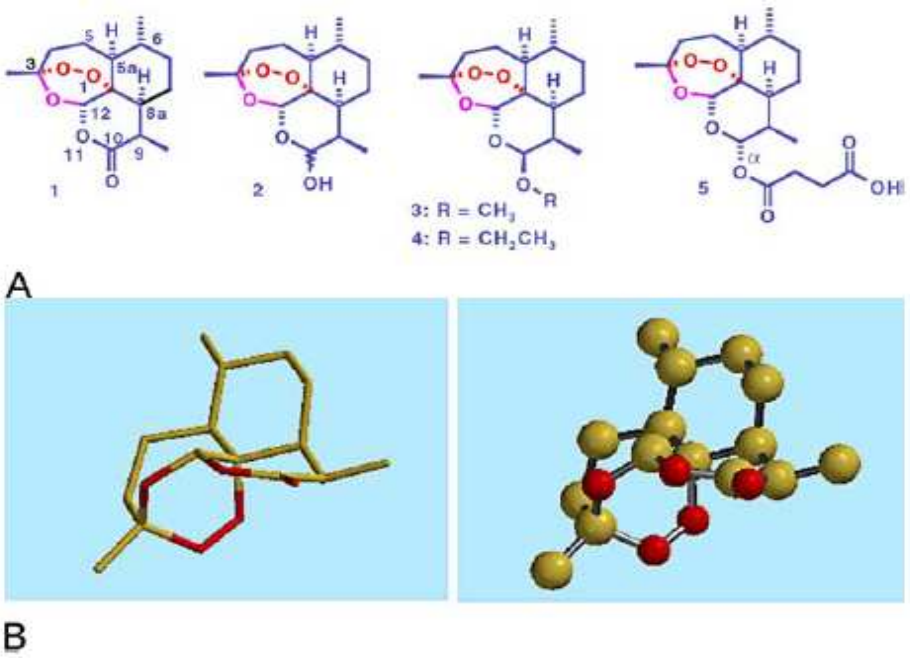


Figure 7 – A - Chemical structure of (1) Qinghaosu or artemisinin and some of its derivatives (2) dihydroartemisinin, (3) artemether; (4) arteether and (5) artesunic acid or artesunate. The Chemical Abstracts numbering system is used. The active pharmacophore is the peroxide bridge, coloured red. The third non-peroxidic oxygen atom, coloured magenta, appears to be important in conferring optimal antimalarial activity. The ensemble of peroxide and non-peroxidic oxygen atoms are incorporated into six-membered ring called a 1, 2, 4-trioxane.

B – Three-dimensional tube, and ball and stick representations of artemisinin. Atoms are colour coded as O red, C tan. H atoms are omitted for clarity.

From: Haynes RK *et al.* 2004, with kind permission.

The artemisinin compounds have antimicrobial activity against several parasites including *Plasmodium* spp., *Schistosoma* spp., *Pneumocystis carinii* and *Toxoplasma gondii*.

In vitro pharmacodynamic experiments in *P. falciparum* show that these compounds are active against a broad spectrum of the life cycle of the parasite but are stage specific; late-stage ring parasites and trophozoites are more susceptible to these drugs than schizonts or small rings [Alin MH *et al.* 1994; Caillard V *et al.* 1992; Geary TG *et al.* 1989; ter Kuile F *et al.* 1993]. They are also gametocytocial [Dutta GP *et al.* 1990; Kumar N *et al.* 1990, Maeno Y *et al.* 1993; Peters W *et al.* 1993; Posner GH *et al.* 1995], due to their activity against both the precursors of the sexual stages and early gametocytes. Liver stages of *P. vivax* and *P. falciparum* are not affected [Antimalaria studies on Qinghaosu 1979]. Artemisinin also

decreased the infectivity of the surviving gametocytes [Chen PQ *et al.* 1994; Targett G *et al.* 2001]. This effect may help diminish transmission rates in areas of low transmission [Price RN *et al.* 1996]. In high transmission areas, however, this effect may not be evident, since rapidly re-infected individuals will continue to maintain a pool of transmissible parasites.

This wide stage specificity of killing gives these drugs a major advantage over the conventional antimalarials. The activity against the later stages of parasite development prevents the releasing of merozoites; this removes or at least attenuates the occasional sharp rise in parasitaemia that normally occurs immediately after treatment.

Artemisinin has been shown to prevent cytoadherence *in vitro*, probably by preventing development to the mature trophozoite stage [Udomsangpetch R *et al*. 1996].

Table 3 – Some pharmacokinetic data of artemisinin and some of its derivatives.
Adapted from Balint GA 2001, with kind permission.

Drug	Absorption	Elimination half-life (hour)	Peak plasma concentration (hour)	Usual oral dose in adults (dose/Kg body weight)
Artemisinin [(1)]	Rapid and incomplete	2-5	<2	20 mg
Arthemether [(2)]	Rapid and incomplete	3-11	3	4 mg
Arteether	Rapid and incomplete	>20	<2	3 mg
Artesunate[(3)]	Rapid and incomplete	<1	<2	4 mg
Dihydroartemisinin	Rapid and incomplete	3,1	0,65	4 mg

Note: (1) Artemisinin is the active parent compound of the plant. Its half-life is intermediate. It is also very safe, and can cross the blood-brain and blood-placenta barriers [de Vries PJ *et al.*1996].

(2) Artemether has the longest life but, at the doses required for treatment, it is the most toxic.
(3)Artesunate is the most active and the least toxic of this group of drugs. It also has the shortest life within the body

The apparent primary disadvantage of artemisinin drugs is that they are characterized by a short half life [Krishna S *et al.* 2004]. Artemisinin drugs are very efficient and fast acting thus

treatment with artemisinin drugs causes reduction of parasite burden below detectable levels without eliminating all parasites and these results in a higher risk of recrudescence [Bjorkman A *et al.* 2005; Krishna S *et al.* 2004]. In addition, a fraction of the parasites exposed to the drug are thought to become dormant and unsusceptible to further dosing until reactivation [Hoshen MB *et al.* 2000]. In order to completely eliminate the parasites avoiding parasite recrudescence and preventing the emergence of resistant *P. falciparum*, combination with other longer-acting drugs is necessary [Olliaro PL *et al.* 2004; Menard D *et al.* 2005]. The combination of artemisinin or one of its derivatives with another drug is named artemisinin combination therapy (ACT).

Artemisinin combination therapies (ACTs) are currently recommended by the World Health Organization (WHO) as the first line antimalarial treatment for *P. falciparum* malaria [Bulletin of the World Health Organization 2005; World Health Organisation. Chemotherapy of malaria and resistance to antimalarials: report of a WHO Scientific Group]. ACTs combine drugs with different modes of action which reduces recrudescences and also reduces considerably the risk of selecting resistant mutants in the parasite population (just the same rationale for combining drugs in the treatment of tuberculosis and HIV-AIDS). Several ACTs have been developed. These include Coartem ®, the combination of artemether with lumefantrine, and the combination of artesunate with amodiaquine, mefloquine or sulfadoxine-pyrimethamine [Balint GA 2001, Bulletin of the World Health Organization 2005; Bunnag D *et al.* 1995, Burk O *et al* 2005, Campbell P *et al.* 2006, Hien TT *et al.* 1993; Olliaro PL *et al.* 2004, Svenson US *et al.* 1998, Svensson US *et al.* 1999].

1.6.1 Artemisinin mode of action

The mode of action of artemisinin-based compounds, in spite of intense scientific activity, is not yet completely understood. Artemisinin and its derivatives are toxic to malaria parasites at nanomolar concentrations, whereas micromolar concentrations are required for toxicity to mammalian cells [Lai H *et al.* 1995, Woerdenbag HJ *et al.* 1993]. One reason for this selectivity is the enhanced uptake of the drug by *P. falciparum* infected erythrocytes to more than 100 fold higher concentrations than do uninfected erythrocytes [Gu HM *et al.* 1984, Kamchonwongpaisan S *et al.* 1994]. This drug uptake is very rapid, reversible, saturable and appears to be partially dependent on metabolic energy [Gu HM *et al.* 1984, Kamchonwongpaisan S *et al.* 1994].

Artemisinin and its derivatives being highly hydrophobic localize in specific parasite membranes artemisinins are present in the parasite limiting membranes [Ellis DS *et al.* 1985], digestive vacuole membranes [Ellis DS *et al.* 1985, Maeno Y *et al.* 1993] and mitochondria [Maeno Y *et al.* 1993]. So the question is once inside the parasite how do artemisinin derivatives really act, and a lot of considerable evidences have been indicating that artemisinins mode of action is mediated by free radicals.

The first clue to its mechanism came from synthetic chemists who demonstrated that the endoperoxide bridge that is part of the molecular structure was fundamental for its antimalarial activity [Brossi A *et al.* 1988] and since peroxides are a known source of reactive oxygen species like hydroxyl radicals and superoxide free radical mode of action was the obvious suggestion and further evidences came from the fact that free radical scavengers antagonised (like alfa-tocopherol, catalase, ascorbate, etc) the *in vitro* antimalarial activity of these drugs and that other free radical generators (like doxorubicin, micanazole, castecin and artemitin) promoted them [Krungkrai SR *et al* 1987] and also that artemisinin treatment of membranes, especially in the presence of heme can cause lipid peroxidation, hemolysis and lysis of infected erythrocytes [revised by Meshnick SR 2002].

Meshnick and collaborators [Meshnick SR *et al.* 1991] on an attempt to clarify artemisinins mode of action showed that artemisinin interacted with intraparasitic heme, and suggested that intraparasitic heme or iron might function to activate artemisinin inside the parasite into toxic free radicals [Meshnick SR *et al* 1991]. One reason that could explain the selective toxicity of artemisinin to the parasites is that the *Plasmodium* parasite is very high rich in heme-iron, derived from the proteolysis of host cell hemoglobin [Meshnick SR *et al.* 1996]. When artemisinins are incubated with heme or iron, they decompose in a fashion that suggests the generation of free radical intermediates also studies using electron paramagnetic resonance

have also shown that the breakdown of artemisinin results in free radicals [Meshnick SR *et al* 1996] during this artemisinin breakdown process ferryl ions (Fe[IV]=O) appear to be formed [Kapetanaki S *et al* 2000]. There are other electrochemical studies that have shown that heme/iron can catalyse the irreversible breakdown of artemisinin derivatives [Zhang F *et al.*1992] and also structure–activity relationship studies have shown a high correlation between antimalarial activities and heme-binding [Meshnick SR *et al* 1996] and between antimalarial activity and protein alkylation [Meshnick SR *et al* 1996]. Also, the predicted pharmacophore or drug receptor from several structure–activity relationship studies seems to resemble heme [Meshnick SR *et al* 1996] and also there are a lot of other theoretical studies that have shown that artemisinin could bind to and react with heme itself [Gu HM *et al.* 1984] furthermore, the presence of the heme polymer, hemozoin, is associated with sensitivity to artemisinins for example artemisinins are inactive against the RC strain of *Plasmodium berghei* [Peters W *et al.* 1986], and the related intraerthrocytic apicomplexan parasite, *Babesia microti* [Wittner M *et al.* 1996],.which both lack hemozoin, yet are most active against schistosomes which also produce hemozoin [Utzinger J *et al.*2001].

Though all these evidences one could not say that artemisinins act like the typical oxidating drugs which cause promiscuous damage to protein, nucleic acids and lipid firstly because, unlike most other oxidant drugs (and oxidizing agents *per si*), artemisinin cannot be cyclically oxidised and reduced causing the cascade effect typical from free radical reactions [Zhang F *et al.*1992] in a way that only one free radical can result from one drug molecule, secondly, all of the oxidant end products observed experimentally were only observed at very high drug concentrations [Berman PA *et al.* 1997], but the drug is effective at much lower concentrations. This is a very strong indication that, artemisinin derivatives must have a more selective toxic effect. One suggestion to the selective toxicity of artemisinins may be the formation of covalent adducts with parasite components, which will then serve as mediators for free radical intermediates. One important alkylation target is heme itself. Artemisinin–heme adducts have been demonstrated in parasite cultures treated with therapeutic concentrations of artemisinin derivatives [Hong YL *et al.* 1994] this means that heme is both an activator and target of the artemisinin derivatives.

The modification that occurs in heme via its ligation to artemisinin could kill the parasite in several ways, firstly, artemisinin or its heme adduct might be able to inhibit hemozoin biosynthesis or cause hemozoin degradation, for example Pandev and co-workers proved that at micromolar concentrations, artemisinin inhibits hemoglobin digestion by malaria parasites and inhibits hemozoin formation [Pandey AV *et al.* 1999] though this observation has only been demonstrated in cell-free conditions, artemisinin treatment of living intraerythocytic *P.*

falciparum in culture caused no change in hemozoin content [Asawamahasakda W *et al.* 1994], suggesting that heme metabolism might not be the major intracellular target. But heme is not the only protein that artemisinin forms adducts with, artemisinins also form covalent adducts with other protein, but not with DNA [Yang YZ *et al* 1993, Yang YZ *et al.* 1994].

The alkylation of specific malaria proteins by artemisinins has been demonstrated [Asawamahasakda W *et al.* 1994] and this could mediate the killing action of artemisinin derivatives since it occurred at therapeutic concentrations of drug. One of the major alkylation targets described in the literature is the malarial translationally controlled tumour protein (tctp protein), a protein that binds heme [Bhisutthibhan J *et al.* 1998], also previous microscopic studies had proven that some of the malarial tctp protein is present in the food vacuole membranes, where it is in proximity to the heme-rich food vacuole [Bhisutthibhan J *et al.* 1998]. Thus, it is likely that the reaction between artemisinin and tctp protein occurs because of an association between tctp protein and heme, however, there is a lack of evidences of tctp or any other malarial protein in association with artemisinin is directly causing the parasite elimination though a variety of ultrastructural studies have been carried out on infected red cells treated with artemisinin derivatives and from those studies it was described that membrane-containing structures, such as the plasma membrane, endoplasmic reticulum, nuclear envelope, food vacuolar membrane and mitochondria appear to be most sensitive to the action of artemisinins [Maeno Y *et al.*1993] thought from these observations a variety of mechanisms of action might be suggested. Another very interesting observation is that when radiolabelled artemisinin derivatives are fed to malaria infected cells, the drug has been found to accumulate in hemozoin and in the membranes of the food vacuole and mitochondria [Maeno Y *et al.*1993]. These observations are consistent with the role of heme in the mechanism of action.

Thought there are as described before many theories to explain artemisinin mode of action one cannot even say what is the cellular target for artemisinin. Tctp protein for the reasons stated before was one of the suggested targets and more recently Krishna and co-workers have indicated that artemisinins might inhibit the sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) of *P. falciparum* (*Pfatp6*) and, therefore, also SERCAs of other *Plasmodium* species because thapsigargin a chemical component that is a potent and selective inhibitor of SERCAs and share some chemical similarities with artemisinins [Eckstein-Ludwig U *et al.* 2003]. Artemisinins, but not other antimalarial drugs, inhibited Pfatp6 protein activity when Pfatp6 was expressed and assayed in *Xenopus* oocytes [Eckstein-Ludwig U *et al.* 2003]. Others have carried out docking simulation studies of artemisinin derivatives to models of the thapsigargin binding site in Pfatp6 protein [Uhlemann AC *et al.* 2005]. Several potential

hydrophobic interactions between side chains of artemisinin derivatives and amino acids of Pfatp6 protein have been identified including Leu263 that seems to modulate artemisinin susceptibility when examined using mutagenesis experiments of malarial SERCAs [Jung M *et al.* 2005, Uhlemann AC *et al.* 2005]. Taken together, there are independent lines of evidence that have been obtained from a range of experimental techniques to suggest that Pfatp6 protein might be the primary target of artemisinins. However, it is suggested that genetic studies are required to support this hypothesis.

More recently, Li and co-workers have suggested that the electron transport chain of *P. falciparum* might be a target for artemisinins [Li W *et al.* 2005]. In support of this idea, when yeast was grown in non-fermentable media (making it dependent on mitochondrial respiration), sensitivity to artemisinin increases, because over expression of some mitochondrial-transport proteins seems to increase sensitivity to artemisinins, it has been suggested that the electron transport chain stimulates the activity of artemisinins, and that these activated artemisinins impede mitochondrial function by depolarizing mitochondrial membrane potential [Li W *et al.* 2005]. The mechanism of this inhibition is unclear but it might be related to the presence of an iron group in the cytochrome center that induces the formation of radicals [Haynes RK *et al.* 2006].

1.7 Drug Resistance

The emergence and spread of parasite resistance to anti-malarial drugs has presented one of the largest obstacles hindering the effective treatment and control of malaria.

The WHO's official definition of malaria parasite resistance dates from 1973; "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" [World Health Organisation. Chemotherapy of Malaria and Resistance to Antimalarials: Report of a WHO Scientific Group].

The WHO 1973 definition of the level of parasite drug resistance remains in use;

A - Sensitive (S): The asexual parasite count reduces to <25% of the pre-treatment level in 48 hours after starting the treatment, and complete clearance after 7 days, without subsequent recrudescence - Complete Recovery.

B - RI Delayed Recrudescence: The asexual parasitaemia reduces to < 25% of pre-treatment level in 48 hours, but reappears between 2-4 weeks.

C - RI Early Recrudescence: The asexual parasitaemia reduces to < 25% of pre-treatment level in 48 hours, but reappears within 2 weeks.

D - RII Resistance: Marked reduction in asexual parasitaemia (decrease >25% but <75%) in 48 hours, without complete clearance in 7 days.

E - RIII Resistance: Minimal reduction in asexual parasitaemia, (decrease <25%) or an increase in parasitaemia after 48 hours.

Though it is important to clarify that clinical treatment failure or increased *in vitro* IC_{50} values alone are not sufficient to prove drug resistance. A parasite isolate should be classified as resistant only after analysis of treatment response parameters (parasite and fever clearance) and treatment success (determination of possible re-infection, in the case of apparent recrudescence) in correlation with the *in vitro* drug sensitivity. Increased IC_{50} values combined with prolonged parasite clearance and treatment failure define a case of confirmed drug resistance [Noedl H 2005]. One might suggest that the first step in the development of clinical resistance may be a decrease in parasite *in vitro* susceptibility, associated with a key mutation in a target enzyme which ultimately results in clinical failure. Thought it is also very important to notice that the parasite response and resistance is not only dependent on the parasite genotype but also involves the general health state and immune status of the patient.

A number of factors influence the likelihood of resistance occurring and the speed with which it spreads. For instance, the mechanism by which the drug works against the parasite is

important; simple modes of action such as simple enzyme inhibition are likely to lead to rapid evolution of resistance, as the number of genetic mutations required to alter enzyme structure is low. This is the case with pyrimethamine resistance, which evolved very quickly after the introduction of the drug, in contrast to the pattern seen with the emergence of chloroquine resistance, which took much longer to evolve, as most studies described chloroquine mode of action has being much more complex than pyrimethamine.

The pharmacokinetic dynamics of drugs are also important in determining the selection pressure for drug resistance. Watkins and Mosobo [Watkins WM *et al.* 1993], for example, showed that the long half-life of sulfadoxine-pyrimethamine was a considerable factor in the selection pressure for resistant mutants, as the drug was present in patients at sub-therapeutic levels for long periods of time. Drugs with high efficiencies of parasite killing, rapid achievement of levels above the minimal inhibitory concentrations and short half-lives, will be the most effective at minimizing the selection pressure for resistant mutants [Winstanley PA *et al.* 2002].

Resistance has been recorded to every anti-malarial currently in use, with the exception of artemisinin and its derivatives. Quinine, the first drug used specifically to treat malaria was used extensively. The first reports of resistance to the drug occurred at the beginning of the 20^{th} century, when Couto (1908) and later Nocht and Werner (1910) reported the treatment of patients who did not respond to quinine treatment [Peters W 1987]. Despite the appearance of quinine resistance, the drug remains remarkably useful today, especially as a first line drug for treating complicated cerebral malaria. In fact, quinine resistance is surprisingly uncommon, and in the few instances it has emerged, it is often associated with parasites that are already resistant to other drugs such as chloroquine and mefloquine [Looareesuwan S *et al.*1990; Meshnick SR 1997; Peters W 1987; Pukrittayakamee S *et al.* 1994]. Chloroquine, itself based on the structure of quinine, was developed in Germany in the early 1940s, resistance to chloroquine was far more forthcoming than with quinine, and the first reports of parasites failing to respond to the drug emerged independently from South America and South East Asia in the late 1950s [Moore DV *et al.* 1961; Young MD *et al.* 1961]. The spread of resistance from these pioneer areas was relatively rapid, and chloroquine resistance is now a major problem throughout the malaria affected areas of the world.

One of the proposed mechanisms for the emergence of drug resistance is through the presence of a drug at sub-therapeutic levels within a population. There can be no doubt that the emergence of chloroquine resistance in South America was facilitated by the policy of distributing chloroquinated salt to the area as part of a well-intentioned control problem. This resulted in a large proportion of the population being exposed to the drug at sub-curative

doses, thus considerably enhancing the chances of selection of chloroquine resistant parasites [Payne D 1988]. The proliferation of chloroquine resistance resulted in the development of mefloquine, a drug that was effective against chloroquine resistant parasites. Initial indications that mefloquine resistance was likely to emerge came, however, in 1977, when resistance was experimentally induced in a rodent malaria parasite [Peters W *et al.* 1977]. Efforts to reduce the possibility of the emergence of resistant parasites in the field by using mefloquine in combination with other drugs (especially pyrimethamine) met with failure, however, and reports of mefloquine resistant parasites emerged throughout the 1980s [Peters W 1998]. Introduced as a first line treatment to Thailand in 1984, substantial resistance had developed within 6 years [Price RN *et al.* 2004].

Concerning the genetic of drug resistance, two main genes have been implicated in chloroquine resistance; the *pfmdr1* (*P. falciparum multi drug resistance* 1 gene) and the *pfcrt* (*P. falciparum chloroquine resistance transporter* gene).

*Pf*mdr1 protein is a membrane protein, belonging to the sub group of ABC-type multidrug transport system. *P. falciparum* gene *mdr1* is localized on chromosome 5 and according to the *P. chabaudi* synteny map the *pcmdr1* (gene homologous on *P. chabaudi* of the *pfmdr1*) gene is localized on chromosome 12.

*Pf*crt protein is a digestive vacuole transmembrane protein, associated to chloroquine resistance. *P. falciparum* gene *crt is* localized on chromosome 7 and according to the *P. chabaudi* synteny map the *pfcrt* gene referred to as *cg10* gene is localized on chromosome 6.

It has been shown that some point polymorphisms in the *pfmdr1* gene can be correlated with chloroquine resistance in some field isolates [Basco LK *et al.* 1995, Cox-Singh J *et al.* 1995, Duraisingh MT *et al.* 1997, Duraisingh MT *et al.* 2000]. Similarly, transfection work has suggested that the gene *pfmdr1* can modulate the sensitivity to chloroquine [Reed MB *et al.* 2000]. However there are other studies found in the literature with parasites collected from the field where no association between point polymorphisms in the gene *pfmdr1* and chloroquine resistance was found [Chaiyaroj SC *et al.* 1999, Cremer G *et al.* 1995, Povoa MM *et al.* 1998]. In addition, the analysis of a genetic cross between a *P. falciparum* chloroquine-resistant clone; Dd2 and a chloroquine sensitive one, HB3, showed that mutations in the gene *pfmdr1* did not genetically segregate with chloroquine resistance [Wellems TE *et al.* 1990]. Later detailed linkage analysis and fine chromosome mapping of the progeny clones of the *P. falciparum* Dd2 x HB3 genetic cross allowed the identification of another gene, the *pfcrt* gene, in which a particular mutation at the amino acid position 76 (K76T) a lysine to a threonine change, that appears to correlate completely with chloroquine resistance among

field isolates of *P. falciparum* [Fidock DA *et al.* 2000]. Babiker HA and colleagues described that the combination of mutation in both *pfmdr1* and *pfcrt* gene loci confer higher chloroquine resistance phenotypes in field populations of *P. falciparum* [Babiker HA *et al.* 2001].

In relation to the genetic of mefloquine resistance, *in vitro* studies on *P. falciparum* have shown that genetic amplification of the *pfmdr1* gene may correlate with both mefloquine and quinine resistance [Cowman AF *et al.* 1994, Peel SA *et al.* 1994]. However, field studies on the association between the *pfmdr1* gene and the parasite response to mefloquine have not provided unanimous results, while some appear to have shown an association between the amplification of the *pfmdr1* gene and mefloquine resistance [Price RN *et al.* 1997, Price RN *et al.* 1999, Wilson CM *et al.* 1993] others as Chaiyaroj SC and co-workers have not found any correlation between the two events [Chaiyaroj SC *et al.* 1999], one possible explanation comes from the fact that the genotype-phenotype associations depends from the parasite geographical origin. In addition and as with chloroquine it has also been demonstrated through genetic crossing and transfection experiments that point mutations in the *pfmdr1* gene may modulate the sensitivity to both mefloquine and quinine in *P. falciparum* [Duraisingh MT *et al.* 2000, Reed MB *et al.* 2000]. In *P. chabaudi* Cravo PV and colleagues [Cravo PV *et al.* 2003] shown that amplification of the *pcmdr1* gene (gene homologous of the *P. falciparum mdr1* gene) is an important event in the generation of mefloquine resistance, paralleling the situation observed by Cowman AF and co-workers and Peel SA and colleagues [Cowman AF *et al.* 1994, Peel SA *et al.* 1994] though other genes are suggested to be also involved.

1.7.1 Resistance to artemisinin

Clinical parasite resistance to artemisinin drugs has not yet been observed, although variations in sensitivity have been described [van Agtmael MA *et al.* 1999] for example various isolates of *P. falciparum* from Vietnam and Thailand have been found to vary in their sensitivity to artemisinins *in vitro* [Brockman A *et al.* 2000; Woitsch B *et al*. 2004; Wongsrichanalai C *et al.* 1997; Wongsrichanalai C. *et al.* 1999]. Differences in the sensitivity level of *P. falciparum* isolates to artemisinins can be due to genetic alterations of the parasite that confer differential sensitivity to these drugs or simply can be due to the natural genetic variation of the parasite in that particular part of the world.

To this stage, several proteins, including a Ca^{2+}-depending SERCA type ATPase protein that in *P. falciparum* is codified by the *pfatp6* gene that is localized in *P. falciparum* chromosome 1, the *P. falciparum* chloroquine resistance transporter protein codified by the *pfcrt* gene, the *P. falciparum* multidrug resistance protein-1 codified by the gene *pfmdr1*, and the translationally controlled tumor protein (*tctp*) that is codified by the gene *pftctp* in *P. falciparum* (this gene being localized in *P. falciparum* chromosome 5), have been implicated in modulation of parasite susceptibility to artemisinin drugs.

Mutations in *pfcrt* gene were associated with increased susceptibility of *P. falciparum* isolates gathered in Asia, Africa and South America [Sidhu AB *et al.* 2002] to artemisinin.

Measurement of the *pfmdr1* gene copy by real-time PCR on filed isolates that were significantly more resistant to mefloquine, quinine, artemisinin and artesunate and more sensitive to chloroquine showed that this isolates had 3 copies of *pfmdr1* gene (in normal conditions *pfmdr1* is a single copy gene), thus, reduced *in vitro* sensitivity to artemisinin and artesunate was correlated to an increased gene copy numbers of the gene *pfmdr1* [Pickard AL *et al.* 2003, Price RN *et al.* 2004]. Price RN and colleagues also associated polymorphisms in the gene *pfmdr1* with an increased artemisinin susceptibility in isolates with a single copy of the gene *pfmdr1*, in this study the N86Y mutation was associated with lower IC_{50}s to mefloquine than in those isolates with a wild-type *pfmdr1* gene. By contrast, the presence of either the S1034C mutation or the N1042D mutation in isolates with single copies of the gene *pfmdr1* was associated with a higher artesunate IC_{50}s than was the wild-type, at both these loci. On the other hand other point polymorphisms of the gene *pfmdr1* have been associated with increased sensitivity to artemisinin [Duraisingh MT *et al.* 2000]. Likewise, the triple mutation in the gene *pfmdr1*, S1034C/N1042D/D1246Y, highly prevalent in South America, was found to enhance parasite susceptibility to mefloquine, halofantrine and artemisinin [Sidhu AB *et al.* 2005]. From all these studies the gene *pfmdr1* appears to be one important

modulator of the parasite susceptibility to artemisinin drugs. Higher copy numbers of this gene predict treatment failure even for chemotherapy with the highly effective combination of mefloquine and 3 days artesunate [Price RN *et al.* 2004]. It seems that there is a correlation between the resistance to artemisinins and to other antimalarials. Since the gene *pfmdr1* has a general importance in antimalarial drug resistance, induction of resistance to one drug may be followed by resistance to other drugs that are not active by the same mechanism [Anderson TJ *et al.* 2005; Duraisingh MT *et al.* 2000; Ferrer-Rodriguez I *et al* 2004; Ngo T *et al.* 2003*;* Pickard AL *et al*. 2003; Price RN *et al.* 2004; Reed MB *et al.* 2000; Sidhu AB *et al.* 2005].

Resistance to artemisinin in *P. yoelii* had been previously been selected by drug pressure and was correlated to a high protein expression level of parasite tctp protein (Translationally Controlled Tumor Protein Homolog) [Walker DJ *et al.* 2000], which has been shown to bind artemisinin [Bhisutthibhan J *et al.* 1998], however, the resistant parasites readily lost resistance once drug-selection pressure was withdrawn [Peters W *et al.* 1999], so no clear association was actually made between artemisinin resistance and the protein tctp, as being a transient phenotype, the genetic involved on this resistance phenotype is not possible, that is why one of the mains objectives of this project was to select artemisinin resistance of stable phenotype.

So in conclusion we can say that we think that the genetic mechanism responsible for artemisinin and its derivatives resistance need clarification. To start the process of clarification it is better to clarify that although studies made directly on *P. falciparum* may provide more incisive information, this presents several limitations starting by the fact that in the artemisinin case there is no artemisinin resistance yet reported, so the work in animal models can circumvented all the human malaria parasite limitations. There are several rodent malaria models available; those will be presented in the following chapter.

1.8 Rodent malaria parasites

The host specificity of human malaria parasites represents a major constraint on the study of malaria as, unlike the other major tropical diseases such as trypanosomiasis and leishmaniasis, the actual causative organisms cannot be maintained in convenient small laboratory animals. The need for suitable laboratory models has resulted in the use of avian and simian parasites and until 1948 these models were the only ones available.

In 1948 the situation changed with the discovery and isolation of a malaria parasite that was capable of infecting laboratory rats and mice [Vincke IH *et al.* 1948]. This parasite, *Plasmodium berghei*, soon became the most intensively studied malaria parasite.

All experiments described within this PhD thesis were carried out using the rodent malaria parasite, *Plasmodium chabaudi. P. chabaudi* belongs to a group of four *Plasmodium* species that infect murine rodents from Central Africa the other species being *Plasmodium vinckei*, *Plasmodium yoelii* and *Plasmodium berghei*. A map of the locations from which the various rodent parasites were isolated is shown in Figure 8.

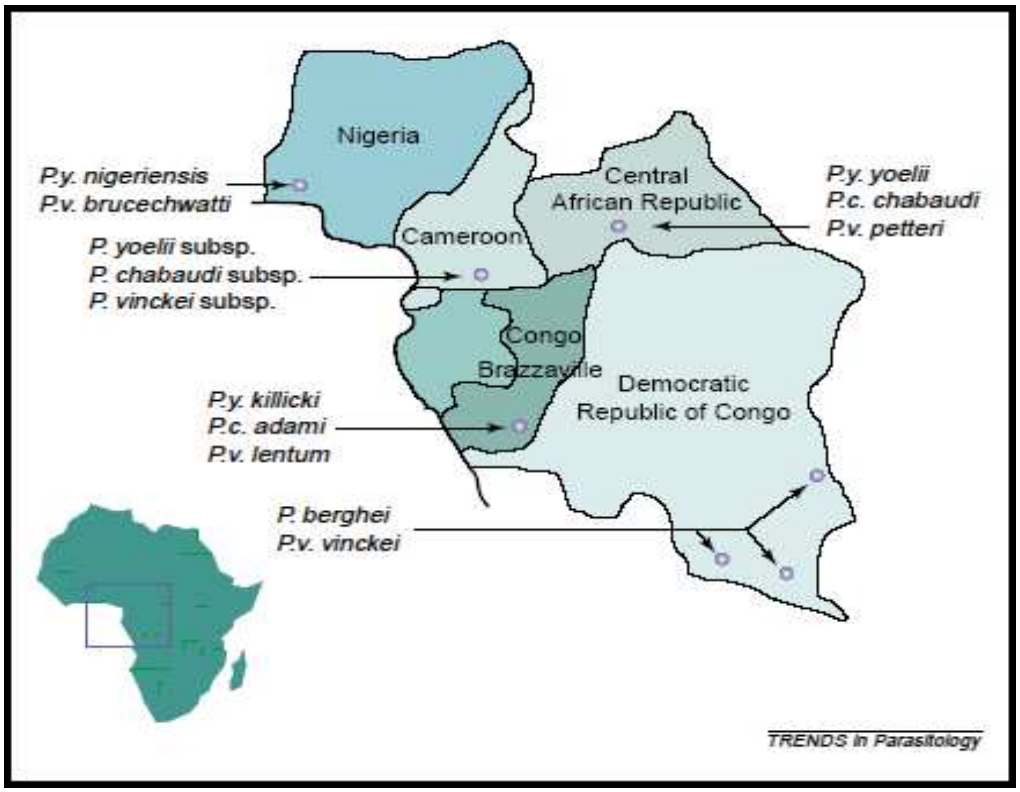


Figure 8 The geographic origins of the rodent malaria parasites.
From Carlton JM *et al.*2001 with kind permission of Dra. Jane Carlton.

The parasites of rodents have received a vast amount of attention both in their own right and as models for human malaria. There are many reasons for using rodent malaria parasites as models for human malaria. The most obvious of these is the ease with which the whole life cycle can be achieved in the laboratory. Apart from the similarities in basic biology between the rodent and human malaria parasites (See Table 4 for details), they share conserved genetics and genome organization, conserved housekeeping genes and biochemical processes, and there is considerable evidence for conservation of the molecular basis of drug-sensitivity and resistance [Janse CJ *et al.* 2004].

Table 4 Some biological similarities and differences between *P. chabaudi* and the human malaria parasite, *P. falciparum*.

Adapted from: Janse CJ *et al.* 2004.

	P. chabaudi	***P. falciparum***
Merozoites per schizont	6-8	8-24
Synchronous blood infection	Yes	Yes
Optimum temperature range mosquito transmission (sporogony)	24-26º C	>26º C
Duration of the asexual blood stage cycle (hours)	24	48
Duration of pre-erythrocytic development	50-58 hours	5.5 - 6 days
Sporozoites in glands at optimum temp. (days after infection)	11-13	10-12

Note: *Plasmodium chabaudi* preferentially parasite mature red blood cells and more closely resemble *P. vinckei* than *P. berghei*.

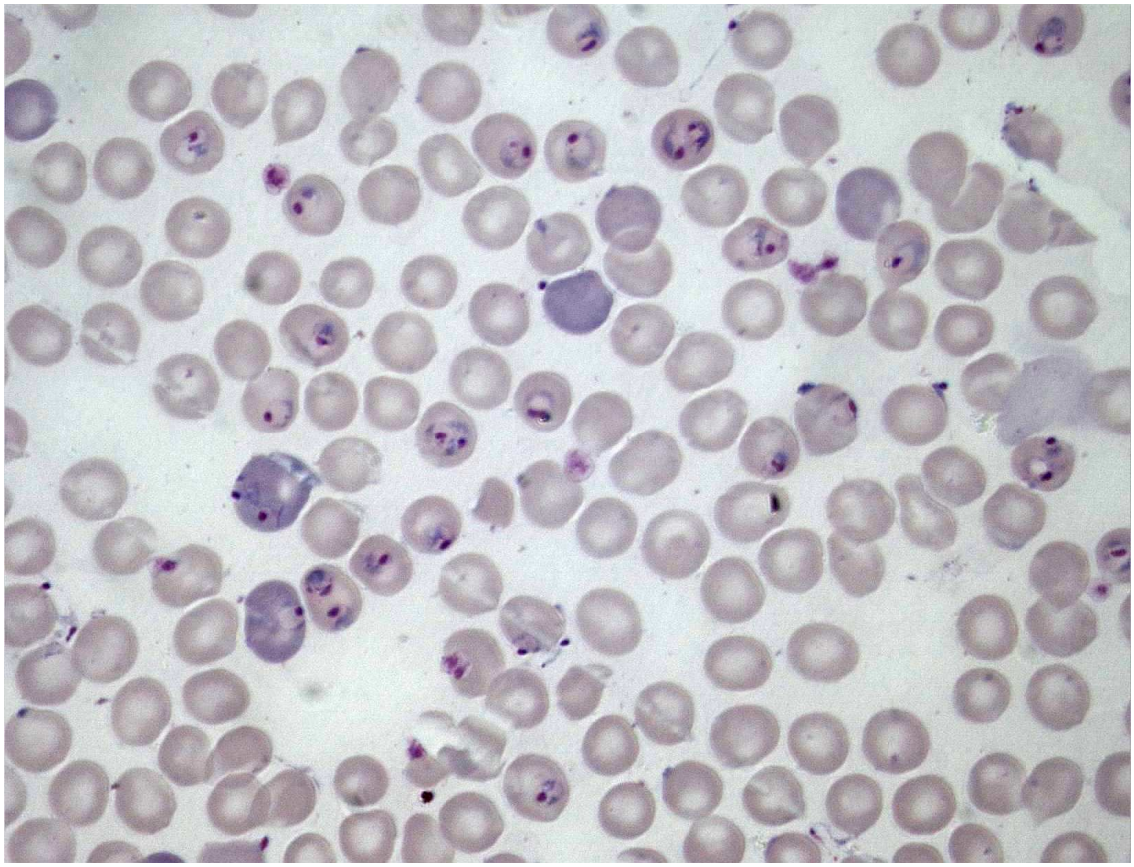


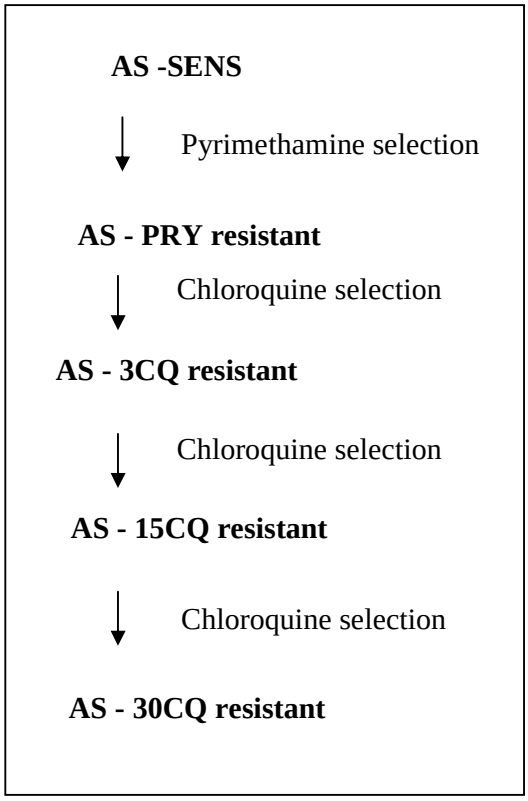
Figure 9 *Plasmodium chabaudi* parasites (trophozoite stage) in mouse peripheral blood.
From www.culleton.org/rodent.html, with kind permission of Dr. Richard Culleton.

Although studies made directly on *P. falciparum* provide more information, this presents several limitations, such as the requirement of working with chimpanzees or humans as hosts for infection, a fact which poses serious ethical problems, which can be circumvented by working with animals models, which make easier the selection of drug-resistant mutants and the identification of genes involved in the resistance by performing genetic crosses.

Of all the rodent malaria models the *Plasmodium chabaudi* model is the most appropriate for studies on the genetics of drug resistance, as *P. chabaudi* is the rodent malaria model that shows most biological similarity to *P. falciparum* because it preferentially parasitizes mature erythrocytes and the schizogony is synchronous [Carlton JM *et al.* 2001]. In addition several clones of this species are already available, which have been selected for resistance to a variety of different drugs (see Table 5 for the *P. chabaudi* clones available for this work). These parasites present the ideal starting material for identifying drug resistance genes since they have been selected from cloned sensitive parasites. In this way, the mutant parasites should have identical genetic backgrounds to the starting sensitive forms except for the genes

determining resistance. Furthermore, for the *P. chabaudi* clones used in this work, an array of amplified fragment length polymorphism (AFLPs) have been previously developed which allows the characterization of all the progeny of genetic crosses between the sensitive and resistant clones and also to pinpoint relevant genes involved in resistant phenotype [Grech K *et al.* 2002]. Using genetic crosses between parents of distinct phenotypes and a recent developed genetic approach, named linkage group selection [Culleton R *et al.* 2005], genes which underlie a particular phenotype can be traced among the cross progeny by the analysis of large numbers of genome-wide genetic markers. Those markers which follow the expression of a phenotype in the recombinant progeny will usually be closely linked to the genes which determine its expression.

Table 5- Clones of *Plasmodium chabaudi* available for this project.



CLONE	DRUG RESPONSE
AS - SENS	Drug sensitive
AS - PYR [Walliker D *et al.* 1975]	Selected from AS - SENS; pyrimethamine-resistant
AS - 3 CQ [Rosário VE 1976]	Selected from AS - PYR; low chloroquine-resistant
AS - 15 CQ [Padua RA 1981]	Selected from AS - 3CQ; intermediate chloroquine-resistant
AS - 30 CQ [Padua RA 1981]	Selected from AS - 15CQ; high chloroquine-resistant

1.8.1 The genetics of rodent malaria parasites

As it is going to be described in more detail later in this thesis, for this research a rodent malaria model; *Plasmodium chabaudi;* was used.

Rodent malaria parasites, such as *P. berghei, P. chabaudi,* and *P. yoelii,* are used as models for *P. falciparum*. Many aspects of the biology, life cycle, and morphology of rodent malaria parasites show a high level of similarity with the human parasites, validating their use as models for human infection. Both species have 14 linear chromosomes and many genes are conserved [Carlton JM *et al.* 1998, Hunt P *et al.* 2004]. There are so many similarities both in gene sequence but also in the arrangement of genes within chromosomes between rodent malaria parasites and *P. falciparum* that using comparative genomics techniques and making use of significant, but partial genome data of the rodent malaria parasites, the construction of a virtual composite rodent malaria parasites genome and its comparison with the *P. falciparum* genome was done, generating what is called a synteny map (in comparative genomics, synteny describes the preserved order of genes between related species) between rodent malaria parasites (*Plasmodium yoelii, Plasmodium berghei* and *Plasmodium chabaudi*) and *P. falciparum* [Carlton JM *et al.* 1998; Janse CJ *et al.* 1994; van Lin LH *et al.* 2000].

To compose the rodent malaria - *P. falciparum* synteny map advances were taken on the release of the complete genome sequence of the human malaria parasite *P. falciparum* and also on the partial genome sequences of the *Plasmodium yoelii, Plasmodium berghei* and *Plasmodium chabaudi* parasites, a genome wide survey was able to be done. This survey was done by merging the sequenced DNA contigs of the three rodent malaria parasites to form composite rodent malaria parasites contigs that cover 90% of the core rodent malaria parasite genomes [Carlton JM *et al.* 2002; Hall N *et al.* 2005]. For rodent malaria parasites, *P. yoelii, P. berghei* and *P. chabaudi* this synteny map was published in 2005 [Kooji TW *et al.* 2005]. See Figure 10 for details.

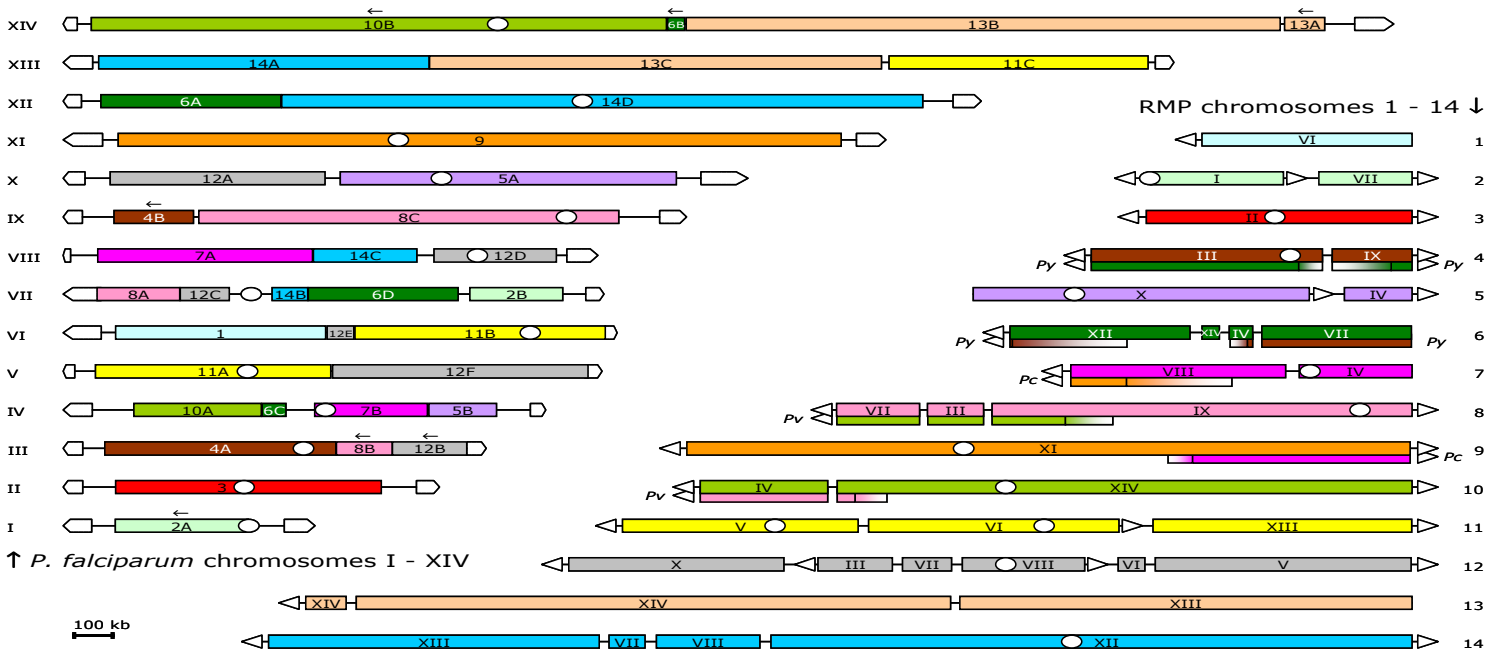


Figure 10 – Rodent malaria genomes synteny map to *P. falciparum.*

Roman numbers represent *P. falciparum* chromosomes numbers (I-XIV). RMP chromosomes, means, rodent malaria parasite chromosomes from 1 to 14. In different colours it is possible to distinguish between the different syntenic blocks, within *Plasmodium* species.

From: Kooji TW *et al.* 2005, with kind permission of Dr. Taco Kooji.

1.9 Linkage Group Selection

As any other living organism, in *Plasmodium* the identification and fully understanding of the genetic mechanisms involved in important phenotypes such as drug resistance is of great importance. In the particular case of drug resistance the knowledge of the genes involved in this phenotype allows epidemiological studies and other long term studies for example as the ones monitoring the resistance of a particular antimalarial in the field. In the case of malaria parasites there are nowadays mainly two genetic methods that can be used to try to locate genes controlling any genetic trait such as drug resistance for example; those are linkage analysis and linkage group selection (LGS).

When using classical linkage analysis to identify the genetic loci involved in drug resistance, the resistant mutants need to be crossed with genetically distinguishable parasites (like AS and AJ strains for example), this genetically distinguishable parasites must differ in a number of genetic markers. These markers can be restriction length polymorphism (RFLPs) or microsatelites for example, and will distinguish the sensitive from the resistant parasites. When using classical linkage analysis, the progeny of the genetic cross obtained needs to be cloned and the resulting cloned progeny will be analysed for linkage of the phenotype, lets consider for the moment drug resistance, with the inheritance of any parental markers either resistance or sensitive. Classical linkage analyses bring to notice groups of markers whose inheritance is linked to the inherence of drug resistance. In the eventual case of having a number of markers considered sufficient a linkage map can be constructed [revised by Carter R *et al* 2007]. Classical linkage analysis has been used before with success to identify regions of the parasite genome that are important for drug resistance as for example the case of Wellems TE and co-workers, Carlton JM and co-workers and Hunt P and collaborators [Carlton JM *et al.* 1998, Hunt P *et al.* 2004b, Wellems TE *et al.* 1991] but only once has it lead to the actually identification of a gene responsible for a particular phenotype, in this case, it was the identification of *pfcrt* has the gene involved in chloroquine resistance [Fidock DA *et al.* 2000, Su X *et al.* 1997]. Due to the fact that for using classical linkage analysis a large number of genetic markers is indeed necessary, it is also necessary to know the position of these markers in the parasite genome and then involves the cloning of a large number of clones from the recombinant progeny and also the genetic characterization of each one, this technique is very laborious and time consuming.

The other technique that enables the discovery of genes controlling biological properties in malaria parasites as stated before is linkage group selection (LGS). LGS is a novel approach developed for malaria parasites in order to identify genes responsible for selectable

phenotypes, as for example drug resistance. LGS was previous validated for finding genes involved in drug resistance by Culleton R and co-workers [Culleton R *et al.* 2005]. LGS can be applied to the genetic analysis of any malaria parasite as long as there are experimental means of infecting mosquitoes with gametocytes from parasites and also means of passaging the genetic progeny of a cross through the liver stage of development and into the blood. LGS has in common to the classical linkage analysis the fact that it is an approach that uses a genetic cross between two unrelated parasites from the same species, one of which is sensitive and the other one is resistant to a particular characteristic that is going to be applied as selective pressure [Culleton R *et al.* 2005], but differs from the traditional approach by avoiding cloning very large numbers of cloned lines from the progeny of a genetic cross [Carter R *et al.* 2007]. In LGS the uncloned progeny of a genetic cross, between a sensitive and a resistant parasite to a particular characteristic and that are genetically distinguishable by a large number of genetic markers such as AFLPs markers for example, is placed under a selection pressure representing the biological property of use, in the case of our project; artemisinin and artesunate resistance. The DNA obtained from the surviving progeny is then screened from the presence of a large number of molecular markers distributed throughout the parasite genome. Prior to the developed of the LGS technique an atlas of a large number of molecular genetic markers distinguishing for example the two different strains AS and AJ was developed [Martinelli A *et al* 2005]. The genetic markers from the sensitive progenitor that are linked to the gene of interest (for example in our case the gene conferring artemisinin resistance) will be under-represented or even eliminated from the progeny of the genetic cross after the drug selection. Finding the genetic position of the markers under selection would allow us an area in the genome where the gene of interest might be located. It is important to noticed that the intensity of reduction of any particular marker is directly proportional to the distance of the gene of interest thus allowing the construction of a selection valley around the genetic area of interest, where the target gene in supposedly located at the valley base [Carter R *et al.* 2007]. See Figure 11 for the resume of the LGS protocol.

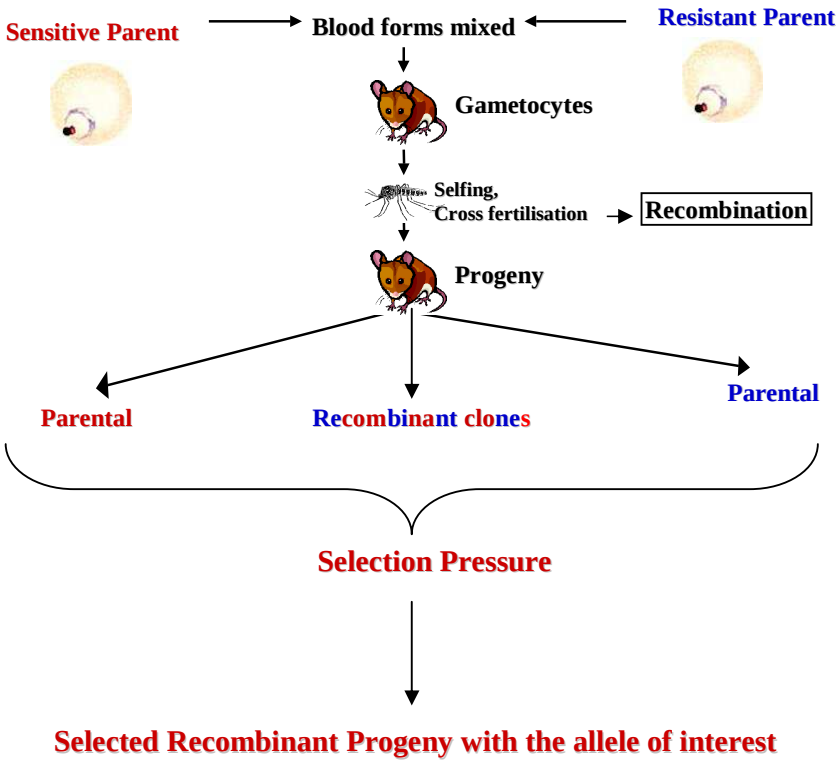


Figure 11 – Schematic representation of the Linkage Group Selection protocol.

1.9.1. Amplified Fragment Length Polymorphism

LGS requires that the two parental clones are distinguished by a large enough number of genetic markers to ensure that some will be linked to the genes of interest. LGS was optimized using amplified fragment length polymorphism (AFLP) a PCR-based method for amplifying DNA fragments from genetically distinct cloned lines of parasites, for example AS and AJ. AFLP is a technique in which large numbers of markers are generated across a genome [Masiga DK *et al.* 2000]. Having a high density of these markers in the genome means that there is a high probability that some of them will be linked to the gene of interest. It has been previously shown that AFLP meets the requirements of LGS both as regards numbers of markers generated in different strains of *P. chabaudi* [Grech K *et al.* 2000] and their quantisation in a mixture of the strains [Martinelli A *et al.* 2004].

The AFLP technique allows the visualization of restriction fragments of DNA. This enables the detection of the variation in DNA between two strains without prior knowledge of the nucleotide sequence. Depending upon the polymorphism between the two strains, large numbers of genetic markers can be produced in a relatively short time [Vos P *et al.* 1995]. AFLP involves cutting genomic DNA into a large number of DNA fragments with two different restriction enzymes (an enzyme called a frequent cutter and another called a rare cutter), thus generating optimal size fragments for visualization on polyacrylamide gels. Using radiation to label one of the primers allows visualization of the products. Genetic differences between strains occurring at a cutting site result in fragments of different sizes which can then be identified on a gel as being present in one strain and absent in the other (See Figure 12 for an example of an AFLP gel).

Polymorphic bands can be produced as a result of polymorphisms between strains at the enzyme recognition sites, polymorphisms between strains at the selective bases used in each PCR, or as a result of insertion/deletion polymorphisms within fragments.

1.10 Aims of the project

This project involves identifying and characterizing genes involved in resistance to artemisinin using the rodent malaria specie *P. chabaudi*, starting by selecting stable artemisinin drug resistance.

To select artemisinin and artesunate mutants using the rodent model, *P. chabaudi*, though prolonged exposure of drug-sensitive lines to low and increasing levels of the drug in mice. Attempts of selecting artemisinin and artesunate mutants in *P. chabaudi* would be carried out though prolonged exposure of drug-sensitive lines to low and increasing levels of the drug in mice; so that the surviving parasites of one lower dose of artesunate and artemisinin would receive an increasing dose of the same drug. These results will be presented on **chapter III**.

After selecting mutant clones resistant to artemisinin and artesunate the objective would be, as a first approach, to study the involvement of *P. chabaudi* gene orthologues *pfmdr1*, *pfcrt*, *pftctp* and *pfatp6*, in the selected mutant clones, previously described as being putative genetic modulators for artemisinin. These results will be presented on **chapter IV**.

The last objective was to perform genetic crosses in mosquitoes with the previously selected and cloned artemisinin and artesunate mutants in *P. chabaudi* (AS-ART and AS-ATN respectively) and the genetic distinctive sensitive parasite line of *P. chabaudi* AJ, and to perform linkage group selection (LGS) on the genetic crosses using artemisinin and artesunate; these results will be presented on **chapter V**.

CHAPTER II

MATERIALS AND METHODS

2.1. Mice

Inbred female CD1 (*Mus musculus*) from Harlan-Tekld Iberica were used for drug tests and selection experiments. Inbred female CBA/CA and C57B1/6J mice (*Mus musculus*) from the University of Edinburgh were used in the rest of the experiments described in this work. All mice used were 4-6 weeks old at the start of the experiments. They were housed in polypropylene cages with sawdust as bedding, and were provided with 41B rat and mouse maintenance diet (Harlan-Tekld) *ad libidum*. Drinking water was supplemented with 0.05% paraminobenzoic acid (PABA), an essential element for the parasite growth, and given *ad libidum*. The cages were kept in a room where temperature was maintained at a constant 25º C ± 3 º C, on a 12-hour light/dark cycle.

2.2. Mosquitoes

Anopheles stepehensi mosquitoes were used for all experiments. Insectaries were maintained on a 12-hour light/dark cycle at 25-27º C temperature and 75-86 % humidity. Larvae were fed on Liquifry™ until 2nd instar and thereafter on ground Tetramin™ fish food, Adult mosquitoes were kept on 10% glucose and 2% PABA-supplemented water solution. Stock adults received weekly rat-blood feeds, which are essential for the production of eggs.

2.3. Parasites

All parasites used in the experiments described here were *Plasmodium chabaudi chabaudi* (referred to as *P. chabaudi* hereafter). See Table 5 and also Table 6.

Table 6 - Parasite clones and lines used in the present work.

CLONE	DRUG RESPONSE
AJ	Drug sensitive, genetically different and distinguishable from AS line
AS-PYR	Derived from AS-SENS; pyrimethamine-resistant
AS-15CQ	Derived from AS-3CQ; resistant to 6 daily doses of chloroquine at 5mg/kg mouse body weight
AS-30CQ	Derived from AS-15CQ; resistant to 6 daily doses of chloroquine at 30 mg/kg mouse body weight

2.4 Preparation of 10^7 standard parasite inocula

10^7 iRBC was established as the standard parasite number to be infected into individual mice at the time of the drug tests, during selection experiments and during preparation of inocula for the production of mixed infections.

The preparation of the inocula for drug tests and for the selection experiments was as follows: the parasitaemia and red blood cell density (RBC/ml) were calculated in the donor mouse, after which the required amount of blood was collected from the mouse tail by calibrated capillary pipette and diluted to a final concentration of 10^7 iRBCs/0.1 ml in a solution of heparinised 1:1 calf serum/mammalian Ringer's solution (Appendix 1). Preparations were kept on ice at all times and an aliquot of 0.1 ml was inoculated into mice.

For the preparation of inocula for the production of mixed infections containing equal proportions of two parasite clones, infections of both clones into a single mouse were induced as described above.

2.5 Cloning

Dilutions for cloning were prepared as in 2.4 except that each mouse was infected either with a mean of 0.5 or 1 parasite. Groups of 50 mice were inoculated in these experiments. If approximately 30% of mice became infected, it could be predicted that 75% of the infections had resulted from a single parasite; a lower percentage of infected mice would indicate higher proportion of pure clones. Cloning was done once drug resistance stability was verified.

2.6 Preparation and administration of artemisinin and artesunate

Artemisinin powder was obtained as a gift from African Artemisia.

Artesunate powder was obtained as a gift from Daphra Pharma.

In the initial tests artemisinin was dissolved in dimethyl sulphoxide (DMSO) and corn oil, and artesunate was dissolved in DMSO, Na_2CO_3 or corn oil, but for further analysis DMSO was always used.

Both artemisinin and artesunate were freshly diluted daily in dimethyl sulphoxide (DMSO) and were kept at room temperature protected from the light. Both drugs were administered to mice by gavage using a lubricated catheter adapted to a 1 ml syringe. Drug doses were expressed as milligrams of drug per kilogram (Kg) of mouse body weight per day. Both drugs

were diluted to a concentration such that the amount of drug corresponding to the desired dose was present in 0.1 ml when given to a mouse weighing 20 grams. At the time of drugging, mice were individually weighed so that the amount of drug given could be adjusted.

2.7 Drug tests

Initially, artemisinin and artesunate drug test trials were carried out on four *P. chabaudi* clones (AS-PYR, AS-15CQ and AS-30CQ), to establish the appropriate drug regimen and a standard test to distinguish between resistant and sensitive parasites as well as parasites with intermediate levels of resistance.

Mice received 10^7 iRBC each by intra-peritoneal (i. p.) injection.

Groups of five mice were prepared; one group was untreated and used as a control for the infection, while the remaining group was drugged three hours after injection, to allow parasites to reach the blood stream. Depending on the particular drug regimen the drug dose was repeated every 24 hours for the desired number of days.

Blood smears from control and artemisinin or artesunate treated mice in the single dose, in the three-day suppressive test and in the five-day suppressive test, were taken on day five post infection and every day thereafter, until the infection peaked or it was clear that no parasites were going to appear. The parasitaemias and number of days for recrudescence of the different parasite lines were compared.

2.8 Artemisinin and artesunate selection experiments: general procedure

Two groups of five (4-6 week old) CD1 mice were inoculated with 10^7 parasites of the clone to be used for selection for increased artemisinin or artesunate resistance.

Three hours after inoculation one of the groups were treated orally with the required doses of artemisinin or artesunate for a total of five days. The remaining group was left untreated and served as a control. Both controls and parasites that survived drug treatment were passaged weekly from the mouse exhibiting the highest parasitaemia into uninfected mice and the treatment repeated. The drug doses were increased according to the parasite response to treatment in the previous passage. To address the possibility that potential increases in drug tolerance could be to due to increased virulence attributed to multiple sub-inoculations, an untreated and unselected parasite line was maintained in parallel and passaged in untreated mice the same number of times as the drug selected line. Following these passages in the

presence of increasing drug concentrations, drug selected parasites showing a significant increase in drug tolerance in comparison with unselected control lines were cloned by the method of limiting dilution described in section 2.5. Cloned parasites were re-tested for their responses to both artemisinin and artesunate.

2.9 Tests to evaluate the stability of drug-resistance

To assess whether artemisinin or artesunate resistance was a genetically stable feature, drug-resistant parasite clones were re-tested for their drug responses after each of three different procedures:

i) Freeze-thaw cycles in liquid nitrogen,

ii) After 12 blood sub-inoculations in mice in the absence of drug treatment and

iii) Transmission through *Anopheles stephensi* mosquitoes.

A measure of resistance in the drug-selected parasite clones was established in the following way. The minimum curative dose (MCD) of each drug was first assessed in drug-selected parasites and untreated control lines. MCD was defined as the minimum dose of each drug that would prevent re-appearance of parasites in all five mice within each treated group at any time during the first 10 days of the follow-up period.

A resistance index was determined using the following equation:

N-fold resistance = MCD drug selected parasites/ MCD drug unselected parasites

2.10. Transmission through *Anopheles stephensi*

For each clone a 30cm³ mosquito cage was set up containing ≈ 200 female *Anopheles stephensi* mosquitoes, 5-7 days old.

Mosquitoes were maintained on glucose and water solution, which was removed 24 hours prior to infective mouse feeds. Blood smears were taken from all infected mice on day 6 post infection, and the presence of gametocytes was confirmed. Mice were then anaesthetized with rohypnol solution (Appendix 1), and attached to cork boards placed on top of the mosquito cages. Mosquitoes were allowed to feed for 30 minutes before the mice were removed. Mice were then killed before awakening from the anaesthesia. Glucose and water solution was placed back into the cages, and egg bowls were provided 2 days post feed. 10 mosquitoes were dissected from each cage 7 days after the feeds, to check for the presence of oocysts.

Fourteen days after the infective feed, mosquitoes from each cage were dissected, and the salivary glands examined for the presence of sporozoites. If the presence of sporozoites was confirmed, the mosquitoes were allowed to feed on a group of two uninfected anesthetised mice. Starting on day 8 and onwards the mice were checked for the presence of parasites in blood.

2.11. DNA extraction

Parasite genomic DNA was extracted when required as follows: parasitized RBCs were harvested from mice under general anaesthesia, when trophozoite stages were most prevalent, into citrate saline (pH 7.2) and passed through a column of fibrous cellulose powder twice (CF11®, WhatmanTM) to remove mouse leukocytes [Homewood CA *et al.* 1976]. The resulting RBC pellet was washed twice in PBS and parasite DNA extracted by overnight incubation in lysis solution (10 mM Tris [pH 8.0], 50 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], proteinase K [1 mg/ml]) at 42ºC. After phenol extraction, DNA was precipitated using propan-2-ol and ammonium acetate (3M) and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH8.0). DNA samples were stored at –20ºC.

2.12. Identification of the *P. chabaudi tctp* and *atp6* genes

The DNA sequences of the *P. falciparum* and *P. yoelii tctp* and *atp6* genes were available online at the NCBI/NIH (National Institute of Health) database (www.ncbi.nih.gov) with the following accession numbers: *pctctp* NP_703454, *pytctp* AF124820, *pfatp6* AB121053 and *pyatp6* AABL01001880. To obtain the *P. chabaudi* orthologues of these genes, these sequences were retrieved and used in BLAST searches against the available *P. chabaudi* sequences (shot gun clones and genomic contigs), deposited at the *P. chabaudi* genome database (www.sanger.ac.uk). The two sequences giving significant hits were retrieved and used to design *P. chabaudi*-specific oligonucleotide primers to amplify overlapping DNA fragments spanning the coding region, introns and both 5'- and 3'-non-coding sequences. These were then used in PCR amplifications containing either *P. chabaudi* genomic DNA or cDNA templates.

2.13. Amplification and sequencing of the *mdr1, cg10, tctp* and *atp6* genes of *P. chabaudi*

Genomic DNA was used as template in 50µl PCR reactions, containing 0.2µM of each oligonucleotide primer, 1x PCR buffer (Promega™), 2.5 mM $MgCl_2$, 0.2mM dNTPs and 0.025U/µl of Taq DNA polymerase. For amplification of the *pcmdr1* and *pccg10* genes, oligonucleotide primers and PCR amplification conditions previously published were used [Cravo PV *et al.* 2003; Hunt P *et al* 2004], based on DNA sequences characterised prior to this study (*pcmdr1* AY123625 and *pccg10* AY304549). These were used in PCR amplifications of AS-15CQ, AS-30CQ, AS-ATN and AS-ART. Negative controls were also prepared, which contained 1µl of sterile distilled water in place of template DNA. Positive controls were prepared using a previously amplified DNA template.

The oligonucleotide primers used for *pcmdr1, pccg10, pctctp* and *pcatp6* are listed in Appendix 2.

All PCR reactions were carried out using a UNO-Thermoblock machine (Biometra).

PCR products were run on a 2% agarose gel in TBE solution and visualized under UV. Products were purified using the QIAquick® PCR Purification Kit from QIAGEN and sequenced using BigDye chain termination v3.1 (Applied Biosystems). The sequencing reactions were analysed by Macrogen®. The primers used in sequencing reactions were those used for the initial amplification of the fragments.

Gene and predicted amino-acid sequences were manually compiled, and then compared between drug selected and unselected clones using an internet-based interface denoted Multiple Sequence Alignment with hierarchical clustering [Corpet F *et al.* 1998], using default alignment parameters (http://prodes.toulouse.inra.fr/multalin/ multalin.html).

2.14. Estimation of copy numbers of the *pcmdr1, pctctp* and *pcatp6* genes

Gene copy number was assessed by RTQ-PCR. We used sequences within a fragment of the Merozoite Surface Protein of *P. chabaudi* gene (*msp1*) (accession no. L22982), [McKean PG *et al.* 1993] as an internal calibrator, since *msp1* is a single copy gene in *P. chabaudi*.

Real-Time Quantitative PCR (RTQ-PCR) was performed using a Roche LightCycler [Meshnick SR 2002].

Ten microliter reactions in LightCycler capillaries (Roche) using FastStart DNA Master SYBR Green I kit reagents (Roche) were used according to the manufacturer's instructions.

Magnesium chloride, primer concentration, denaturating, annealing and elongation rates and times were varied to determine the optimum conditions under which only the specific amplicon was produced. The average amplification efficiencies of the reactions were closely matched because small differences can result in large errors in quantification between samples. The average amplification efficiency, E, was determined from the slope of the standard curves produced in each experiment using the equation E=10-1/slope. PCR reactions where the amplicon doubles at every cycle have an optimal efficiency of 2.0 compared to reactions where no amplification occurs and efficiency is 1.0. All samples were analysed in replicate within each LightCycler run. The fluorescence signal produced from the amplicon was acquired at the end of the polymerisation step at 72ºC.

The gDNA samples used were the same as those used for gene amplification and sequencing. The purity and quantity of DNA was determined by fluorimetry, UV spectrophotometry and electrophoresis of serially diluted ethidium-bromide stained samples on agarose gels. Standards consisting of 10-fold serial dilutions of parasite genomic DNA in the range of 60-0.006 ng were used as quantification standards for the LightCycler calibration curve for each RTQ-PCR experiment.

Maximum recovery filter tips (AXYGEN® Scientific) and "No Stick" microtubes (Alpha Laboratories) were used in these experiments.

The analysis of relative gene expression data was performed using the $2^{-\Delta\Delta Ct}$ method described in detail by Livak and Schmittgen [Livak KJ *et al.* 2001].

Thus, the average Cycle threshold (Ct) was calculated for both control (*pcmsp1*) and target genes and the ΔCt ($Ct_{\text{target gene}} - Ct_{\text{msp-1}}$) was determined. ΔΔCt was calculated for the relative quantification of the target gene; $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{msp\text{-}1})_{\alpha} - (Ct_{\text{target gene}} - Ct_{msp\text{-}1})_{\beta}$, where α = resistant sample and β = sensitive sample. After validation of the method, results for each sample were expressed in N-fold changes in α target gene expression, normalised to msp-1 relative to the expression of β, according to the following equation: amount of target = $2^{-\Delta\Delta Ct}$.

The oligonucleotide primers used for *pcmsp1*, *pcmdr1*, *pctctp* and *pcatp6* are listed in Appendix 3 together with the PCR conditions used to amplify the fragments.

2.15 Production of cross progeny - Overview of procedure

To produce a genetic cross between two strains, the two parental clones are inoculated into mice to produce a mixed infection. Infections of both AS-ART/AS-ATN and AJ clones were induced in mice. Absolute numbers of parasites per volume of blood were worked out at peak

parasitemia for both infections by multiplying the parasitaemia recorded by thin blood smear against the numbers of red blood cells per volume of blood of each mouse (calculated by flow cytometry; Beckman Coulter). A volume of blood containing known parasite numbers was then taken from both AS-ART/AS-ATN and AJ infected mice by incision of the distal portion of the tail, and collected in glass capillaries. This blood was then mixed to produce a 50/50 mixture of the two clones by parasite number, and diluted to a concentration of 1×10^7 parasites per 0.1 ml with 50% ringers solution, 45% heat-inactivated calf serum and 5% 200 units/ml heparin solution. This solution was kept on ice, and administered intra-peritoneally to each experimental mouse in 0.1 ml aliquots.

The mixed infections were followed by microscopy for the presence of gametocytes. When gametocytes are present in the infection, mosquitoes are allowed to feed on the mice, and the sporozoites present in the salivary glands of infected mosquitoes 14 days later are inoculated intra-peritoneally into mice.

Previous work determined the optimum day post mouse inoculation to achieve infections in mosquitoes. The results of this work showed that feeding mosquitoes on mice at day 6 post-inoculation produced the greatest number of infected mosquitoes. All mosquito feeds in this experiment, therefore, were carried out at day 6 post mouse-infection.

2.15.1 Mosquito feeds

Six 30cm³ mosquito cages were set up containing ≈ 200 female *Anopheles stephensi* mosquitoes, 5-7 days old. Mosquitoes were maintained on glucose and water solution, which was removed 24 hours prior to infective mouse feeds. Blood smears were taken from all infected mice on day 6 post infection, and the presence of gametocytes was confirmed. Mice were then anaesthetized with rohypnol solution (Appendix 1), and attached to cork boards placed on top of the mosquito cages. Mosquitoes were allowed to feed for 30 minutes before the mice were removed. Mice were then killed before awakening from the anaesthesia. Glucose and water solution was placed back into the cages, and egg bowls were provided 2 days post feed. 7 days after the feeds 10 female mosquitoes from each cage were dissected, to check for the presence of oocysts.

2.15.2 Infection of mice from sporozoites

Fourteen days after the infective feed, mosquitoes from each cage were dissected, and the salivary glands examined for the presence of sporozoites. If the presence of sporozoites was confirmed, all the female mosquitoes from the cage were dissected, and the salivary glands removed. These were placed in a glass tube containing 50% Ringer's solution (Appendix 1), 45% heat-inactivated calf serum and 5% 200 units/ml heparin solution. The glands were then gently crushed using a pestle and mortar in order to release the sporozoites. This solution was kept on ice, and injected intra-peritoneally into mice in 0.1 ml aliquots. Thin blood smears were taken from infected mice daily, and parasitaemias recorded.

2.15.3 Preparation of artemisinin and artesunate

Artemisinin and artesunate solutions were prepared by dissolving artemisinin and artesunate powder in dimethylsulfoxide (DMSO), the exact same way has during the selection procedure. This was administered to mice orally in 0.1 ml volumes using a lubricated catheter. The dose of artemisinin used was 25 mg/kg for five days and the dose of artesunate used was 5 mg/kg for five days. At the time of drugging, mice were individually weighed so that the amount of drug given could be adjusted accurately to each mouse.

2.16 Linkage Group Selection

2.16.1 Selection of cross progeny

When the sporozoite-induced infections reached peak parasitaemia (10%–15%), the parasites were harvested, pooled, and inoculated into a group of mice (designated the "non-passaged" group containing 3 -5 mice depending on the experiment). Each mouse in the group received 1 x 10^7 parasites. This initial experimental group was left untreated, and the parasites were harvested at peak parasitaemia for AFLP analysis to provide a reference point for markers analyzed in the subsequent treatment groups.

For the AS-ART x AJ cross, parasites were pooled and sub-inoculated from the "non-passaged" group into two further groups of mice, one of which was treated with artemisinin ("ART treated" group), and the other left untreated ("untreated" group).

For the AS-ATN x AJ cross, parasites were pooled and sub-inoculated from the "non-passaged" group into two further groups of mice, one of which was treated with artesunate ("ATN treated" group), and the other left untreated ("untreated" group). These two groups provided the material for the comparison of markers between drug-treated and untreated parasite populations. Artemisinin was administered orally at a dose of 25 mg/kg of mouse body weight daily at 24-h intervals for 5 days, starting 3h after parasite challenge. Artesunate was administered orally at a dose of 5 mg/kg of mouse body weight daily at 24-h intervals for 5 days, starting 3h after parasite challenge. Both the artemisinin-treated and artesunate-treated and the untreated blood-stage cross progeny were allowed to grow to peak parasitaemia (30%–40% for untreated, and 20%–30% for treated), at which point the blood was harvested. Two samples of parasite DNA were prepared for AFLP and other molecular analyses by pooling separately the blood from the treated and untreated mice.

2.16.2 Amplified Fragment Length Polymorphism (AFLP) analysis

2.16.2.1 Preparation of parasite DNA from experimental groups

When infections reached peak parasitaemia, blood was extracted from all mice in each group by severance of the brachial artery, pooled, and prepared for DNA extraction. Blood was filtered in order to remove any mouse lymphocytes or other nucleated cells, by passing it twice through a 5 ml column of powdered cellulose (Sigma) washed with citrate saline. Blood was then filtered through Plasmodipur™ filters (Euro-Diagnostica) twice. The filtrate was centrifuged for 5 mins at 3000 rpm and the supernatant removed, leaving a pellet of packed cells. 0.15% saponin in Phosphate Buffered Saline (PBS) was added to promote cell lysis cells. After lysis of erythrocytes occurred (associated with a change in colour of the solution from bright red to burgundy colour), PBS was added in excess to prevent parasite lysis. This solution was then centrifuged again at 4000 rpm for 5 minutes and washed twice in PBS. Supernatant was discarded and pellets stored at -70°C. Three thick blood smears were taken, one prior to filtration, one after cellulose filtration and one after Plasmodipur™ filtration in order to determine the efficiency of host cell removal at each stage.

The frozen pellet was re-suspended in 0.4 ml buffer A (Appendix 1), and 10 µl of 10% SDS and 50 µg Proteinase K (Sigma) were added. The pellet was left at 37°C overnight after which an equal volume of 1:1 phenol/chloroform mixture was added, mixed for 3 min, and centrifuged at 5,000 g for 1-2 minutes. The upper aqueous layer was transferred to a fresh

tube. The step was repeated 2-3 times. Then an equal volume of chloroform was added, and the tube was centrifuged as before for 1-2 minutes. The upper aqueous layer was removed to a fresh tube. This procedure was repeated once before an equal volume of ether was added, the solution centrifuged as before for 1-2 minutes and the upper layer removed. The remaining ether was left to dry in the air. Three volumes of absolute ethanol (0º C) and 1/10th volume of 3M sodium acetate (pH 5.2) were added to the dried pellet, and the tube was mixed and placed on ice for 15-45 minutes to precipitate the DNA. The tube was then centrifuged at 10,000 g in a Speed Vac (Savant) for 10 minutes and the ethanol mixture removed. The tube was again centrifuged at 10,000 g for 5-10 minutes to remove final traces of ethanol and the pellet was resuspended in 100-200 µl of TE buffer (pH 8.0) (see Appendix 1) and left at 37°C for 10 minutes before storage at -20º C.

2.16.3 AFLP analysis

0.5µg of parasite genomic DNA was cut using two enzymes. Firstly, 10 U of EcoRI (MBI Fermentas, recognition sequence: G↓AATTC) were added and DNA incubated at 37°C for 1 h, then 5 U of TruI/MseI (MBI Fermentas, recognition sequence: T↓TAA) were added and DNA incubated for further 3 h at 65°C.

The digestion stage was performed in a 40 µl solution containing 2X Y+/ Tango buffer (Promega). The fragments were then ligated with adapters matching the cut ends produced by the enzymes.

All primers used were provided by MWG-Biotech UK Ltd. Adapters disrupted the cutting site recognised by the enzymes in order to prevent cutting of the adapters from the DNA fragment.

These adapters also provided a recognition site for primers.

-MseI adapters: MeI.a1 and MeI.a2

-MeI.a1: 5'-GACGATGAGTCCTGA**G**-3'

-MeI.a2: 3'-TACTCAGGACT**C**AT-5'

-EcoRI adapters: EoI.a1 and EoI.a2

-EoI.a1: 5'-CTCGTAGACTGCGTAC**C**-3'

-EoI.a2: 3'-CATCTGACGCATG**G**TTAA-5'

Bold letters indicate base substitution to disrupt enzyme cutting site.
Adapters were prepared by adding equimolar amounts of both strands and then performing the following procedure:

- Heat adapters at 94°C for 5 mins, then cool to 21°C for 5 mins
- Heat adapters at 72°C for 60 s., then cool to 21°C for 5 mins
- Heat adapters at 65°C for 60 s., then cool to 21°C for 5 mins.

Adapters were then diluted to 50 pmol/µl. 10 µl of ligation mixture were then added to 40 µl of digested DNA and this incubated at 37°C for 3h, then overnight at 15°C. Ligated material was diluted 1:10 in TE buffer (pH 8.0) (Appendix 1) and stored at -20°C.
A preliminary PCR amplification was performed involving the use of "non-selective" primers (meaning primers with no extra nucleotides added at their 3' ends extending beyond the adapters sequence) matching the adapters:

- Non-selective EcoRI primer: 5'-GACTGCGTACCAATTC-3'
- Non-selective MseI primer: 5'-GATGAGTCCTGAGTAA-3'

A 20µl PCR solution containing 0.32 µM of each of the non-selective primers, 1 µl template DNA, 0.4 U Taq polymerase (Promega), 1X Mg-free PCR Buffer (Promega), 1.5 mM MgCl2, and 0.2mM of all 4 dNTPs was set up. The following cycles were performed:

- 94°C for 60 s., then
- 94°C for 30 s.
- 56°C for 60 s.
- 65°C for 60 s.

repeat the three steps for 20 cycles

The resulting PCR material was diluted 50-fold in TE buffer (pH 8.0) and stored at - 20°C.
Selective amplification was performed using radiolabelled primer (EcoRI-primer) and selective primers (meaning primers with a selective extension at the 3'-end, in order to reduce the number of fragments amplified). For radiolabeling, 2.5x Kinase buffer (Promega), 20U T4 polynucleotide Kinase (Promega), 100 µCi [γ-^{33}P] ATP / [γ-^{32}P] ATP (ICN) and 500 ng of oligonucleotide primer were incubated in a 20 µl solution at 37°C for 60 min. The reaction

was stopped by adding 1 µl of 0.1 M EDTA pH 8.0 and heating at 70°C for 10 min. The mixture was then made up to a volume of 50 µl by adding sterile, distilled water.

Primer purification was performed using TE Micro Select-D, G-25 microcentrifuge spin columns produced by Eppendorf-5 Prime Inc.

Hot PCR with the radiolabelled primer was performed as follows: 0.32 µM of the selective MseI primer, 0.05 µM of the labelled selective EcoRI primer, 1 µl template DNA, 0.4 U Taq polymerase (Promega), 1X Mg-free PCR Buffer (Promega), 2.5 mM $MgCl_2$, and 0.2mM of all 4 dNTP's were added to a 20µl total volume PCR solution.

PCR conditions for this stage were:

- 94°C for 60 s., then
- 94°C for 30 s.
- 65°C for 60 s., annealing temperature is reduced at each cycle by 0.7°C for the next 12 cycles, then remained at 56°C for the remaining 23 cycles
- 65°C for 60 s.

repeat the preceding three steps for 35 cycles

The PCR products were mixed with 20 µl of loading dye specific for poly-acrylamide sequencing gels (Anachem), then heated at 99°C for 3 min and immediately cooled on ice. 5µl of each sample was loaded onto a 5% denaturing polyacrylamide gel (5% acrylamide, 0.25% methylene bisacryl, 7.5 M urea in 50mM Tris/50mM Boric acid/1mM EDTA). 500 µl of a 10% Ammonium Persulfate solution (APS) and 100µl of TEMED (Sigma) were added to 100 ml of gel solution and the gel cast using a SequiGen 38x50 cm gel apparatus (BioRad). Electrophoresis was performed at 110 W for 2 h in 1x TBE buffer. Gels were then dried in a vacuum gel drier (model 583, Bio Rad) and exposed overnight in phosphorimager screens (Fuji) at -70°C. Results were visualised on an autoradiography film (Kodak XAR-5). They were developed in an automatic autoradiographer developer (Exograph).

As an example of an AFLP gel and the band nomenclature see Figure 12.

Thus, and just as an example, the marker AJAG02CA denotes the second largest (that is where the 02 cames from) AJ-specific band (thus beginning with AJ) obtained using *Eco*RI primers with AG (the first pair of nucleotide letters, like the forward primer) as additional "selective" 3'-nucleotides, and CA as "selective" nucleotides on the *Mse*I primers (the second pair of nucleotide letters, like the reverse primer).

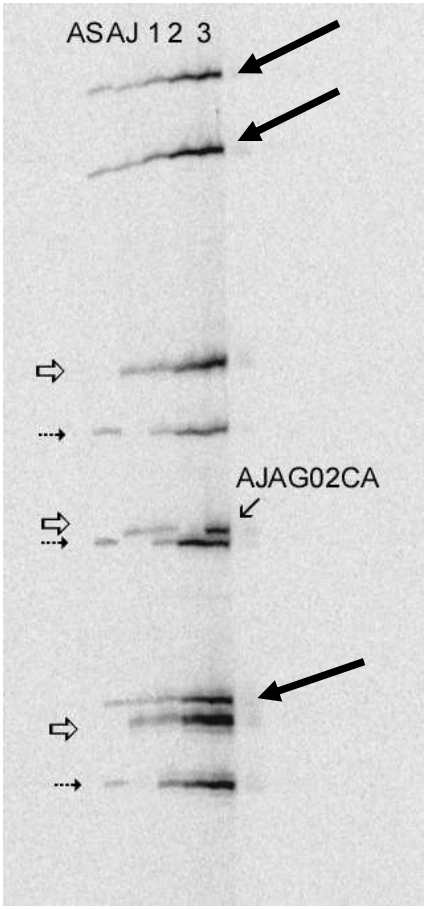


Figure 12 - An example of an AFLP gel.

In this particular gel we can see the results of bands generated with *Mse1* (CA selective bases, so called forward primer) + *EcoR1* (AG selective bases, so called reverse primer). One of the markers of the sensitive parent (AJAG02CA) was absent in the treated population and is then presumably closely linked to the locus conferring resistance.

Each one of the lanes is labelled as followed;

AS: denotes DNA extracted from single AS-SENS clone infection (in our project the AS alleles are the resistant ones).

AJ: denotes DNA extracted from single AJ clone infection (in our project the AJ alleles are the sensitive ones).

1: denotes DNA extracted from the unpassaged pooled group.

2: denotes DNA extracted from the treated pooled group (in the case of our project the AS-ART x AJ cross was treated with ART and the AS-ATN x AJ cross was treated with ATN).

3: denotes DNA extracted from the untreated pooled group.

Each lane represents a DNA sample obtained from the pooled DNA of all mice on each experimental group.

Polymorphic markers are bands that are different for AS or AJ. AS specific markers (thus resistant) are marked with a broken arrow (⇢), and AJ specific markers (thus sensitive) with an open arrow (⇨), bands marks with an arrow (→) represent a non-polymorphic marker (there are no differences between AS and AJ within this marker). Adapted from Culleton R *et al*. 2005 with kind permission of Dr Richard Culleton.

2.16.4 Measurement and comparison of the intensity of AFLP markers

This technique makes uses of a large number of molecular genetic markers distinguishing two strains of malaria parasites, the AFLP markers. These markers can be visualised as bands on a polyacrilamide gel. Most of the bands that will appear on a gel will be present in both the parasites and are called non-polymorphic bands and some are unique of one of the clones in our case either AS - resistant or AJ - sensitive. Once the selection pressure is applied in the genetic progeny of a cross, in the case of this project, artemisinin or artesunate pressure, the parasites that carry the allele that is sensitive (in this case the AJ allele) will be removed, and from an experimental point of view this disappearance is visible by the decrease in intensity or complete disappearance of an AFLP band specific for the sensitive allele (AJ band). Thus the progeny after selection is then screened for markers. Specially those from the sensitive parent that would either be significantly reduced or absent because these markers should be linked to the locus or loci under selection, in our case under artemisinin or artesunate selection. Polymorphic markers (between AS-ART and AJ and between AS-ATN and AJ, here simplified to AS and AJ), meaning those that are different between AS and AJ, were named and placed in the AFLP map, to denote specificity of the polymorphic band meaning the size of the band (relative to other polymorphic bands in the same gel lane) and the selective bases used, this would make it very straightforward the identification of a particular band to the AFLP map already developed by Martinelli A and collegues [Martinelli A *et al.* 2005]. As stated before we were looking for the disappearing of AJ sensitive markers on the treated group (meaning artemisinin or artesunate treated group) in comparison of the untreated group, each one of these markers decreased in intensity or disappeared is predicted to lay in what we call a selection valley that can then be associated to a particular gene underling the resistant phenotype or at least responsible for given so type of selective advantage over the sensitive parasites. An example of an AFLP gel is represented in Materials and Methods Figure 12.

As described in the materials and methods chapter, each marker band intensity was measured with PhosphorImager and IMAGEQUANT software (Molecular Dynamics). For each marker of interest, either AS or AJ, an intensity index (II) was calculated by taking the intensity of a polymorphic marker (either AS or AJ) and compare it to a no polymorphic marker, making sure that this is done using the same PCR material applied in the same polyacrilamide gel. Each II is then converted to a relative intensity index (RII) either unselected RII_u or selected RII_s. The RII_u is calculated by making a ration between the II obtained for the unselected

material and the II obtained for the parental (either AS if we are looking at an AS specific markers or AJ is we are looking for an AJ marker). The RII_s is calculated by making the ratio between the II obtained for the selected material and the II obtained for the parental (either AS or AJ) [Martinelli A *et al.* 2004].

To compare between different markers a comparative Intensity (CI) of the polymorphic markers was calculated. This comparative intensities are defined as the RII of an AFLP marker in the cross progeny selected in "treated" mice (RII_t), divided by the RII of the marker of the cross progeny grown in a parallel "untreated" group of mice (RII_{ut}), and expressed as a percentage:

$$\mathbf{CI = (RII_t/RII_{ut}) \times 100.}$$

Keeping in mind that the objective is to identify the genomic loci under drug selection either by artemisinin or artesunate that would correspond to a selection valley associated to the resistant phenotype, AFLP markers with low CIs were identified and their position according to the previously define genetic linkage map [Martinelli A *et al*. 2005] were noted.

2.16.5 Assignment of AFLP markers to locations in a *P. chabaudi* genetic linkage map.

Markers had previously been ordered on a genetic linkage map of *P. chabaudi* [Martinelli A *et al.* 2004, Martinelli A *et al.*2005] obtained from previously generated crosses between AS-derived clones and AJ strains of *P. chabaudi* [Carlton JM *et al.* 1998; Rosario VE 1976; Walliker D *et al.* 1975].

A total of 674 AFLP markers were typed for each uncloned progeny analysed in this work, and were subsequently assigned to linkage groups using the previously generated map [Martinelli A *et al.* 2005].

A total of 674 AFLP markers were typed for each of 28 cross-progeny clones, and were subsequently assigned to linkage groups using the Map Manager QTX software [Manly KF *et al.* 2001]. A total of 44 RFLP markers characterized in a previous study [Carlton JM *et al.* 1998] were used as genetic anchors to allow the assignment of the various linkage groups to chromosomes. In total, 11 chromosomes could be identified, while 12 linkage groups of as yet unknown assignment remain, which include the three remaining chromosomes (chromosomes 2, 4, and 14).

2.16.6 Sequencing of AFLP markers, and their location on the *Plasmodium falciparum* genome

AFLP bands that appeared to be under selection were excised from acrylamide gels using a sterile scalpel. The gel fragments were then soaked in an Eppendorf tube in 50 µl of autoclaved, distilled water (sdH_2O) overnight.

Gel slices were centrifuged at 12000g prior to removal of the liquid phase containing the DNA. DNA was precipitated using 1/10 volume of 3M sodium acetate (pH 5.2) and 3 volumes of ice-cold absolute ethanol. The solution was placed at -20°C for at least 1 h. Thereafter the tubes were spun for 30 minutes at high speed. The liquid phase was removed and the DNA pellet washed twice in 70% ethanol, before air drying. The pellet was then dissolved in 50 µl TE buffer (pH 8.0).

The extracted DNA fragment was amplified by PCR, using the same selective AFLP primers and PCR conditions that produced it.

Sequencing reactions were carried out using the protocol described, below in section 2.16.7.

The sequences of markers were then physically mapped in the *P. falciparum* genome (sequence data for *P. falciparum* were obtained from the Sanger Centre website, which can be accessed at www.sanger.ac.uk_Projects_P_falciparum), using BLAST searches.

2.16.7 AFLP band sequencing and purification of PCR products

Sequencing PCR reactions were set up using the ABI PRISM Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit, (PE Applied Biosystems). PCR reactions were prepared to a final volume of 10µl, containing the following reagents: 4µl template DNA (100-250ng), 4µl Terminator Ready Reaction Mix, 1µl primer (3.2pmol), 1µl sterile distilled water

Sequencing PCR conditions were as follows;

Denaturing	95°C for 30 seconds	
Annealing	50°C for 20 seconds	x 25 cycles
Extension	60°C for 4 minutes	

Following the sequencing PCR, the products were purified by precipitation with sodium acetate and 95% ethanol, and washed in 70% ethanol.

For sequencing of purified PCR products, internal primers covering the extreme 5' and 3' ends of the fragments were used. All fragments were sequenced in opposite directions.

Sequencing results were analysed using the SeqED V 1.0.3 software (Applied Biosystems Inc., 1992). The programme allows the visualisation of chromatograms of the sequenced DNA.

So as a summary: traditional linkage analysis of individual cross progeny clones, is labour-intensive and expensive, as it involves the genotypic and phenotypic characterization of individual clones from a cloned progeny of a genetic cross. Without the generation of an extremely large number of recombinant clones it has a poor resolution, which makes the actual identification of the underlying genes extremely difficult unless strong candidates are already suspected. There is an inverse relationship between the size of the locus within which possible target genes may be located and the number of recombinant clones that must be generated [Wellems TE *et al.* 1991].

LGS, used in this project, has enabled the discovery of genes controlling biological properties in malaria parasites to be greatly accelerated [Culleton R *et al.* 2005; Martinelli A *et al.* 2005]. LGS differs from the traditional approach to genetic analysis of malaria parasites by not requiring individually characterisation of very large numbers of clones from the progeny of the cross [Carter R *et al.* 2007]. LGS characterises the uncloned progeny of a genetic cross (between a resistant and sensitive parasite to the particular parasite characteristic in study) by measuring the proportion of parental polymorphic markers at genome-wide loci (AFLP), before and after drug treatment. Markers from the sensitive parent that are linked to the gene underlying the resistance phenotype will be under-represented or eliminated after drug treatment, forming a "selection valley". In the case of this project, the AJ markers correspond to the markers from the sensitive parent; those markers will be under represented or eliminated after artemisinin and artesunate treatment.

As Carter R and colleagues so clearly summarise [Carter R *et al.* 2007], LGS analysis depends upon several distinct experimental components.

A) The crossing of two genetically distinct lines of malaria parasite by preparing a mixed infection of gametocyte-producing blood-stage parasites in a mouse, allowing mosquitoes to feed upon the mixture, with consequent parasite development and invasion of sporozoites to salivary glands;

B) The subjection of the progeny of the cross to a selection pressure (in the case of this research, ART or ATN pressure). This drug selection is applied to the blood stage parasites in mice infected with the cross progeny sporozoites.

C) The screening of the uncloned, selected cross progeny with quantitative markers, such as quantitative real-time PCR, quantitative AFLP and proportional sequencing. So before the LGS technique could be applied the development of quantitative markers covering as much the parasite genome as possible is necessary. Prior to this project quantitative AFLP were optimized and developed for being use with AS and AJ parasite lines from *P. chabaudi* [Culleton R *et al.* 2005; Martinelli A *et al.* 2005].

The location of the genetic markers in a *Plasmodium* genome database with the expectation that markers linked to genes controlling the target of drug selection will form a selection valley containing the locus of the selected genes. A genetic linkage map, in the case of this project already previous developed by Martinelli A and co-workers [Martinelli A *et al.* 2005], a genome sequence database, in the case of our model *P. chabaudi* sequence though not fully complete with a big coverage and a complete syntenic map developed prior to this project [Kooij TW *et al.* 2006] allowed us to map genes and markers genetically and physically, thus allowing identification of loci under selection. Then mutations in suspected candidate genes within the locus can be identified by comparative sequencing of genes from both the resistant mutant and its sensitive progenitor [Carter R *et al.* 2007].

2.17 Experiments with genetic crosses of AS-ART and AJ or AS-ATN and AJ

To analyse the efficacy and composition of each of the genetic crosses and the progression of the drug selection proportional sequencing was used. Proportional sequencing was used with DNA samples from sporozoites and mice blood samples before and after drug selection.

2.17.1 Blood collection for Proportional sequencing analyses

5 µl of blood were removed from each infected mice daily using a glass capillary. The blood was placed in an Eppendorf tube which contains 2 drops of citrate saline solution. The samples were then spun in a microcentrifuge at 10,000 rpm for 2 minutes, and the supernatent removed. The resulting pellet was frozen at -70°C.

2.17.2 Extraction of parasite DNA from blood or sporozoites

Frozen blood pellets and frozen sporozoites crushed were thawed at room temperature, and the DNA extracted using the InstaGene™ Matrix (Bio-Rad), following the manufacturers handbook protocol.

2.17.3 Determination of the proportions of clones in genetic crosses by proportional sequencing

2.17.3.1 Principle of proportional sequencing

The blood and sporozoite samples taken from each mouse or from the mosquitoes salivary glands were analysed using Proportional Sequencing. This technique allows the determination of the percentages of each parasite in a mixture on a given day of the infection. By applying this technique it was possible to determine the proportions of two parasites in mixed infections over the course of an infection.

To directly quantify the proportions of the sensitive (AJ) and resistant (AS-ART and AS-ATN) parasites present in the selected and unselected mixed infections, DNA was amplified by nested PCR at the *dhps* locus using primers common to non-polymorphic sequences from both parental alleles. The resulting PCR products were purified and sequenced. Sequencing results were analyzed using the SeqED v 1.0.3 software (Applied Biosystems, Inc., 1992), which allows the visualization of chromatograms of sequenced DNA and the quantitation of individual fluorescent peaks.

The relative heights of peaks at the polymorphic sites in these genes can be used as an index of the relative proportions of the AJ and AS-ART and AS-ATN parasites in each sample. With five replicate samples, and with reference to a calibrated series of mixtures between AS and AJ [Cheesman SJ *et al.* 2003], it is possible to estimate the percentage of parasites carrying the AS or AJ alleles of each gene with a standard error of <3% [Hunt P *et al* 2005].

A detailed description of the Proportional Sequencing technique is available elsewhere [Hunt P *et al.* 2005]. Briefly, the technique exploits the fact that during a PCR reaction, DNA is amplified in proportion to the initial template. If DNA containing a mixture of both AS and AJ type alleles is amplified in the same reaction, then the proportions of these clones will remain constant throughout the PCR. This means that the amplified PCR product will contain the same proportions of AS-ART or AS-ATN and AJ DNA as the template DNA that was

amplified. When this DNA is sequenced, it is possible to determine the proportions of the clones in a population by measuring the proportions of a single base that differs between them.

For example it is possible to distinguish AJ and AS-ART or AS-ATN parasites by 4 single nucleotide polymorphisms on the *pcdps* gene. As an example the gene and the polymorphisms will be presented on Figure 13.

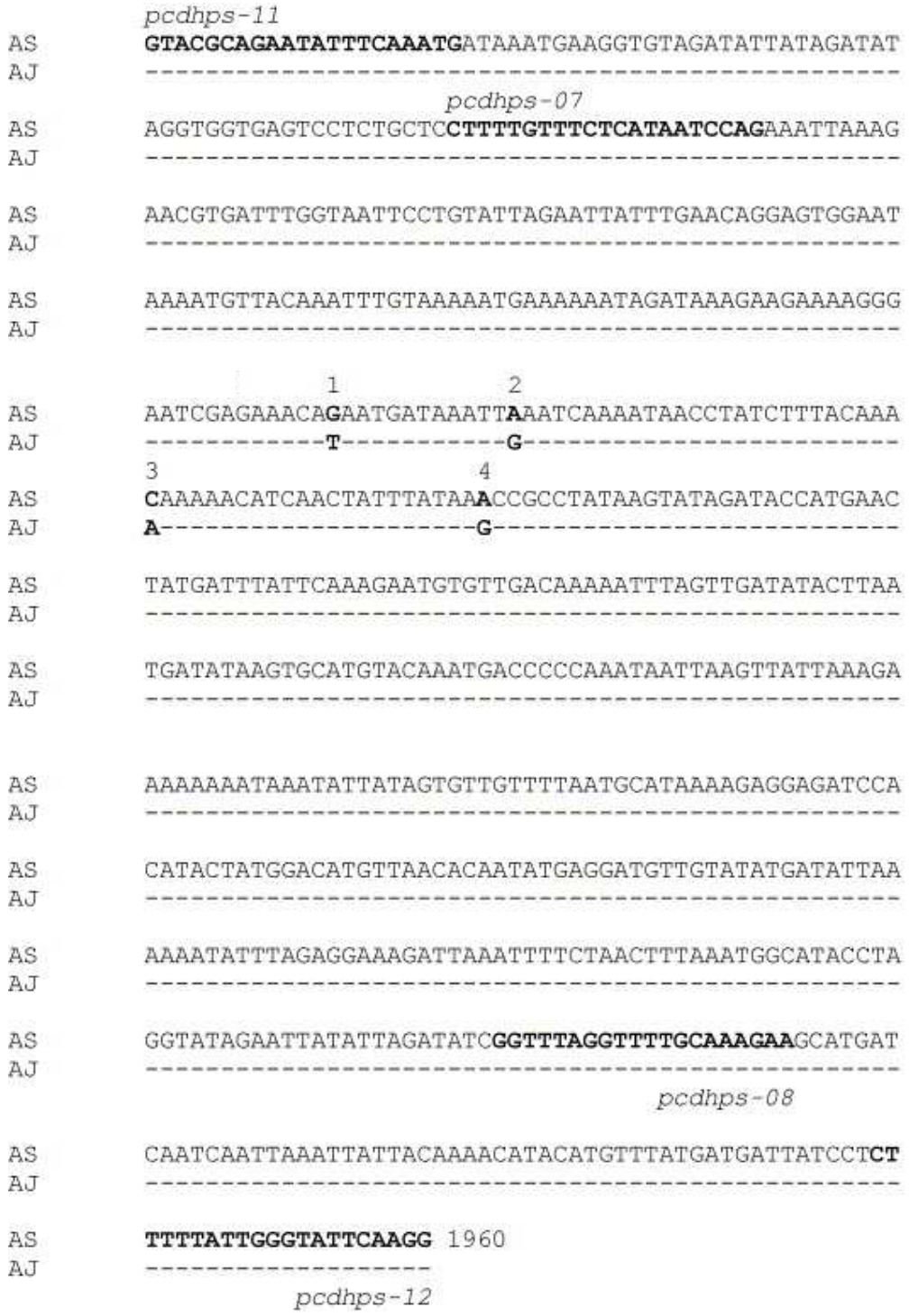


Figure 13- The four polymorphisms on the *pcdhps* gene that allowed differentiating between strains AS and AJ. Numbers 1-4 correspond to the polymorphisms. Primers are presented in bold.
From Hunt P *et al.* 2005, with kind permission of Dr. Paul Hunt.

When both these parasites are present in an infection, both bases will be present at positions indicated in Figure 13 with numbers 1-4 in the sequence. By measuring the proportions of

each base (by comparing the size of the peaks on a sequence chromatograph) it is possible to determine the proportions of each parasite in the mixture.

2.17.3.2 Proportional sequencing - PCR reagents and reaction conditions

Extracted parasite DNA was subjected to a nested PCR, amplifying a portion of the *dhps* gene that contains single nucleotide polymorphisms between AS-ART and AS-ATN and AJ. The oligonucleotide primers used were as follows:

***pcdhps*:**

Outer PCR

pcdhps –11 : **GTACGCAGAATATTTCAAATG**

pcdhps –12 : **CTTTTTATTGGGTATTCAAGG**

Inner PCR

pcdhps –07 : **CTTTTGTTTCTCATAATCCAG**

pcdhps –08 : **GGTTTAGGTTTTGCAAAGAA**

Individual PCR reactions were carried out in 50µl volumes containing 10pM of both oligonucleotides, 1 X PCR buffer, 1 X dNTP solution, 1 unit *Taq* DNA polymerase (Boehringer Mannheim), and 20µl of extracted DNA solution from for the outer PCR and 1µl of the resulting PCR product for the inner PCR. Negative controls were also prepared, which contained 1µl of sterile distilled water in place of template DNA. Positive controls were prepared using a previously amplified DNA template.

PCR reactions were carried out using an UNO-Thermoblock machine (Biometra), under the following standard conditions;

Denaturing	95°C for 1 minute	x 30 cycles
Annealing	52°C for 1 minute	
Extension	65°C for 1 minute	

PCR conditions were the same for both rounds of the nested reaction.
PCR products were run and visualized on a 1.5% agarose gel in TBE solution. The resulting PCR products were then purified using the QIAGEN QIAquick^{TM} PCR purification kit, following the manufacturer's instructions.

2.17.3.3 Proportional sequencing – Sequencing and purification of PCR products

Sequencing PCR reactions were set up using the ABI PRISM Big Dye^{TM} Terminator Cycle Sequencing Ready Reaction Kit, (PE Applied Biosystems). PCR reactions were prepared to a final volume of 10µl, containing the following reagents: 4µl template DNA (100-250ng), 4µl Terminator Ready Reaction Mix, 1µl primer (3.2pmol), 1µl sterile distilled water
Sequencing PCR conditions were as follows;

Denaturing	95°C for 30 seconds	x 25 cycles
Annealing	50°C for 20 seconds	
Extension	60°C for 4 minutes	

Following the sequencing PCR, the products were purified by precipitation with sodium acetate and 95% ethanol, and washed in 70% ethanol.
For sequencing of purified PCR products, internal primers covering the extreme 5' and 3' ends of the fragments were used. All fragments were sequenced in opposite directions.

2.17.3.4 Proportional sequencing – Analyses of sequencing results

Sequencing results were analysed using the SeqED V 1.0.3 software (Applied Biosystems Inc., 1992). The programme allows the visualisation of chromatograms of the sequenced DNA. The relative proportions of the two strains in each sample were determined by measuring the two peaks associated with the single nucleotide polymorphism that confers pyrimethamine resistance. It was possible, therefore, to calculate the percentages of each allele of a gene in each sample. For an example of a gel for proportional sequencing for the gene *pcdhps* see Figure 14.

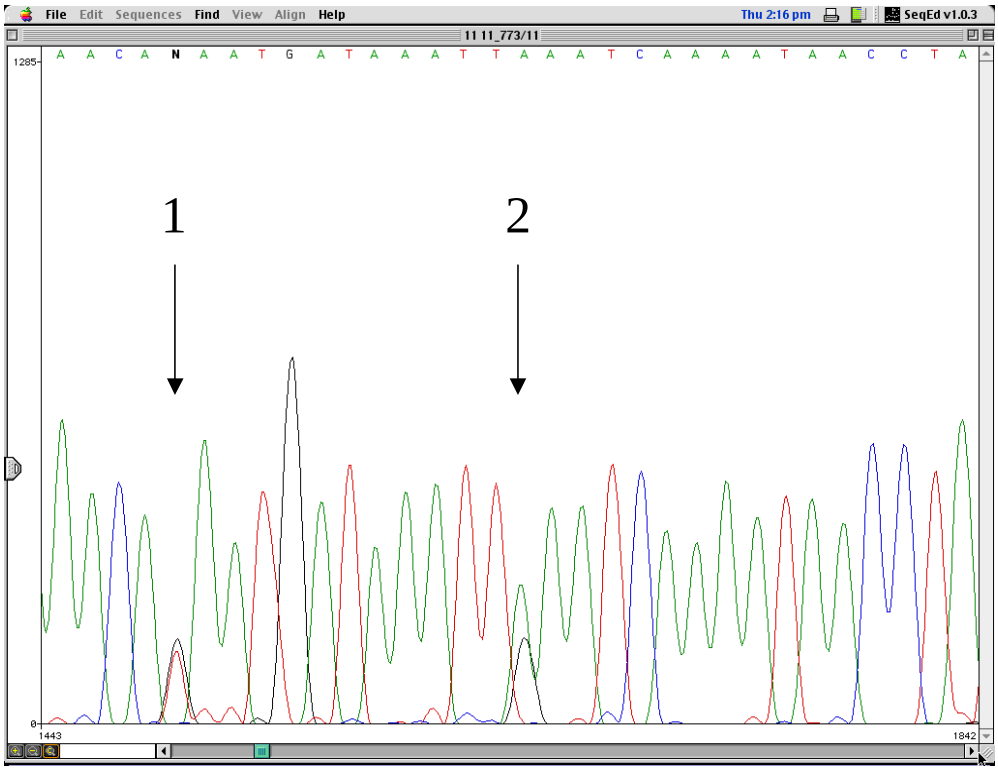


Figure 14 – Example of an electropherogram for proportional sequence analysis of two polymorphisms on the *pcdhps* gene. Numbers 1 and 2 represent a polymorphism number; both numbers correspond to the polymorphism number on Figure 13.

From: Hunt P *et al.* 2005 with kind permission of Dr. Paul Hunt.

2.18. Amplification and sequencing of the *ubp-1* gene of *P. chabaudi*

The amplification and sequencing of *ubp-1* gene has done using the same protocol as the one described for *mdr1, cg10, tctp* and *atp6* genes, and this protocol is already described in 2.13.

Genomic DNA was used as template in 50µl PCR reactions, containing 0.2µM of each oligonucleotide primer, 1x PCR buffer (Promega™), 2.5 mM $MgCl_2$, 0.2mM dNTPs and 0.025U/µl of Taq DNA polymerase. These were used in PCR amplifications of AS-15CQ, AS-30CQ, AS-ATN and AS-ART. Negative controls were also prepared, which contained 1µl of sterile distilled water in place of template DNA. Positive controls were prepared using a previously amplified DNA template.

The oligonucleotide primers sequence is presented in Appendix 4.

All PCR reactions were carried out using a UNO-Thermoblock machine (Biometra).

PCR products were run on a 2% agarose gel in TBE solution and visualized under UV. Products were purified using the QIAquick® PCR Purification Kit from QIAGEN and sequenced using BigDye chain termination v3.1 (Applied Biosystems). The sequencing reactions were analysed by Macrogen®. The primers used in sequencing reactions were those used for the initial amplification of the fragments.

Gene and predicted amino-acid sequences were manually compiled, and then compared between drug selected and unselected clones using an internet-based interface denoted Multiple Sequence Alignment with hierarchical clustering [Corpet F *et al.* 1998], using default alignment parameters (http://prodes.toulouse.inra.fr/multalin/ multalin.html).

RESULTS

The results of the present work are divided into three chapters:

- **Chapter III** describes the results of experiments designed to select artemisinin and artesunate resistant parasites. These experiments also involved evaluation of certain resistance features such as stability.

- **Chapter IV** describes the results of experiments designed to analyse the involvement of previously described putative genetic modulator for artemisinin and artesunate resistance, the *P. chabaudi* genes *mdr1*, *cg10*, *tctp* and *atp6.*

- **Chapter V** is concerned with the results of experiments on genetic crosses between an artemisinin resistant (AS-ART) and a sensitive (AJ) cloned strain of *P. chabaudi* and an artesunate resistant (AS-ATN) and a sensitive (AJ) cloned strain of *P. chabaudi*. Presented here are also the results of LGS experiments conducted with the uncloned recombinant progenies of the genetic crosses.

RESULTS

CHAPTER III

EXPERIMENTS FOR DRUG SELECTION OF ARTEMISININ AND ARTESUNATE RESISTANCE

This chapter presents the results of experiments to select artemisinin and artesunate resistance in *P. chabaudi*, of stable phenotype, of cloning the parasite lines obtained and in the characterization of the resistance obtained, including its stability.

3.1. Introduction

The objective of this part of the project was to select artemisinin and artesunate mutants in the rodent malaria *P. chabaudi,* through prolonged exposure of drug-sensitive lines to low and increasing levels of the drug, administered to mice.

Table 7 depicts the maximum doses tolerated by the progenitor parasite lines as established in preliminary work.

Table 7 – Maximum doses tolerated by the progenitor parasite lines used to select for resistance.

P. chabaudi	**Maximum dose for artemisinin (mg/kg/day)**	**Maximum dose for artesunate (mg/kg/day)**
AS-15CQ	1.2	2
AS-30CQ	4	1.6

3.2 Artemisinin drug selection

P. chabaudi clone AS-30CQ was exposed to gradually increasing concentrations of artemisinin, during several consecutive passages in mice, starting with a drug dose of 4 mg/kg/day, previously determined to be subcurative for these parasites (data not shown). See Table 7.

The drug selection procedure is described in more detail in the Materials and Methods Chapter (see Point 2.8) and summarized in Figure 15.

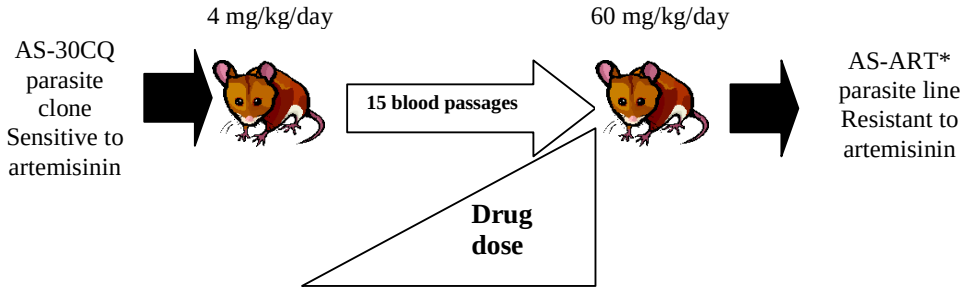


Figure 15 – A schematic representation of the artemisinin selection procedure. AS-ART* is uncloned.

Figure 16 represents the stepwise increase in drug dose during the artemisinin selection procedure.

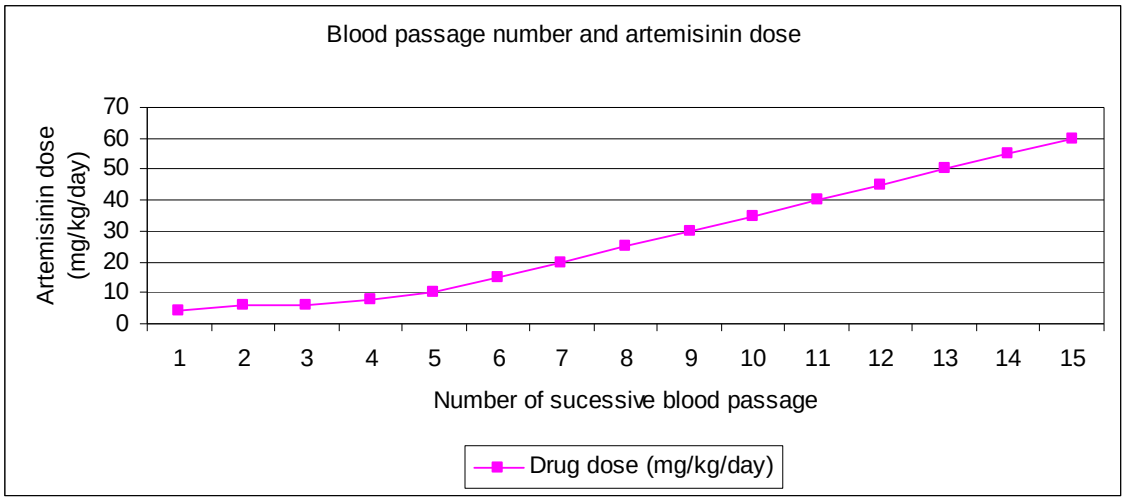


Figure 16 – The increase of the artemisinin dose (mg/kg/day), tolerated by AS-30CQ clone, during 15 blood passages.

Figure 16 shows the stepwise increment on the artemisinin dose during selection, starting by a 4 mg/kg/day dose of artemisinin, this dose being the maximum dose tolerated by the initial parasites, AS-30CQ.

The drug resistance selection process lasted for approximately 27 weeks (approximately 7 months); meaning that during this period the *P. chabaudi* parasite clone AS-30CQ was kept under increasing drug pressure until a concentration of 60 mg/kg/day, at the end of which resistance seemed to have been selected. Due to time constrains the selection process was finished at this stage where stability of resistance was assayed.

In the first four passages under drug pressure, the total increment on the artemisinin dose was of only 6 mg (from first passage to passage number 10) only from passage number five onwards we were able to increase 5mg of ART on each passage.

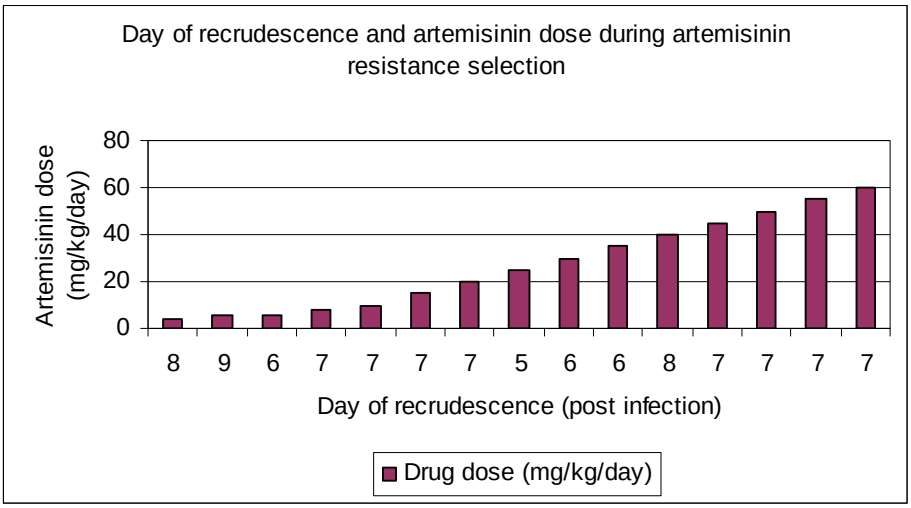


Figure 17 – The day of recrudescence on each passage under selection and artemisinin dose (mg/kg/day).

Figure 17 shows the stepwise increment on the artemisinin dose during selection, and time of recrudescence.

The day of recrudescence was considered during the selection procedure as an indication on the parasite sensitivity to artemisinin. However, after the five passages, it was noticed that the time of parasite presence under pressure remained roughly the same; though drug pressure was increased a few fold.

At the end of the selection procedure, assays were carried out to confirm that a parasite resistant line to artemisinin was obtained. This was established by the determination of the minimum curative dose (MCD) of each drug from the selected and original sensitive parasite populations.

MCD was defined as the minimum dose of each drug that would prevent recrudescence within each treated group at any time during the first 10 days of the follow-up period.

A resistance index was determined using the following equation:

N-fold resistance = MCD drug selected parasites/ MCD drug unselected parasites

Table 8 – N-fold resistance, using the equation above and calculated for each passage under artemisinin pressure. The MCD drug pressure for the 1st passage is 4 mg/kg/day, which was the initial drug dose tolerated by the AS-30CQ clone used for drug selection.

Number of blood passage under artemisinin pressure	MCD drug selected parasites (mg/kg/day)	N-fold resistance (MCD drug selected parasites/ MCD drug unselected parasite)
1	4	1
2	6	1.5
3	6	1.5
4	8	2
5	10	2.5
6	15	3
7	20	3.5
8	25	3.8
9	30	4
10	35	4.5
11	40	5
12	45	7.5
13	50	10
14	55	12.5
15	60	15

At the 15th passage, when due to time constrain limitations the selection procedure was stopped, the parasite line obtained, denoted AS-ART*, (* uncloned parasite population). AS-ART* has an N-fold resistance of 15 when compared to the initial parasite clone and was cloned by the method of limiting dilution [Rosario V, 1976]. See Section 3.4.

Cloned parasites were re-tested for their responses to artemisinin.

3.3 Artesunate drug selection

To select for artesunate resistance, *P. chabaudi* clone AS-15CQ, was exposed to gradually increasing concentrations of artesunate during several consecutive passages in mice, starting with a sub curative drug dose of 2 mg/kg/day. See Table 7.

The procedure was the same as for artemisinin drug selection and is described in more details in materials and methods (See Section 2.8) and previous on Section 3.2 and a schematic representation of the protocol can be seen on Figure 18.

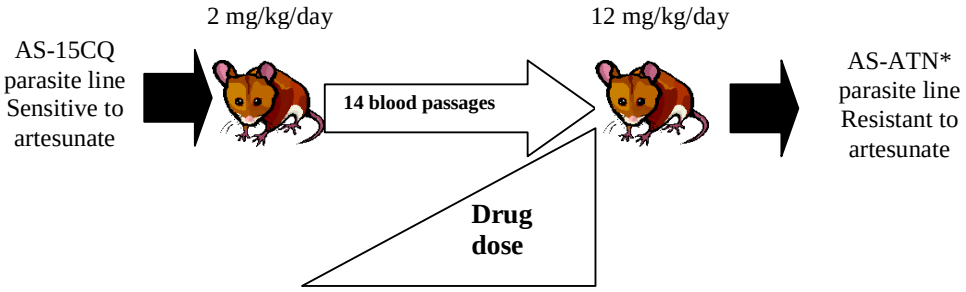


Figure18 – A schematic representation of the artesunate selection procedure. AS-ATN* is uncloned.

Figure 19 represents the stepwise increase in drug dose during the artesunate selection procedure.

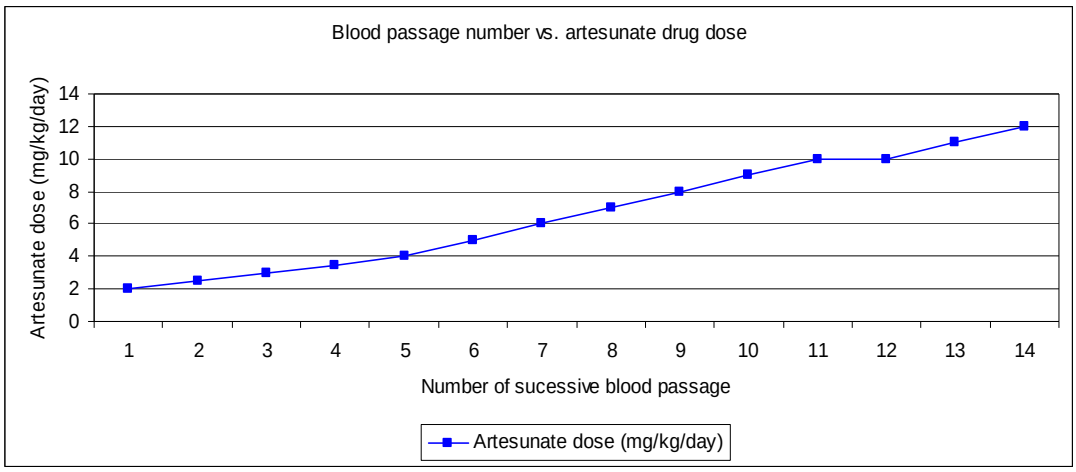


Figure 19 – The increase of the artesunate dose (mg/kg/day), tolerated by AS-15CQ clone, during 14 blood passages.

Figure 19 shows the stepwise increment on the artesunate dose during selection, starting by a 2 mg/kg/day dose of artesunate, this dose being the maximum dose tolerated by the initial parasites, AS-15CQ.

The drug resistance selection process finished at the 14th passage when the parasite tolerance had increased to 12 mg/kg/day, due to time constrains the selection process was finished at this stage where stability of resistance was assayed.

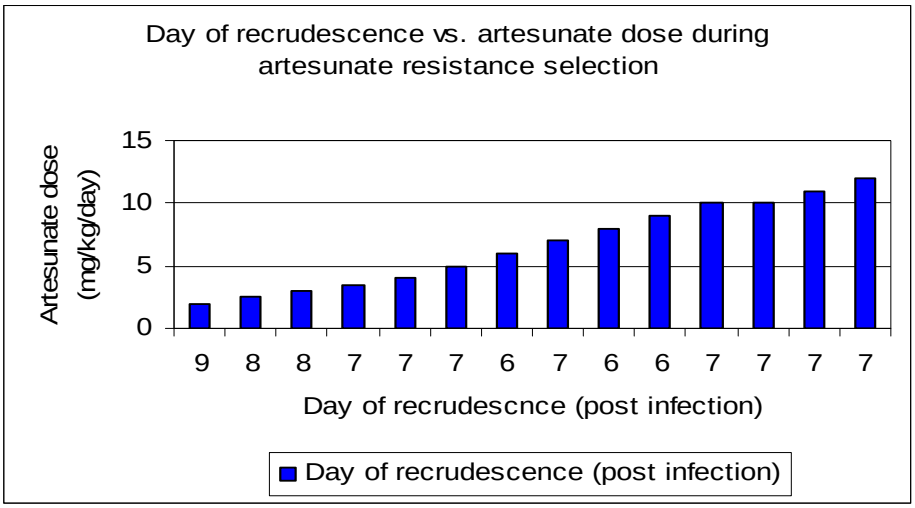


Figure 20 – The day of recrudescence under selection and artesunate dose (mg/kg/day).

Figure 20 shows the stepwise increment on the artesunate dose during selection versus the day of recrudescence.

As stated before the time of recrudescent parasites appearing in the blood of treated mice was used during the selection procedure as an indication on reduced susceptibility to artesunate. Here, and in a different fashion from artemisinin, the selection of artesunate resistance was carried out in a more consistent increase of the drug pressure within roughly the same period of time. It is speculated that this is related to the metabolism of the drug. Thus, being a second generation derivative of artemisinin, it is more active than artemisinin itself in the process of eliminating the parasites.

The minimum curative dose (MCD) for artesunate was calculated the same way as for artemisinin.

Table 9 – N-fold resistance calculated for each passage under artesunate pressure. The MCD drug unselected parasites is 2 mg/kg/day, which was the initial drug dose tolerated by the AS-15CQ clone initially used for drug selection.

Number of blood passage under artesunate pressure	MCD drug selected parasites (mg/kg/day)	N-fold resistance (MCD drug selected parasites/ MCD drug unselected parasite)
1	2	1
2	2.5	1.2
3	3	1.5
4	3.5	2
5	4	2.2
6	5	2.5
7	6	2.5
8	7	3
9	8	3.5
10	9	4
11	10	4.5
12	10	5
13	11	5.5
14	12	6

At the 14th passage at the end of the selection procedure, the parasite line obtained, denoted AS-ATN*, (* uncloned parasite population). AS-ATN* has an N-fold resistance of 6 when compared to the initial parasite clone and was cloned by the method of limiting dilution [Rosario V, 1976]. See following Section 3.4.

Cloned parasites were re-tested for their responses to artesunate.

3.4 Cloning of resistant parasites

At the end of the selection procedure, it was considered that a significant level of resistance to both to artemisinin and artesunate had been obtained. The N-fold level of resistance was calculated. The artemisinin resistant parasite line AS-ART* was 15 fold more resistant than the initial clone AS-30CQ; the artesunate resistant parasite line AS-ATN* was 6 fold more resistant to ATN than the initial clone AS-15CQ.

Both parasite populations were cloned by the limiting dilution method. (See Section 2.5 for details). The cloning procedure is extensively described at Rosario V 1976.

From the cloning procedure of the parasite line AS-ART*, from the 50 mice inoculated, five mice developed patent parasitemias (10 % infection rate) from which parasites were harvested and frozen in liquid nitrogen

From the cloning procedure of the parasite line AS-ATN*, from the 50 mice inoculated, seven mice developed patent parasitemias (14% infection rate) from which parasites were harvested all frozen in liquid nitrogen.

Of these, one clone from each line was chosen on the basis of their faster growth rate, for further studies, including stability assays and genetic crosses and analysis. The artemisinin and artesunate resistant clones were designated *P. chabaudi* AS-ART and AS-ATN respectively. See Figure 21 for details.

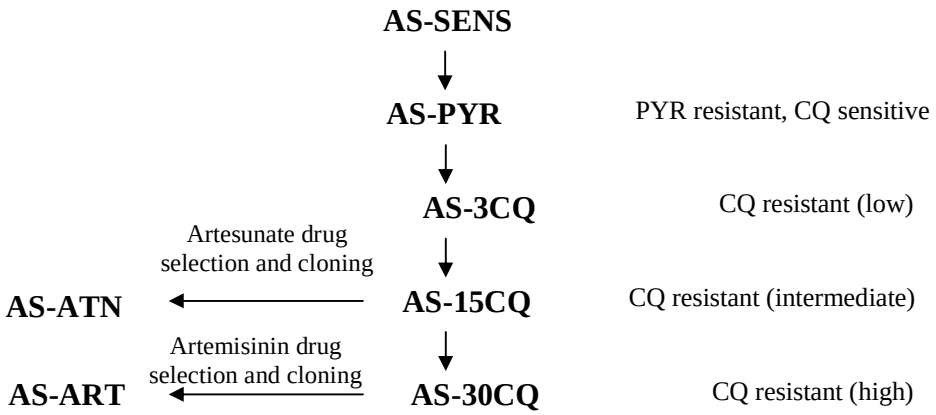


Figure 21 – A schematic representation of the clones and parasite lines of *Plasmodium chabaudi* used in this project.

AS-ART and AS-ATN were tested for their response to artemisinin and artesunate respectively, immediately after cloning. AS-ART and AS-ATN clones were inoculated into naïve CD1 mice and then re-tested for their susceptibility to artemisinin and artesunate, by comparing their response to each drug with that of non-treated and passaged the same number of times as the parasites under selection, control parasites, AS-30CQ and AS-15CQ respectively.

AS-ART and AS-ATN were shown to retain the same phenotype as that of the drug-resistant population from which they had been derived, under the same drug pressure of respectively 60 mg/kg/day treatment for 5 days with artemisinin, or a 12 mg/kg/day treatment for 5 days with artesunate. These parasites were then used in subsequent studies to investigate further whether the observed drug resistance was stable. These results will be presented on Section 3.5.

3.5 Drug resistance stability tests

Once resistance to artemisinin and artesunate were selected and cloned stability of the resistant phenotype, under absence of drug pressure was assayed.

Drug-resistant parasite clones, AS-ART and AS-ATN were re-tested for their drug responses after each of three different procedures:

i) Freeze-thawing cycles in liquid nitrogen,

ii) 12 continuous sub inoculations in mice in the absence of drug treatment and

iii) Transmission through *Anopheles stephensi* mosquitoes into new mice.

N-fold resistance index was calculated as it has been previously described.

3.5.1. Resistance stability after liquid nitrogen preservation (deep-freezing)

To check for resistance stability after liquid nitrogen preservation, resistant clones were frozen down in liquid nitrogen and then thawed, and re-tested for resistance.

Briefly infected blood is drawn, when the majority of asexual parasites are at the ring stage of development, into heparinised syringes. The red blood cells are pelleted by centrifugation (2000 rpm for 5 minutes) and the majority of the plasma is removed, two volumes of deep-freeze solution (Appendix 1) are added to one volume of the packed red blood cells with constant mixing in a drop wise fashion. Such material, inoculated i. p. into a mouse, after any period of storage, induced an infection, which became patent from 7-14 days after inoculation. The parasites growing in this fashion were evaluated for their response to artemisinin or artesunate in comparison with unselected control parasites, the same way as during the drug resistance selection.

AS-ART and AS-ATN parasite clones phenotypes drug resistance remained unchanged in both cases. The two cloned lines retained a 15 and 6 times N- fold resistant phenotype respectively. The parasites response to the drug was the same after freezing and thawing. Stability of resistance was therefore, confirmed.

3.5.2. Resistance stability after blood passages in the absence of drug pressure

AS-ART and AS-ATN cloned parasites were subjected to 12 passages in untreated mice, after which they were tested for their drug responses.

Briefly, 10^7 infected red blood cells were established as the standard parasite number to be infected into individual mice. Infections in the absence of drug pressure were maintained by passaging resistant parasites twelve times in CD1 mice i. p. route. Treatment was 60 mg/kg/day for 5 days for ART and as 12 mg/kg/day for 5 days for ATN.

Once drug tests were preformed after the twelve passages in the absence of drug treatment, it was verified that the resistant phenotype maintained because resistant parasites recrudesced as expected while sensitive parasites did not.

Resistant clones retained resistance to the corresponding drug.

3.5.3. Resistance stability after cyclical transmission through mosquitoes

After confirming the stability of the resistance phenotype after cloning, freezing and thawing of these clones, in the absence of drug pressure, it was necessary to demonstrate that this genetic trait was transmissible through mosquitoes.

The procedure for cyclical transmission through mosquitoes was previously described by Landau and colleagues [Landau I *et al.* 1966].Briefly, infected splenectomised rats, in which gametocytes were present, were exposed to mosquitoes, which had been starved for 24-48 hours. The infected rodents were exposed to mosquitoes for a varied length of time (1-2 hours) depending on the feeding performance of the mosquitoes. Seven to ten days later a small number of mosquitoes were dissected in order to count the number of oocysts, which had developed on each midgut. Fifteen and, eventually, seventeen days after the blood meal, when sporozoites were present in the salivary glands, an uninfected mouse was exposed to the mosquitoes for transmission of the malaria infection. Patent blood infections could be detected after 4-8 days in blood smears from the mice. Resistant clones successfully transmitted in this fashion were tested for their response to artemisinin or artesunate in comparison with unselected control parasites.

Mice infected with AS-ART or AS-ATN were used to feed *A. stephensi* mosquitoes. AS-ART was successfully transmitted through mosquitoes on two separate occasions, and the resulting

blood forms that developed in mice were renamed AS-ARTA and AS-ARTB. The N-fold resistance index of AS-ARTB were then assessed in parallel to the, untreated but passaged control line, AS-30CQ (this line was also transmitted through mosquitoes at the same time, as a control). AS-ARTB showed an N-fold resistance index of 15-fold to artemisinin relative to AS-30CQ. Thus, artemisinin resistance remained stable after transmission of the resistant parasites through mosquitoes. AS-ARTA was not tested and the parasites where deep-frozen. For simplicity and clarity on further analysis during this project, AS-ARTB clone was renamed AS-ART.

In a similar fashion, AS-ATN was also subjected to mosquito transmission and showed a 6-fold increase in the N-fold resistance to artesunate relative to sensitive control; therefore we also consider artesunate resistance to be stable after mosquito transmission. See Figure 22 for a summary of the drug selection / stability tests procedure.

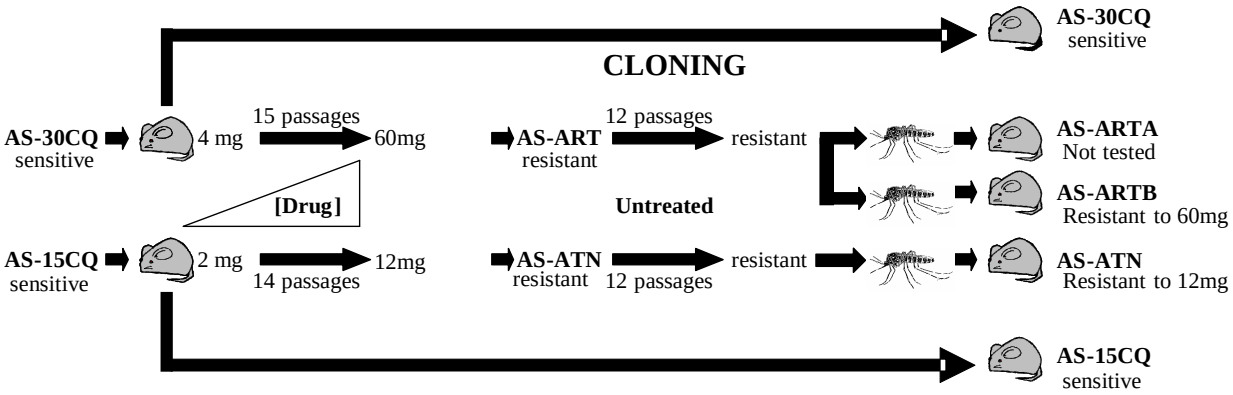


Figure 22 – A schematic representation of the artemisinin and artesunate selection procedure.

Clone AS-30CQ and parasite line AS-15CQ were passaged in the absence and presence of gradually increasing doses of drug (artemisinin and artesunate, respectively). Initial drug sensitivities (4 mg/kg/day or 2 mg/kg/day) decreased (to 60 or 12 mg/kg/day) after 15 or 14 passages, respectively. The drug responses after cloning, passage in the absence of drug ("untreated") and transmission through mosquitoes remained unchanged. Control selection procedures in the absence of drug are also shown.

Adapted from: Afonso A *et al.* 2006.

The artemisinin and artesunate resistance phenotypes were unaltered after passaging in the absence of drug pressure, after freezing and thawing and after transmission through laboratory mosquitoes (Figure 22). Thus artemisinin and artesunate resistance obtained in our rodent model *P. chabaudi* is stable, indicating that resistance is likely to be genetically encoded.

3.6 Test for cross-resistance between artemisinin and artesunate clones

In order to evaluate whether the mechanisms of resistance to artemisinin and to artesunate share similar features, the responses of AS-ART to artesunate and AS-ATN to artemisinin were tested, resistant clones were cross tested with each other's selective drugs. Artemisinin at chosen dose was given to the artesunate resistant clone (AS-ATN) and artesunate at chosen dose was given to the artemisinin resistant clone (AS-ART).

For these tests several drug doses were initially tested for both resistant clones, the maximum dose of artemisinin tolerated by the artesunate resistant clone (AS-ATN) is 32 mg/kg/day for five days and the maximum dose of artesunate tolerated by the artemisinin resistant clone (AS-ART) is 8 mg/kg/day (data not shown). AS-ART showed a five-fold increase in the MCD to artesunate relative to AS-30CQ while AS-ATN showed a greater than ten-fold increase in the MCD to artemisinin, relative to AS-15CQ. These tests therefore revealed that both clones showed cross-resistance and that in both parasites there were greater increases in artemisinin resistance (15–26 fold) than for artesunate resistance (5–6 fold). The demonstrated cross-resistance suggests some sharing of genetic or pathways features in the parasites clones described here. Thought the existence of cross-resistance suggests that resistance to artemisinin and artesunate do share similar features, at least in the parasites clones described in this project.

Table 10 - N-fold resistance of *P. chabaudi* AS-ATN and AS-ART. The absolute and relative (N-fold) drug sensitivities of AS-ATN and AS-ART after blood passage in the absence of treatment, freeze/thaw and mosquito transmission are given. MCD – minimum curative dose.
From: Afonso A *et al.* 2006.

P. chabaudi	**MCD ATN (mg/kg/day)**	**MCD ART (mg/kg/day)**	**N-fold ATN**	**N-fold ART**
AS-15CQ	2	1.2	-	-
AS-ATN	12	32	6	26
AS-30CQ	1.6	4	-	-
AS-ART	8	60	5	15

3.7 Discussion

Drug resistance genetic basis can be study using various methodologies. A correct and complete method to study drug resistance genetics depends on firstly selecting drug resistance mutants of stable phenotype, because only the presence of a stable phenotype guarantees that resistance is genetically encoded and not a physiological adaptation to the constant presence of drug pressure. Drug resistance can be selected *in vitro* using for example chemical mutagenesis or *in vivo* by drug pressure either using a single very high dose like the method used to select PYR resistance or by progressively increasing the drug concentration. The latter situation is the one that most closely mimics the actually scenario taking place in natural parasite populations. Therefore, an appropriate method that has been used for selecting and understanding drug resistance is the utilization of genetically stable resistant mutants selected through drug pressure, originated from cloned sensitive parasite lines. Both the original drug-sensitive and the selected drug-resistant parasites should be genetically identical, (or isogenic), except for any mutations involved in resistance; such mutations can then be pinpointed using different approaches.

This project represents the first study where malaria parasites with genetically stable and transmissible resistance to the antimalarial drugs artemisinin and artesunate were selected and used in genetic crosses.

The reasons for obtaining stable resistance where others have previously tried unsuccessfully are not clear. One reason could be the use of the rodent model *P. chabaudi*, which had been used before to select for pyrimethamine, chloroquine and mefloquine resistance. The existence of parasite clones with an accumulation of drug resistance genetic markers may create a favourable background for the selection of artemisinin and artesunate resistance.

Two parasite lines were selected and cloned: AS-ART is 15 times more resistant to artemisinin than its progenitor parasite line, AS-30CQ and AS-ATN which is 6 times more resistant to artemisinin than its progenitor parasite line, AS-15CQ.

In this work the utilization of approximately the same number of blood passages, during a similar time period under drug pressure, produced an N-fold resistance for artesunate inferior to the N-fold resistance for artemisinin and this is not explainable.

Metabolization differences between the two drugs may have had an intrinsic difference in the selective pressure within the host, but drug concentration studies were not carried out.

A very interesting finding during this part of the project was the cross resistance between the selected resistant clones, giving a very strong indication that resistance to ART and ATN share similar features.

The successful selection of these parasites and the fact that they can be transmitted through *Anopheles sp.* mosquitoes is a significant achievement for two main reasons: a) these observations demonstrate that malaria parasites are genetically and biologically capable of sustaining stable resistance to artemisinins which had been selected through drug pressure. Consequently, it is also conceivable that, in human malaria, artemisinin resistance may appear in the future due to extensive and/or inappropriate drug usage; once it does, it may spread in the parasite population and become established; b) the resistant *P. chabaudi* parasites reported here could then be used to investigate the genetic determinants of resistance to these drugs.

It was possible to generate ART or ATN resistance from two different parasite clones, AS-30CQ and AS-15CQ which were already resistant to chloroquine and pyrimethamine. Efforts to generate ART or ATN resistance from the very original chloroquine sensitive clone (AS-PYR), were abandoned, because this clone showed, under the same drug pressure methodology, high susceptibility to the treatments (data not shown). As stated before, this may be a result of a genetic ability, if not potentiation of the parasite ability to generate mutations in response to drug treatment (called the "Accelerated Resistance to Multiple Drugs" (ARMD) phenotype) [Rathod PK *et al.* 1997] which might have occurred during the generation of previous drug selective methods, including chloroquine.

Alternatively, it is possible that the ART resistance phenotype is only expressed in chloroquine resistant clones, almost as resistance to CQ is required for the selection of ART and ATN.

This suggests the presence of functional interactions between the pathways underlying chloroquine and artemisinin resistance or that resistance to cloroquine genetically creates genetic conditions which facilitate the appearance of ART and ATN resistance. These questions have significant relevance to the practical lifetime of a drug in areas where resistance to other drugs (or to chloroquine specifically) is prevalent.

The inspection of mutation or mutations underlying resistance to ART and ATN, will be described in Chapter IV (known or suspected mutations) and in Chapter V using high-throughput comparative genomic studies based on genome-wide approaches, Linkage Group Selection (LGS) (new mutations) [Culleton R *et al.* 2005].

RESULTS
CHAPTER IV

ANALYSIS OF THE PUTATIVE GENETIC MODULATORS FOR ARTEMISININ AND ARTESUNATE RESISTANCE

4.1. Sequencing of *P. chabaudi mdr1, cg10, tctp* and *atp6* genes, in the selected mutant clones.

The first objective of this research work was the selection resistant parasites in the rodent malaria model, *P chabaudi*, to artemisinin and artesunate and this was achieved. At this stage in order to see if there were differences in the previously described putative genetic modulators for artemisinin and artesunate resistance, *P. chabaudi* genetic homologues of the *P. falciparum mdr1, crt, tctp* and *atp6,* between the sensitive and the resistant parasites, DNA was extracted was extracted from the parasites AS-15CQ and AS-30CQ, and from their related artemisinin (AS-ART) and artesunate (AS-ATN) resistant clones.

4.1.1 Isolation of the *mdr1, cg10, tctp* and *atp6 P. chabaudi* orthologues

Prior to the present work, the *P. chabaudi* homologues of the *P. falciparum* genes *pfmdr1* and *pfcrt,* which are respectively *pcmdr1* and *pccg10* had been identified by Cravo PV and colleagues and Hunt P and co-workers [Cravo PV *et al.* 2003; Hunt P *et al* 2004]. Thus, for PCR amplification of the *pcmdr1* and *pccg10* genes, oligonucleotide primers and PCR amplification conditions previously published were used [Cravo PV *et al.* 2003; Hunt P *et al* 2004], based on DNA sequences characterised prior to this study (*pcmdr1* AY123625 and *pccg10* AY304549).

The DNA sequences of the *P. falciparum* and *P. yoelii tctp* and *atp6* genes were available online at the NCBI/NIH (National Institute of Health) database (www.ncbi.nih.gov) with the following accession numbers: *pctctp* NP_703454, *pytctp* AF124820, *pfatp6* AB121053 and *pyatp6* AABL01001880. To obtain the *P. chabaudi* orthologues of these genes, these sequences were retrieved and used in BLAST searches against the available *P. chabaudi* sequences (shot gun clones and genomic contigs), deposited at the *P. chabaudi* genome database (www.sanger.ac.uk). The two sequences giving significant hits were retrieved and used to design *P. chabaudi*-specific oligonucleotide primers to amplify overlapping DNA fragments spanning the coding region, introns and both 5'- and 3'-non-coding sequences. These were then used in PCR amplifications.

Pcmdr1 gene has a nucleotide sequence identity of 75.0% to *P. falciparum* and 78.1% amino acid identity to *P. falciparum.* [Hunt P *et al.* 2004]. Its accession number is AY123625 and it encodes a protein of 1416 amino acids and its coding region is of 4248 base pairs.

For the *P. chabaudi cg10* gene, there is a nucleotide sequence identity of 68.6% and amino acid sequence identity of 75.1% within the predicted coding region when compared to *P. falciparum crt* gene. [Hunt P *et al.* 2004].

To investigate whether the *P. chabaudi* orthologue of *Pftctp* gene play a role in the resistance to artemisinin derivatives in our model, *P. chabaudi* specific database sequences (www.sanger.ac.uk/cgi-bin/blast/submitblast/p_chabaudi) were used to design oligonucleotide primers for amplifying *pctctp*. *Pctctp* gene has an 84% identity with *pftctp* gene in relation to nucleotides and a 90 % identity to pftctp in relation to amino acid. This high degree of identity is a confirmation that the gene has been corrected isolated from the database. [Afonso A *et al.* 2006].

PCR amplifications were initially carried out using genomic DNA from the drug-sensitive parasite clone AS-SENS, according to the gene sequence and primer sequence presented at the appendix 2, the *P. chabaudi tctp* gene was first characterised in the drug-sensitive parasite AS-SENS (Genbank accession number AY304545).

In AS-SENS, the gene is encoded by a continuous open reading frame containing 516 b.p., which translates into a predicted peptide of 171 amino acids [Afonso A *et al.* 200].

In order to identify possible point mutations in *tctp* gene, in artemisinin (AS-ART) and artesunate (AS-ATN) resistant parasites, in comparison to progenitors, DNA from AS-15CQ, AS-30CQ, AS-ART and AS-ATN was used to sequence the entire coding region of the *pctctp* gene. See Figure 25 for details in the extent of DNA sequence analysed.

Pcatp6 gene has a 72% identity with *pfatp6* gene in relation to nucleotides and a 72 % identity to pfatp6 in relation to amino acid. This high degree of identity is a confirmation that the gene has been corrected isolated from the database. [Afonso A *et al.* 2006]. The *atp6* gene has a Genbank accession number of DQ171938. In *P. chabaudi* the gene was verified to be constituted of 3539 nucleotides, comprised of three exons interrupted by two introns one of 92 nucleotides and the other of 90 nucleotides.

The *pcatp6* gene has two introns (intron 1: 92 bps; intron 2: 90 bps) located near the 3´end of the gene and in identical positions to the predicted introns in the *P. falciparum* gene (See Figure 26 for details). The full coding sequence translates into a predicted protein of 1118 amino acids. . Due to the fact that *pfatp6* has been pointed as the target for artemisinin the non-coding region that lay in the proximal region of the gene was also checked for gene mutations, because in the case of existing these mutations could eventually affect the level of transcription. However, no differences in nucleotide sequence between the resistant clones and their sensitive progenitors were found in 4kb of nucleotide sequence upstream or in 1 kb downstream of the gene.

The gene sequence and the primers used for amplification of all of the above genes are presented in Appendix 2.

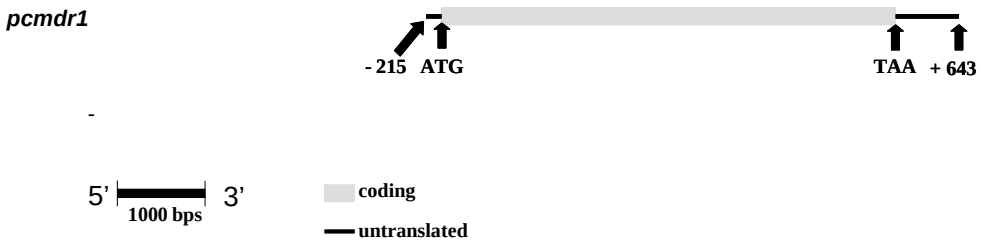


Figure 23 – Extent of sequence analysed for the gene *pcmdr1* for the clones AS-15CQ, AS-30CQ, AS-ART and AS-ATN. Numbers refer to nucleotides 5' to 'start' codon ATG (negative) or 3' to termination codon (positive). Adapted from Afonso A *et al.* 2006

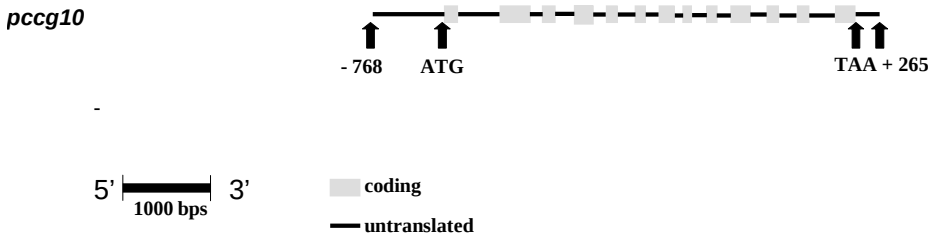


Figure 24 – Extent of sequence analysed for the gene *pccg10* for the clones AS-15CQ, AS-30CQ, AS-ART and AS-ATN. Numbers refer to nucleotides 5' to 'start' codon ATG (negative) or 3' to termination codon (positive). Adapted from Afonso A *et al.* 2006.

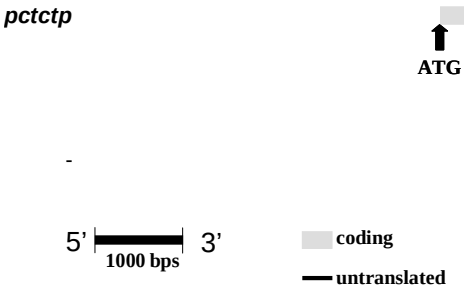


Figure 25 – Extent of sequence analysed for the gene *pctctp* for the clones AS-15CQ, AS-30CQ, AS-ART and AS-ATN. Numbers refer to nucleotides 5' to 'start' codon ATG (negative) or 3' to termination codon (positive). Adapted from Afonso A *et al.* 2006.

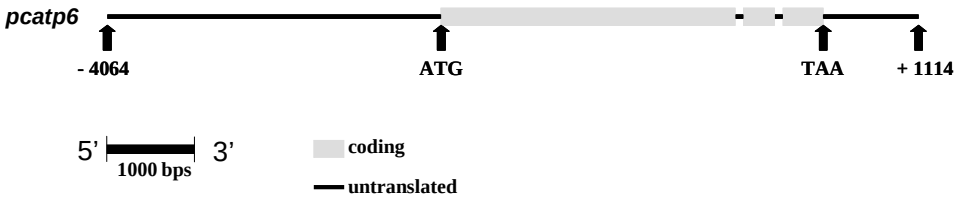


Figure 26 – Extent of sequence analysed for the gene *pcatp6* for the clones AS-15CQ, AS-30CQ, AS-ART and AS-ATN. Numbers refer to nucleotides 5' to 'start' codon ATG (negative) or 3' to termination codon (positive). Adapted from Afonso A *et al.* 2006.

4.1.2. Sequence comparisons of *P. chabaudi mdr1, cg10, tctp* and *atp6* in the selected mutant clones and their sensitive progenitors.

AS-ATN, resistant to artesunate and its progenitor parasite line AS-15CQ and AS-ART, resistant to artemisinin and its progenitor clone AS-30CQ were all sequenced twice in both directions (sense and antisense) for all genes described above. Pairwise comparisons were made between sensitive and resistant clones as well as the sequence published in the *P. chabaudi* database. The result revealed that the sequence of all genes was identical between all parasites and between the database sequences, showing that no mutations had occurred during the selection of drug resistance. In other words, the genes do not appear to be involved in chemoresistance in the rodent malaria model *P. chabaudi*.

4.2. Estimation of gene copy numbers of the *pcmdr1, pctctp* and *pcatp6* genes, in the selected mutant clones.

Besides gene mutations, changes in copy numbers may also be responsible for drug resistance. Therefore, gene copy number of *pcmdr1, pctctp* and *pcatp6* on the previously selected and cloned artemisinin and artesunate mutants in *P. chabaudi* (AS-ART and AS-ATN respectively) were studied. At this stage the gene *pccg10* was not analysed for gene copy number change due to the fact that there were no references found in the literature that account for changes in the gene copy number of either *pfcrt* or its *P. chabaudi* homologue *pccg10.*

The gene copy number can be analysed by various methods, the method of using real-time quantitative PCR (RTQ-PCR) has been extensively validated for the use in malaria parasites. We found that the most robust method for copy number determination by real-time PCR is the comparative Ct ($2^{-\Delta\Delta Ct}$) method [Livak KJ *et al.* 2001]. While requiring an endogenous control and a calibrator, meaning a gene from which previous knowledge is needed for the exact gene number (for example *msp-1* gene that is known for sure to be a single copy gene in *P. chabaudi)* and a sample that is use as a control for that gene, it differs from relative standard method by relying on equal PCR efficiencies with the target and the endogenous control genes. The $2^{-\Delta\Delta Ct}$ method is described in detail by Livak and colleagues [Livak KJ *et al.* 2001]. Briefly for the ΔΔCt calculation to be valid, the efficiency of the amplification of target and reference gene must be approximately equal. The control gene was *msp-1* and the target genes were *mdr1, tctp* and *atp6* genes. The average Ct was calculated for both control and target genes and the ΔCt ($Ct_{\text{target gene}} - Ct_{msp\text{-}1}$) was determined. Different plots of the log DNA dilution versus ΔCt were made and whenever the slope was close to zero the efficiencies of the target and reference genes were similar, the ΔΔCt was calculated for the relative quantification of the target gene; $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{msp\text{-}1})_{\alpha} - (Ct_{\text{target gene}} - Ct_{msp\text{-}1})_{\beta}$, where α = ART or ATN resistant sample and β = AS-30CQ or AS-15CQ samples respectively.

After the method was validated the results for each sample were expressed in N-fold numbers in α gene copy number with normalization to the *msp-1*gene copy number according to the equation: gene number of target = $2^{-\Delta\Delta Ct}$ [Livak KJ *et al.* 2001].

After optimisation of all the RTQ-PCR conditions we aimed to investigate if ART and ATN resistance could be related to changes in the gene copy number of *mdr1, tctp* and *atp6* genes. For this the N-fold gene number was evaluated and *mdr1, tctp* and *atp6* genes were normalised to *msp-1* gene, in artemisinin resistant parasites *P. chabaudi* AS-ART relative to

artemisinin sensitive *P. chabaudi* AS-30CQ from which this parasite line was obtained and artesunate resistant *P. chabaudi* AS-ATN relative to artesunate sensitive *P. chabaudi* AS15CQ from which this parasite line was obtained. Three independent experiments were carried out; the mean N-fold values of the experiments are presented in Figure 27. There were no changes in the gene copy number of *mdr1*, *tctp* and *atp6* genes detected (Figure 27), meaning that during the selection for artemisinin and artesunate resistance the genes *pcmdr1*, *pctctp* and *pcatp6* did not suffer any gene copy number change.

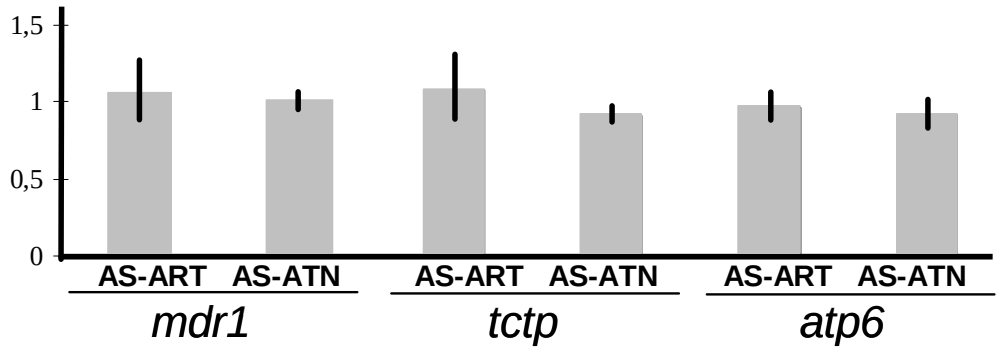


Figure 27 – Relative differences (N-fold) in gene copy number between artemisinin (AS-ART) and artesunate (AS-ATN) resistant parasites and their sensitive progenitors, AS (30CQ) and AS (15CQ) respectively.
The y-axis represents the mean N-fold of gene number (grey bars) and standard deviations (vertical lines) at 95% confidence interval of each of the genes under study normalised against *msp-1*, generated after three independent assays.
From: Afonso A *et al* 2006.

4.3. Discussion

4.3.1. Summary

The previously described putative genetic modulators for artemisinin and artesunate drug resistance, *pcmdr1, pcg10, pctctp* and *pcatp6* that are *P. chabaudi* gene homologues of *P. falciparum mdr1, crt, tctp* and *atp6,* respectively, were fully isolated and analysed for their coding regions. In the case of the gene *atp6* gene an area of 4kb upstream and 1 kb downstream was also analysed, in both the resistant clone lines, AS-ART and AS-ATN in relation to their sensitive progenitors AS-30CQ and AS-15CQ respectively and found to be not mutated.

Collectively, the above data allowed us to conclude that in our model *P. chabaudi* the *pcmdr1, pcg10, pctctp* and *pcatp6* genes are not involved in artemisinin or artesunate resistance by gene point mutation.

Besides looking for point mutations, the genes *pcmdr1, pctctp* and *pcatp6* were also investigated for their involvement in artemisinin and artesunate resistance by inspecting putative changes in gene copy number. This allowed us to conclude that the phenotype of artemisinin and artesunate resistance in our model, *P. chabaudi,* is also not associated with an increase in the gene copy number of the same genes.

4.3.2. General Discussion

This study has shown that no changes in nucleotide sequence or copy number for *pcmdr1, cg10, tctp or atp6* genes were found in the artemisinin or artesunate resistant parasites when compared to their sensitive progenitors.

We consider, however, that other genetic mechanisms such as protein turnover and/or post-translational modifications of the gene products may account for the involvement of these genes in the resistance phenotype. However the investigation of these putative mechanisms was not contemplated in this study.

In malaria, the genetic mechanisms underlying drug resistance have been extensively studied for most antimalarials, but are not fully understood, except for resistance to pyrimethamine. Resistance to this drug has been to shown to be conferred by cumulative single nucleotide mutations in the *dhfr* gene [Sirawaraporn W *et al.* 1997] in *P. falciparum* and in all rodent malaria models analyzed. Artemisinin has been used in the field for centuries without resistance ever being registered, so one might expect a complex mechanism for resistance, not as simple as that for pyrimethamine.

In our study in a rodent malaria model, we selected for high levels of drug resistance, which suggests the involvement of more than one gene.

The evidences supporting the role for pfatpase6 protein in artemisinin sensitivity in *P. falciparum* arise from the interaction of artemisinin in an *ex vivo* heterologous system (*Xenopus* oocytes) [Eckstein-Ludwig U *et al.* 2003] which may reflect a histological, physiological and biochemistry point of view which may be difficult to interpret in light of the human malaria parasite. Evidence for the involvement of this gene in modulating artemisinin susceptibility also came from the fact that recent evidence indicates that mutations in the *pfatpase6* gene may correlate with varying degrees of *in vitro* responses of *P. falciparum* to artemisinin derivatives [Jambou R *et al*. 2005]. In this case the pfatpase6 protein S769N, A623E and E431K polymorphisms were associated with an increased mean in the IC50 for artemisinins [Jambou R. *et al.* 2005]. The importance of these findings is unclear however, since: i) the correspondence between reduced sensitivity of the field isolates to artemisinins and the presence of the different polymorphisms was incomplete and ii) there was a region-specific association of these polymorphisms with varying degreees of susceptibility correlating highly (but not completely) with the S769N mutation in French Guiana but not in Senegal or Cambodia. In addition, later studies found no association between mutations in the *pfatpase6* gene and the sensitivity of field isolates to artemisinins from Tanzania [Mugittu K *et al.* 2007] and São Tomé and Principe [Ferreira ID *et al.* 2007].

Interestingly, it may be possible that another gene closely link to the *atp6* gene may be involved in the resistance to this drug, which could explain the strong but incomplete association observed in French Guiana [Jambou R. *et al*. 2005]. In actual fact, a similar scenario has already been described before with the chloroquine resistance determinant in *P. falciparum* which had been mapped by linkage analysis to a segment on chromosome 7 where two candidate genes *cg1* and *cg2* with complex polymorphisms initially linked to the chloroquine resistance phenotype where found [Duraisingh MT *et al.* 2000a, Fidock DA *et al.* 2000a]. More detailed analysis within that region later revealed a different, but closely linked gene, *pfcrt*, to be the major determinant of chloroquine resistance.

Finally, it is relevant to mention that, if *pfatpase6* turns out to be the major modulator of artemisinin responses in *P. falciparum*, it is conceivable that the rodent malaria *P. chabaudi* may reveal alternative mechanisms of resistance to those of *P. falciparum*, as is the case with chloroquine resistance [Hunt P *et al.* 2004].

RESULTS

CHAPTER V

EXPERIMENTS USING LINKAGE GROUP SELECTION AS AN ATTEMPT TO IDENTIFY THE LOCUS OR LOCI INVOLVED IN ARTEMISININ AND ARTESUNATE RESISTANCE

5.1 Introduction

As described in the previous chapter (Results – Chapter IV) sequence analysis of genes with potential involvement in modulating parasite responses to artemisinin derivatives, was carried out for the *P. chabaudi* orthologues of *pfatp6* [Eckstein-Ludwig U *et al.* 2003; Jambou R *et al.* 2005; Uhlemann AC *et al.* 2005], *pfcrt* [Sidhu AB *et al.* 2002], *pfmdr1* [Ferrer-Rodríguez I *et al.* 2004; Price RN *et al.* 2004; Reed MB *et al.* 2000; Sidhu AB *et al.* 2005], and *pftctp* [Bhisutthibhan J *et al.* 1998; Walker DJ *et al.* 2000]. Sequencing of AS-ART and AS-ATN and their progenitors showed that there were no mutations or copy number changes in these genes (data published Afonso A *et al.* 2006).

This chapter describes the application of Linkage Group Selection (LGS) as an approach to identify the genetic locus or loci involved in artemisinin and artesunate resistance in *P. chabaudi*.

Some of the results described below have been accepted for publication [Hunt P *et al.* in press at the *Molecular Microbiology*].

5.2 Production of cross progeny

The procedure to obtain a cross progeny have been already described (See Chapter II - Materials and Methods).

Three independent genetic crosses were obtained using AS-ART and AJ and two independent genetic crosses were obtained using AS-ATN and AJ.

One of the conditions for a correct and useful utilization of LGS for identifying genetic locus involved in a particular phenotypic trait, is that large numbers of recombinant progeny clones are present both in the progeny prior to the selection, and post selection. For that reason and in order to increase the number of recombinants, the uncloned progeny from these crosses (called “unpassaged”) were pooled in equal proportions (equal number of parasites) and passaged through the two treated and untreated groups. See Figure 28 for details on the experimental groups.

For further analysis pooled crosses were used. Those pooled crosses were named super crosses. The three genetic crosses obtained between AS-ART and AJ were pooled together in a super cross that for clarity was named AS-ART x AJ. The two genetic crosses obtained between AS-ATN and AJ were polled together in a super cross that for clarity was named AS-ATN x AJ.

Each cross was not analysed individually during this project.

5.3 Selection of cross progeny

5.3.1 General procedure

When the sporozoites-induced infections (so called “unpassaged”) reached parasitaemias of between 10%–15%, the parasites were harvested for AFLP analysis (providing a reference point for markers analysed in the subsequent treatment groups), pooled, and inoculated (each mouse in each group received 1 x 10^7 parasites) into two groups of mice, one drug treated with either artemisinin or artesunate (so called “treated”) and the other left untreated (so called “untreated”). See Figure 28 for details.

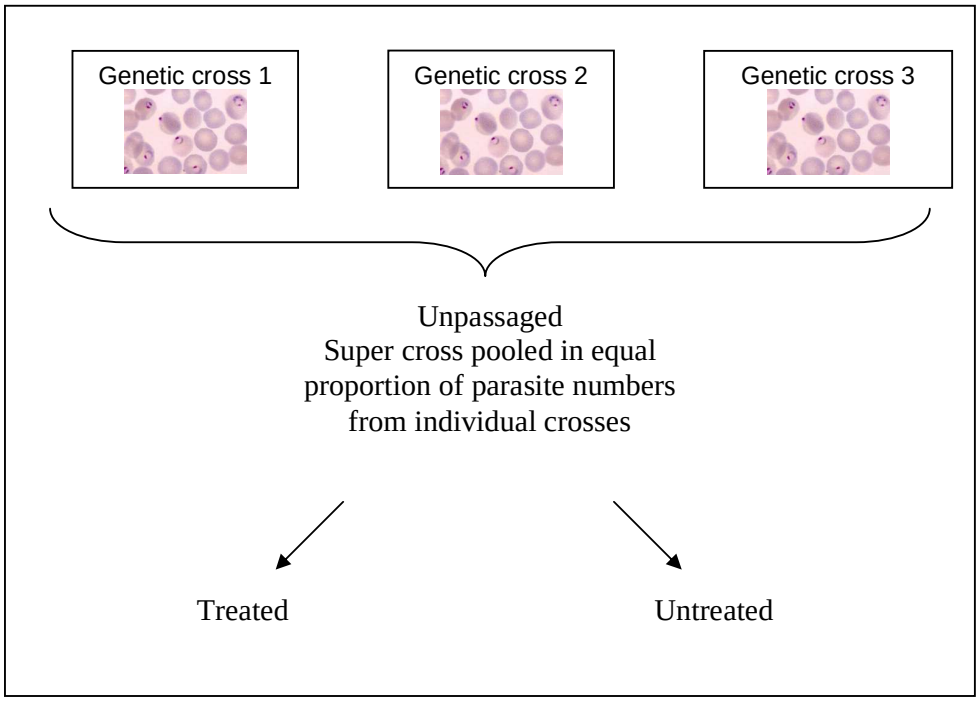


Figure 28 - Schematic representation of the selection of the pooled cross progeny (LGS) experiment using previously generated individual crosses between AJ and AS-ART

For the pooled cross AS-ATN x AJ only two individual crosses were used.

Adapted from Culleton R, 2005 with kind permission from Dr. Richard Culleton.

5.3.2 Results

We passaged the pooled uncloned cross progeny in the presence and in the absence of drug treatment. The dose of artemisinin used for LGS in the treated group of the cross AS-ART x AJ was 25 mg/kg of mouse body weight daily for 5 consecutive days, starting 3h after parasite challenge. The dose of artesunate used for LGS in the treated group of the cross AS-ATN x AJ was 5 mg/kg of mouse body weight daily for 5 consecutive days, starting 3h after parasite challenge. These doses were chosen as a likely compromise between a very good selection, that would eliminate most sensitive progeny and a dose that allowed the maintenance of a still high number of recombinants.

Both the artemisinin or artesunate treated and the untreated blood-stage cross progeny were allowed to grow to peak parasitaemia (30-40 % for untreated approximately day 8 post infection and 20-30% for treated approximately day 10 post infection), at which point the blood was harvested, and DNA was extracted for future analysis. See Figures 29 and 31: each point on these figures corresponds to a group of five mice; means were calculated and standard deviations are presented in the graphs.

5.3.2.1 Selection of the pooled AS-ART x AJ cross

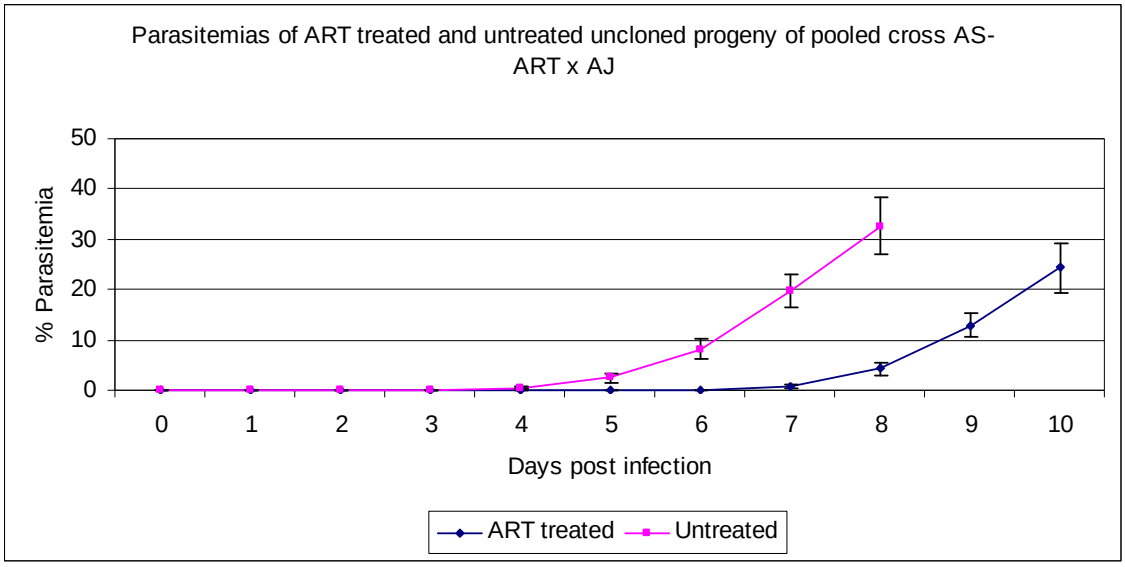


Figure 29 - Parasitaemia curves for the "untreated" and "artemisinin treated" uncloned cross progeny of the AS-ART x AJ cross. Each point corresponds to a mean parasitemia of an experimental group of five mice and the vertical bars are standard deviations.

The dose of artemisinin used for LGS of the cross AS-ART x AJ was 25 mg/kg of mouse body weight daily at 24-h intervals for 5 consecutive days, starting 3h after parasite challenge.

Parasitaemia curves for both artemisinin treated and untreated groups are shown in Figure 29. The ART treated group show a delay in the parasite grow due to drug administration, though it does not show a very marked inhibition of parasite growth when compared to the untreated group, meaning there is not a very big delay on the parasite recrudescence of the treated group indicating presence of relative high amounts of parasites carrying the resistant allele (AS allele) in the polled cross used AS-ART x AJ.

Before engaging in the genome wide analysis using AFLP, the proportions of AS and AJ alleles were measure to get information on the quality and genetic composition of the cross relative to the number of sensitive and resistant alleles,

Quantitation of the proportion of each allele on the genetic crosses allowed us to evaluate the quality of the selection by calculating the percentage of the resistant allele (AS allele).

Proportional Sequencing [Hunt P *et al.* 2005] estimates the proportions of alleles of a gene in a mixture by measuring the heights of the peaks in DNA sequence gel electropherograms which correspond with the nucleotides at the polymorphic sites under study (See example Figure 13, 14 and 30).

For analysing the composition of the progeny of the genetic cross AS-ART x AJ (the unpassaged, ART treated and untreated experimental groups), proportional sequencing of alleles of the *dhps* gene was performed.

Each DNA sample (one from AS-ART x AJ and other from AS-ATN x AJ untreated group) was prepared with the blood of all mice in each experimental group pooled together. Each individual measurement was repeated 5 times from the PCR stage (meaning 5 different PCR amplifications using the same DNA sample) and a mean %AS (AS concentration in the mixture) was calculated for each polymorphism and for each different PCR amplification for the *dhps* gene. On Table 11 we can se the mean %AS for each one of the four polymorphism (named for simplicity 1-4 see Figure 13 and 30 for correspondence) obtained from the quintuplicate analysis for each experimental group (unpassaged, untreated and ART treated) and for each group another mean was calculated using each mean value obtained for each polymorphism each will then gives us the mean %AS on each experimental group. These values serve the purpose to quickly evaluate the quality of the genetic cross and its selection, prior the extended AFLP analysis. The approximate %AS composition for each polymorphism was calculated by the relative peak height corresponding to the nucleotide from the AS DNA sequence, normalized with a non polymorphic allele. The data normalised treatment is described in detail by Hunt P and colleagues [Hunt P *et al.* 2005].

Proportional sequencing protocols are described before (see Materials and Methods - Chapter 2) and in great detail in Hunt P *et al*, 2005.

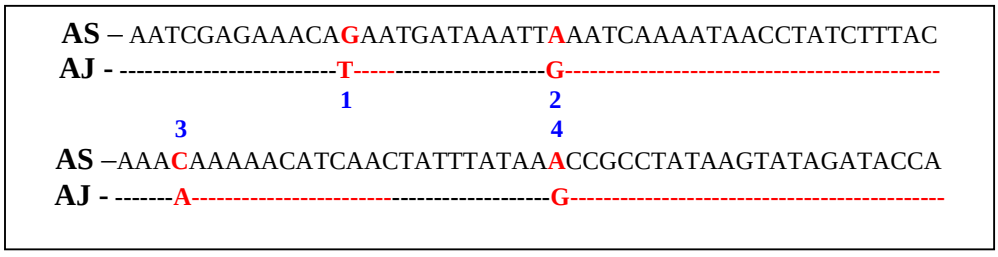


Figure 30 – Detail of the *pcdhps* gene sequence with indication of polymorphisms in red and bold, 1-4 numbers in blue are the numbers used in Tables 11 and 12 to indicate each one of the polymorphism between AS and AJ. For entire sequence of the gene please check Figure 13.

Table 11 – Proportional sequencing results for each group of the selection procedure for the genetic cross AS-ART x AJ ("unpassaged", "untreated" and "artemisinin treated") for all the polymorphisms of the *dhps* gene analysed. There are four polymorphisms between AS and AJ strains for the gene *dhps*, the mean %AS coming from the quintuplicate measurements was calculated for each polymorphism and finally a mean %AS was calculated as a mean between the four %AS means.

	***dhps* gene (%AS)**				**%AS for the *dhps* locus**
	1	2	3	4	Mean
Unpassaged group	43.4	42	31.4	30.4	36.8
Untreated group	13.4	15.8	25.8	16.3	17.8
ART treated group	42.8	39.8	37.9	36.4	39,2

From the results presented on Table 11 we can observe that though the unpassaged group was not submitted to any kind of drug selection, the %AS does not correspond to the 50% initially inoculated (the initial inoculum was 50% AS and 50% AJ parasites, calculated by parasite numbers), which can be explained by the fact that in previous experiments (data not shown) AJ parasites overgrew AS, AJ is a faster grower in comparison to AS.

As expected under drug treatment, and due to the selective pressure applied, AS overgrows AJ which carried the sensitive alleles, this was an expected result and gives us an indication that the selection pressure is being efficient.

5.3.2.2 Selection of the pooled AS-ATN x AJ cross

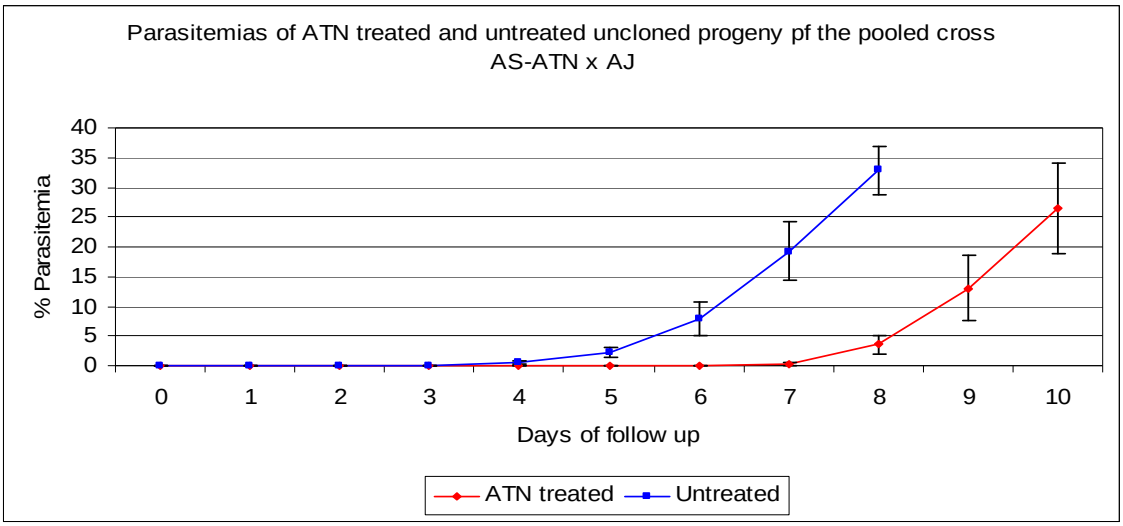


Figure 31 - Parasitaemia curves for the "untreated" and "artesunate treated" uncloned cross progeny of the AS-ATN x AJ cross. Each point corresponds to a mean parasitemia of an experimental group of five mice and the vertical bars are standard deviations.

The dose of artesunate used for LGS of the cross AS-ATN x AJ was 5 mg/kg of mouse body weight daily at 24-h intervals for 5 consecutive days, starting 3h after parasite challenge.

Parasitaemia curves for both artesunate treated and untreated groups for the AS-ATN x AJ pooled cross are shown in Figure 31. The ATN treated group show a delay in the parasite grow due to drug administration, thought it does not show a very marked inhibition of parasite growth when compared to the untreated group, meaning there is not a very big delay on the parasite recrudescence of the treated group indicating presence of relative high amounts of parasites carrying the resistant allele (AS allele) in the polled cross used AS-ATN x AJ.

Table 12 – Proportional sequencing results for each group of the selection procedure for the genetic cross AS-ATN x AJ ("unpassaged", "untreated" and "artesunate treated") for all the polymorphisms of the *dhps* gene analysed. There are four polymorphisms between AS and AJ strains for the gene *dhps*, the mean %AS coming from the quintuplicate measurements was calculated for each polymorphism and finally a mean %AS was calculated as a mean between the four %AS means.

	***dhps* gene (%AS)**				**%AS for the *dhps* locus**
	1	2	3	4	Mean
Unpassaged group	21.9	13.4	24.8	31.7	22.9
Untreated group	30.5	23.5	21.6	17.9	23.4
ATN treated group	7.6	11.4	17.5	16.8	13.3

As expected under drug treatment, and due to the selective pressure applied, AS overgrows AJ which carried the sensitive alleles, this was an expected result and gives us an indication that the selection pressure is being efficient.

5.4 AFLP analysis

5.4.1 General procedure

Once the selection pressure is applied in the genetic progeny of a cross, the parasites that carry the allele that is sensitive (in this case the AJ allele) will be removed, and from an experimental point of view this disappearance is visible by the decrease in intensity or complete disappearance of an AFLP band specific for the sensitive allele (AJ band). Thus, we were looking for the disappearing of AJ sensitive markers on the treated group (artemisinin or artesunate treated) in comparison to the untreated group (an example of an AFLP gel is represented in Materials and Methods Figure 12).

Keeping in mind that the objective is to identify the genomic loci under drug selection either by artemisinin or artesunate that would correspond to a selection valley associated to the resistant phenotype. AFLP markers with low CIs were initially identified and positioned according to the previously defined genetic linkage map of Martinelli A *et al.* 2005. Subsequently, markers under selection were sequenced in order to confirm their location though comparative genomics, making use of a rodent malaria genetic sinteny map.

5.4.2 Results

We determined the CIs of 206 AJ specific AFLP markers and 119 AS specific AFLP markers. A table showing raw data from all markers (AS and AJ specific markers) comparative indices (CI) between all treatment groups is included in Appendix 5.

When in the genetic cross AS-ART x AJ, the ART treated group was compared to the untreated group, 36 of the 206 AJ specific markers showed CIs of less than 0.5 (meaning a reduction of more than 50 % on the band intensity when comparing selected and unselected). These are shown in Table 13, along with their derived positions as located on the genetic linkage map, assigned by Martinelli A and colleagues [Martinelli A *et al.* 2005]. Those positions only correspond to their localization on the genetic map, prior from sequencing of each individual band, which then will eventually allowed a physical position on the parasite chromosome. The markers that were further analysed are represented in grey in the table.

By assigning AFLP markers to the *P. chabaudi* genetic linkage map, it was possible to generate graphs showing the change in marker intensities between treatment groups as they appear across the *P. chabaudi* genome.

5.4.2.1 AFLP analysis for the AS-ART x AJ

Table 13 - CI of AJ specific AFLP markers under selection in the ART treated group, compared to the untreated group for the AS-ART x AJ genetic cross, and their *P. chabaudi* chromosomal locations according to the genetic linkage map.
Group 2, 12, 16 and 38 are linkage groups that could not be assigned to any particular chromosome. No data means there is not any data for the chromosomal position of this marker.
Unlinked means a marker that is not linked to any chromosome or group. The marker name, when a marker shows reduction in both crosses AS-ART x AJ selected with ART and AS-ATN x AJ selected with ATN is coloured in orange. In grey, AJ specific AFLP markers that showed high reduced CI that were sequenced.

Marker name	Comparative intensities (CI)	*P. chabaudi* chromosomal location in genetic linkage map according to Martinelli A *et al.* 2005
AJAC03TA	0.4797	Chromosome 1
AJTT05AT	0.1252	Chromosome 6
AJTA01CA	0.4483	Chromosome 7
AJGA03AC	0.4496	Chromosome 7
AJTG02AT	0.4702	Chromosome 7
AJAG03AG	0.3000	Chromosome 8
AJTT02AT	0.1500	Chromosome 8
AJAT01TA	0.3230	Chromosome 8
AJTG03AA	0.4667	Chromosome 8
AJAC03AT	0.3185	Chromosome 8
AJGA01TT	0.2667	Chromosome 10
AJAG03AC	0.2500	Chromosome 10
AJAC02AC	0.3077	Chromosome 10
AJAC02AA	0.1667	Chromosome 10
AJGA03AG	0.2195	Chromosome 10
AJGA01CA	0.2027	Chromosome 10
AJGA02CA	0.2797	Chromosome 10
AJTT03AT	0.1538	Chromosome 11
AJTT04AT	0.1364	Chromosome 12
AJAT02CT	0.4141	Chromosome 12
AJTA01AG	0.3622	Chromosome 13
AJAA01TA	0.4000	Group 2
AJAG02AT	0.3333	Group 2
AJGA01GA	0.3770	Group 2
AJAC05AT	0.4903	Group 2
AJTA03AC	0.3617	Group 2
AJAC01TT	0.4379	Group 2
AJGA02AC	0.4398	Group 12
AJTG04AT	0.4706	Group 16
AJTT01AT	0.1488	Group 33
AJTA02AG	0.2857	Group 38
AJAC01TG	0.4528	Group 38
AJGA01TA	0.3182	Group 38
AJAC04.5AT	0.3600	No data
AJTC02AG	0.4528	No data
AJGA03AT	0.4505	Unlinked

Figure 32 shows the CI of AJ specific AFLP markers between the ART treated and the untreated group. It was found that all of the AJ markers which were most reduced under ART

pressure lie in chromosomes 8 and 10 (in the graph with a red arrow) of *P. chabaudi* and groups 2 and 38 (unassigned).

Figure 33 shows the same graph but for the AS specific AFLP markers. No markers showed significant reductions in the CI between the ART treated and untreated group, except in the case of three markers (represented in the graph by a red arrow) which seeme to be found under selection, although the reason for this is unclear.

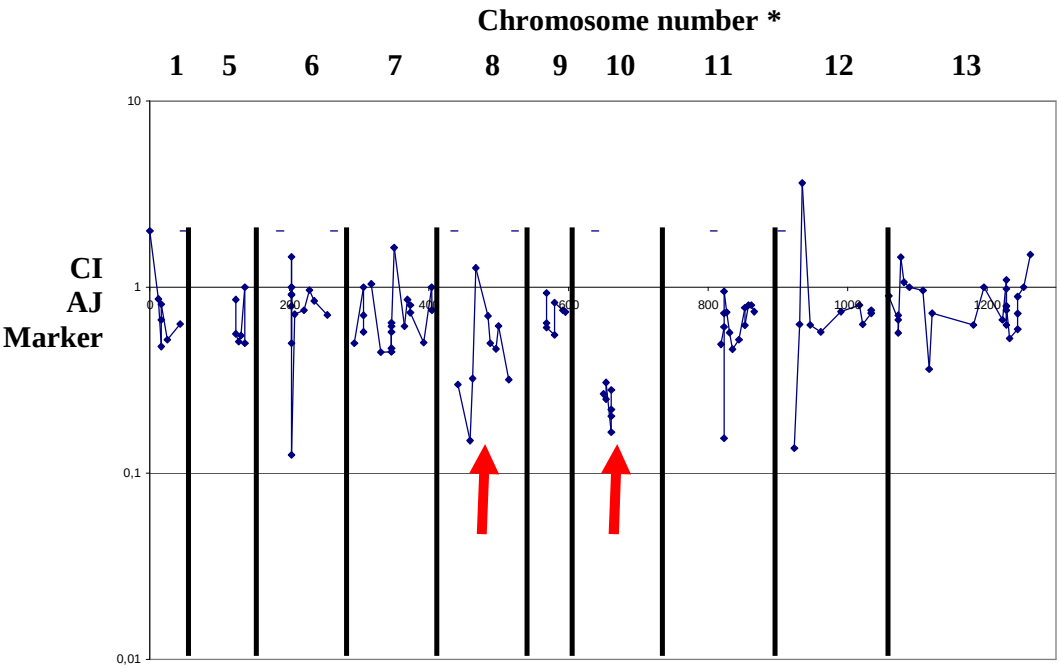


Figure 32 - Comparative Intensities (CI) of AJ (sensitive parent) specific AFLP markers in the ART treated group compared to the untreated group, for the AS-ART x AJ cross.

P. chabaudi chromosome numbers (Chromosome number *) correspond to the markers chromosomal location in genetic linkage map according to Martinelli A *et al.* 2005 (prior to individual band sequencing).

Markers are positioned on the X axis according to their positions in cM along the chromosomes. Each point corresponds to an AJ-AFLP specific marker and their position along the chromosome. The gaps correspond to areas without any marker.

The two red arrows indicate the chromosomes where there is a bigger AJ specific marker selection thus a bigger drug selection.

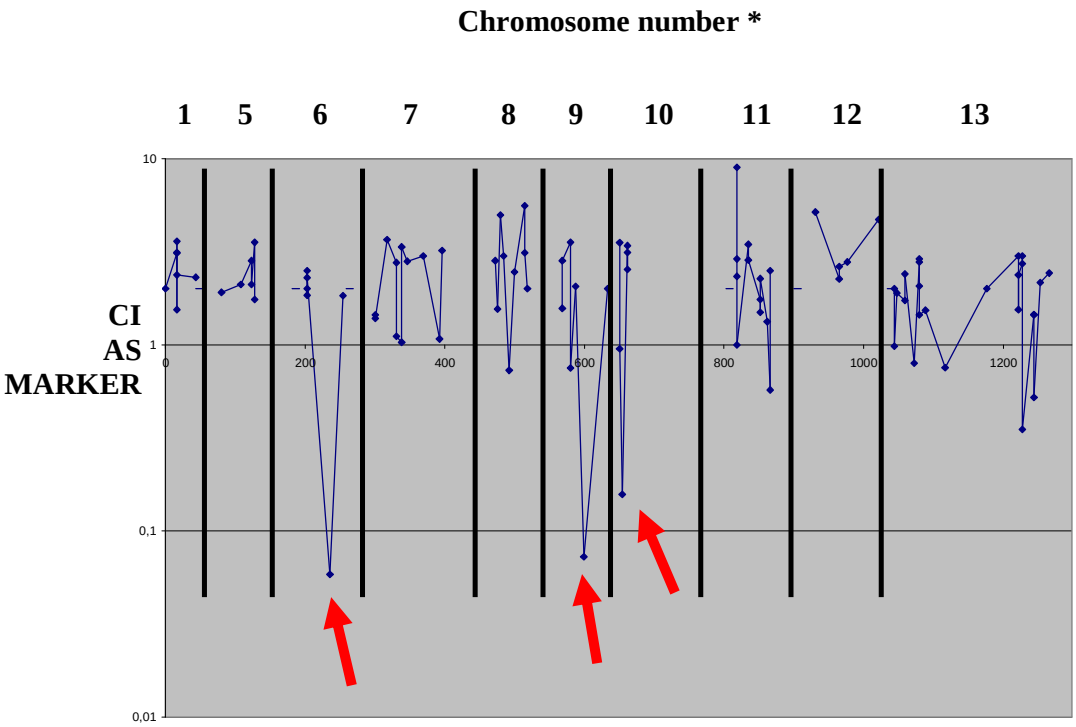


Figure 33 - Comparative Intensities (CI) of AS (resistant parent) specific AFLP markers in the ART treated group compared to the untreated group, for the AS-ART x AJ cross.

P. chabaudi chromosome numbers (Chromosome number *) correspond to the markers chromosomal location in genetic linkage map according to Martinelli A *et al.* 2005 (prior to individual band sequencing).

Markers are positioned on the X axis according to their positions in cM along the chromosomes. Each point corresponds to an AS-AFLP specific marker and their position along the chromosome. The gaps correspond to areas without any marker.

5.4.2.2 AFLP analysis for the AS-ATN x AJ

Once AFLP analysis was done for the AS-ART x AJ cross we wished to investigate whether similar loci were involved in the selection of the pooled AS-ATN x AJ cross after artesunate treatment. 13 of the 206 AJ markers showed CI of less than 0.5 (meaning a reduction of more than 50 % on the band intensity when comparing the treated and the untreated groups). These are shown in Table 14, along with their derived positions as located on the linkage map.

Only four markers were reduced on both the pooled AS-ART X AJ selected with ART and this one, pooled AS-ATN x AJ selected with ATN, those were: AJAG03AC located on *P. chabaudi* chromosome 10, AJTA01AG located on *P. chabaudi* chromosome 13, AJAC05AT a marker that belongs to group 2 and finally AJGA03AT a marker that is unlinked (represented in orange in Tables 13 and 14).

Though only one marker on chromosome 10 (AJAC02AC) was significantly reduced in this cross, all the AFLP markers from this chromosome are represented in Table 14 in grey, due to the fact that although their CI indices in this pooled AS-ATN x AJ were reduced to border line values to the cut level of 0.5 of CI those same markers on *P. chabaudi* chromosome 10 were significantly reduced in the pooled cross AS-ART x AJ.

Table 14 - CI of AJ specific AFLP markers under selection in the ATN treated group, compared to the untreated group for the AS-ATN x AJ genetic cross, and their *P. chabaudi* chromosomal locations according to the genetic linkage map.

Group 2 and 20 are linkage groups that could not be assigned to any particular chromosome.

Unlinked means a marker that is not linked to any chromosome or group.

The marker name, when a marker shows reduction in both crosses AS-ART x AJ selected with ART and AS-ATN x AJ selected with ATN is coloured in orange.

In grey, AJ specific AFLP markers that showed high reduced CI that were sequenced.

Marker name	Comparative intensities (CI)	*P. chabaudi* chromosomal location in genetic linkage map according to Martinelli A *et al.* 2005
AJTC01TG	0,4549	Chromosome 8
AJGA01TT	0,6667	Chromosome 10
AJAG03AC	0,5556	Chromosome 10
AJAC02AC	0,3571	Chromosome 10
AJAC02AA	0,6667	Chromosome 10
AJGA03AG	0,6667	Chromosome 10
AJGA01CA	0,5316	Chromosome 10
AJGA02CA	0,6866	Chromosome 10
AJTT02GA	0,4545	Chromosome 11
AJTT01CT	0,0313	Chromosome 12
AJTA01AG	0,4097	Chromosome 13
AJAG02TT	0,4444	Group 2
AJAC05AT	0,4419	Group 2
AJGA01GA	0,3684	Group 2
AJTC02TA	0,4885	Group 20
AJAT02CT	0,4426	Group 20
AJGA03AT	0,4865	Unlinked
AJTC01AT	0,4750	Unlinked
AJTT03GA	0,4362	Unlinked

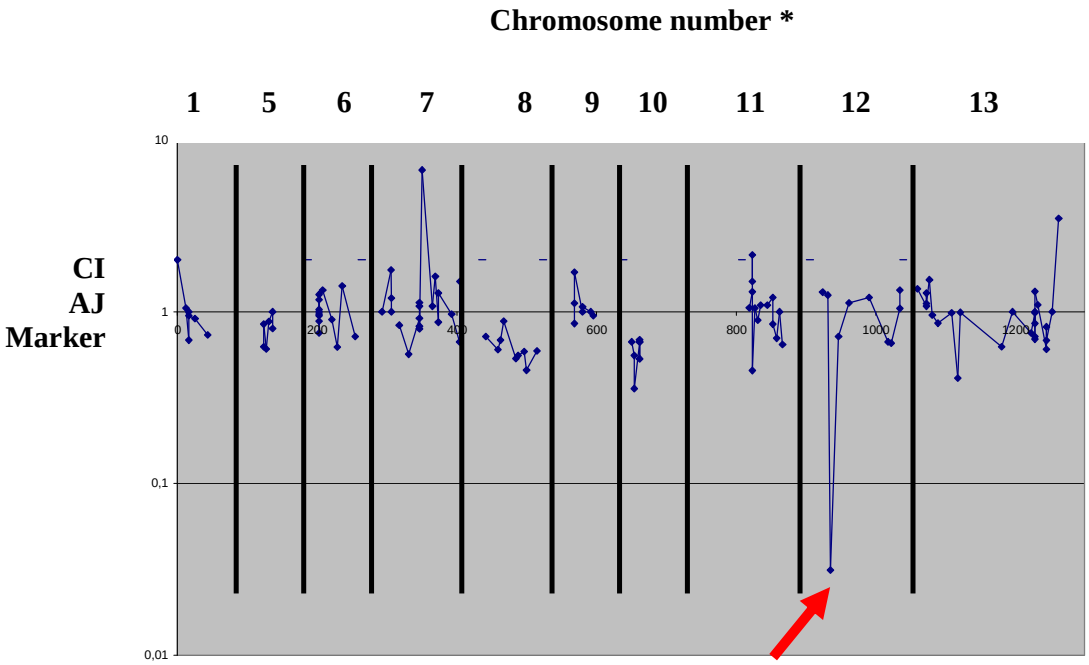


Figure 34 - Comparative Intensities (CI) of AJ (sensitive parent) specific AFLP markers in the ATN treated group compared to the untreated group, for the AS-ATN x AJ cross.

P. chabaudi chromosome numbers (Chromosome number *) correspond to the markers chromosomal location in genetic linkage map according to Martinelli A *et al.* 2005 (prior to individual band sequencing).

Markers are positioned on the X axis according to their positions in cM along the chromosomes. Each point corresponds to an AJ-AFLP specific marker and their position along the chromosome. The gaps correspond to areas without any marker.

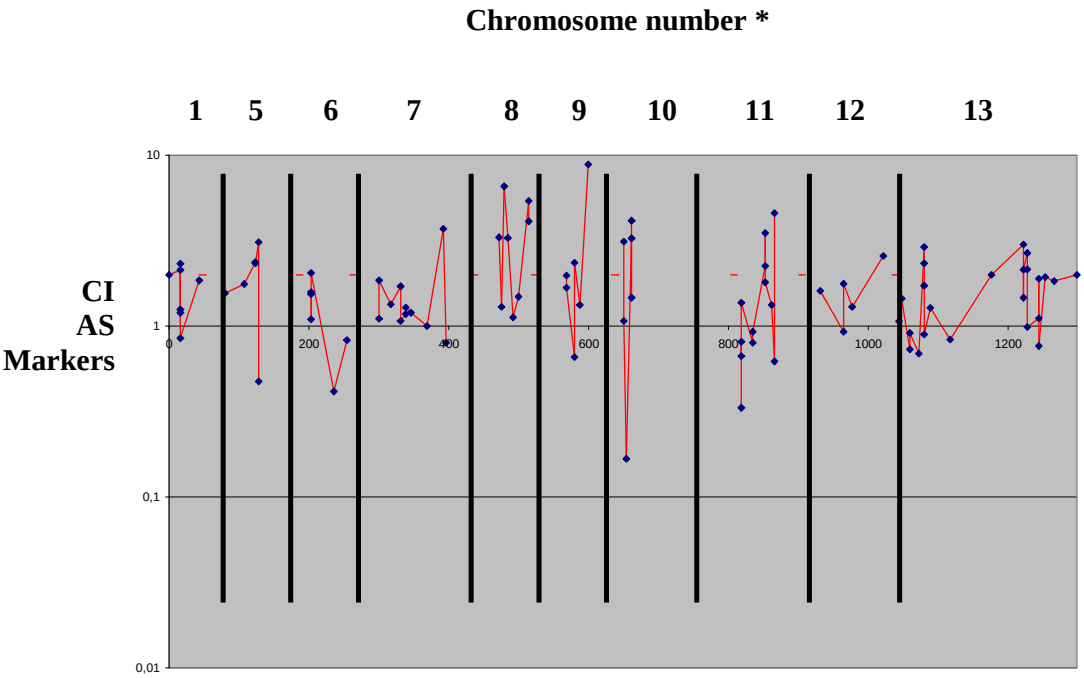


Figure 35 - Comparative Intensities (CI) of AS (resistant parent) specific AFLP markers in the ATN treated group compared to the untreated group, for the AS-ATN x AJ cross.

P. chabaudi chromosome numbers (Chromosome number *) correspond to the markers chromosomal location in genetic linkage map according to Martinelli A *et al.* 2005 (prior to individual band sequencing).

Markers are positioned on the X axis according to their positions in cM along the chromosomes. Each point corresponds to an AS-AFLP specific marker and their position along the chromosome. The gaps correspond to areas without any marker.

5.4.2.3 AFLP analysis comparison between pooled crosses AS-ART x AJ and AS-ATN x AJ

As summary of the AFLP analysis on the two pooled crosses:

A – There is a general reduced pattern of selection in the AS-ATN x AJ pooled cross in relation to the AS-ART x AJ that may potentially be attributed to a reduced number of recombinants in the AS-ATN x AJ cross;

B – There are four main genetic loci under strong selection, according to the *P. chabaudi* genetic linkage map defined by Martinelli A and colleagues [Martinelli A *et al.* 2005] on chromosomes 8 and 10 and two unlinked groups 2 and 33. The AFLP bands from these loci were sequenced and results will be presented in the next section.

5.5 Sequencing and mapping of AFLP bands under selection

5.5.1 General procedure

The genetic linkage map [Martinelli A *et al.* 2005] shows the position of markers upon the *P. chabaudi* genome as determining from the characterisation of 28 clones of a cross between AS and AJ.

The way the genetic linkage map was prepared and published by Martinelli A and colleagues [Martinelli A *et al.* 2005] may not reflect exactly the correct physical linkage in the genome, thus and to be absolutely sure about the genetic and chromosomic position of these markers sequencing is necessary. In this context, with the objective of obtaining the exact correct physical position of those markers in the genome and mapping their physical chromosomal/gene position all AFLP markers were sequenced. The strong synteny between *P falciparum* and the rodent malaria parasites [Carlton JM *et al.* 1998; Hunt P *et al.* 2004] and the synteny map compiled by Kooij TW and colleagues [Kooji TW *et al* 2006], allowed us to physically localize the markers in relation to genes closely linked.

AFLP bands that appeared to be under selection were sequenced and the corresponding sequences in the *P. falciparum* genome were identified [Hunt P *et al*. 2004b]. The sequences obtained in the *P. falciparum* database were then used in BLAST searches against the *P. chabaudi* database in addition to the genome-wide previously described syntenic map [Kooij TW *et al*. 2006]. See Table 15 for details.

5.5.2 Results

All the data from AFLP markers sequencing is summarized in Table 18. Though there were other markers under selection either by artemisinin or artesunate (see Tables 16 and 17), their sequences are not presented there due to the fact that they were either not possible to assigned unambiguously to any particular area in *P. chabaudi* genome or due to poor sequence quality. So as a result summary from the AFLP sequencing analysis:

A – Three AJ specific markers were found to be under selection on *P. falciparum* chromosome 6 that are allocated, using the *P. falciparum* rodent malaria synteny map, on *P. chabaudi* chromosome 1;

B – Two AJ specific markers were found to be under selection on *P. falciparum* chromosome 12 and one on *P. falciparum* chromosome 13 that are all allocated, using the *P.falciparum* rodent malaria synteny map, on *P. chabaudi* chromosome 14;

C – Two AJ specific markers were found to be under selection on *P. falciparum* chromosome 9 that are allocated using the *P. falciparum* rodent malaria synteny map, on *P. chabaudi* chromosome 8;

D – Four AJ specific markers were found to be under selection on *P. falciparum* chromosome 7 and one on *P. falciparum* chromosome 1 all of them are allocated using the *P. falciparum* rodent malaria synteny map, on *P. chabaudi* chromosome 2 (see also Figure 36).

Mostly due to the evidence presented above that all AJ specific AFLP markers on *P. chabaudi* chromosome 2 were under strong selection further analysis was only done on this chromosome. The other *P. chabaudi* loci under selection; chromosome 1, 8 and 14 will be analysed within the scope of other projects.

Table 15 - Physical and genetic mapping of the AFLP markers with low CI.Most of the AJ specific AFLP markers that were found to have CIs under 0,5 are presented. Their names, CIs and physical localization according to Martinelli A *et al.* 2005, and the gene annotation got from the *P. chabaudi* database is also presented. Their chromosomal position in the *P. falciparum* genome and their correspondence after using the correspondences facilitated by the use of the synteny map are also presented.Pfxx means the chromosome number in *P. falciparum*. Na – Not possible to assign. Adapted from: Hunt P *et al.* in press at the Molecular Microbiology, 2007, with kind permission of Dr. Paul Hunt.

AFLP Marker	**Comparative Intensities (CI)**	***P. chabaudi* linkage group according to Martinelli A et al. 2005**	**Gene name**	***P. falciparum* genomic locus**	***P. chabaudi* chromosome location according to synteny from Kooij TW et al. 2006**
AJAT01TA	0.3230	Chromosome 8	hypothetical	Pf09	8
AJAC03AT	0.3185	Chromosome 8	hypothetical	Pf09	8
AJGA01TT	0.2667	Chromosome 10	hypothetical	Pf07	2
AJAG03AC	0.2500	Chromosome 10	Insufficient sequence for analysis	Na	Na
AJAC02AC	0.3077	Chromosome 10	Insufficient sequence for analysis	Na	Na
AJAC02AA	0.1667	Chromosome 10	Ubiquitin carboxy-terminal hydrolase	Pf07	2
AJGA03AG	0.2195	Chromosome 10	hypothetica	Pf07	2
AJGA01CA	0.2027	Chromosome 10	hypothetical	Pf01	2
AJGA02CA	0.2797	Chromosome 10	hypothetical	Pf07	2
AJAA01TA	0.4000	Group 2	hypothetical	Pf12	14
AJAG02AT	0.3333	Group 2	Translation initiation factor-2, putative	Pf13	14
AJAC05AT	0.4903	Group 2	hypothetical	Pf12	14
AJGA01TA	0.2857	Group 38	hypothetical	Pf06	1
AJAC01TG	0.4528	Group 38	hypothetical	Pf06	1
AJTA02AG	0.3182	Group 38	hypothetical	Pf06	1

5.6 Further investigation of markers under selection on chromosome 2

From a detailed observation of the synteny map in Figure 10, it is clear that *P. chabaudi* chromosome 2 is syntenic with two blocks of the *P. falciparum* genome, on chromosomes 1 and 7 [Kooij TW *et* al. 2006]. One should also note that the fragment from *P. falciparum* chromosome 1 in inverted on *P. chabaudi* chromosome 2. See Figure 36 for details.

When we joined the syntenic *P. falciparum* block of approximately 330 kb from chromosome 1 with the syntenic *P. falciparum* block of approximately 220 Kb from the end portion of *P. falciparum* chromosome 7 we obtained a region of 550 Kb in *P. falciparum* that is expected to be most similar in size in *P. chabaudi* due to the big similarities already published between genome sizes of *P. falciparum* and rodent malaria parasites [Carlton JM *et al*. 2002]. The approximately 150 Kb difference between the described 700 Kb size of *P. chabaudi* chromosome 2 and the 550 Kb obtained by the synthenic map can be due to the fact that telomeres and genes from the chromosomes extremities due to the fact that are very variable are not included in the syntenic map between *P. falciparum* and *P. chabaudi.*

An interesting finding concerning the genetic localization of the markers found to be under selection and their position on *P. falciparum* genome is that the gene that has been mostly suggested and described as the target for artemisinin, the *SERCA-type* Ca^{2+}*-dependent atpase* gene [Eckstein-Ludwig U *et al.* 2003] is genetically localized very near the markers under selection.

The AJ specific marker AJAC02AA annotated as an *ubiquitin carboxyl-terminal hydrolase, putative* gene, for simplicity named *ubp-1,* was sequenced and two major mutations were subsequently detected between different parasite clones as described in the ensuing sections.

Briefly, ubiquitin hydrolases promote the catalysis of the hydrolysis of non terminal peptide linkages in oligopeptides or polypeptides. Ubiquitin hydrolases are involved in the hydrolysis of esters, including those formed between thiols such as dithiothreitol or glutathione and the C-terminal glycine residue of the polypeptide ubiquitin [Hall N *et al.* 2002].

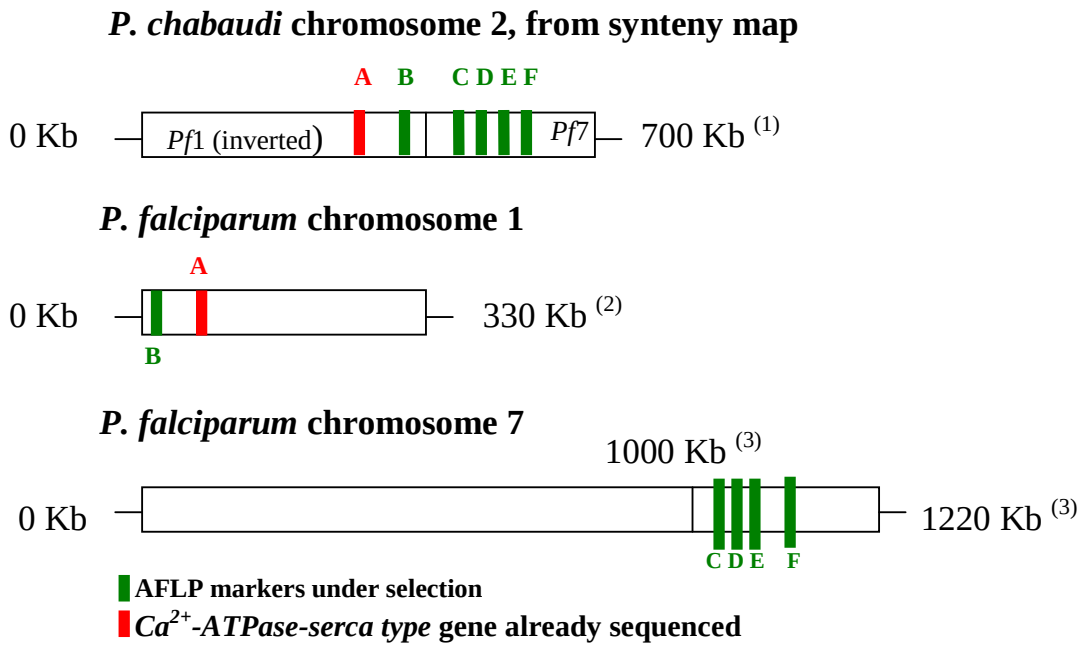


Figure 36 - *P. chabaudi* chromosome 2 with syntenic blocks represented, *P. falciparum* chromosome 1 and *P. falciparum* chromosome 7.

In red (A) the *Ca^{2+}-ATPase-serca type* gene.

In green the AFLP markers that were sequenced.

Legend for AFLP markers:

B – AJGA01CA

C – AJAC02AA

D – AJGA03AG

E – AJGA02CA

F – AJGA01TT

See Table 18 for details on the AJ specific AFLP markers.

Note (1): The dimension of *P. chabaudi* chromosome 1 is approximately 750 Kb. This result was obtained from pulse-field gel electrophoresis analysis of *P. chabaudi* clones AS and AJ by Carlton JM [Carlton JM, personal communication].

Note (2): 330 Kb is the dimension of the *P. falciparum* synthenic block. The published size of this chromosome is 644 Kb. 330 Kb does not include telomeres and the chromosome extremities where genes like *strevor, rifins* and *cir* are found.

Note (3): 1220 Kb approximately the dimension of the *P. falciparum* chromosome 7 defined by the synteny map the first synthenic block is approximately 1000 Kb long and the second synthenic block is approximately 220 Kb long. The published size of this chromosome is 1352 Kb.

5.6.1 Identification of *ubp-1* gene mutation on *P. chabaudi* chromosome 2

The entire sequence, primers used for its amplification, predicted protein sequence and gene and protein alignments relative to the *ubp-1* gene are presented in Appendix 4.

Due to the fact that a mutation was found between the DNA sample from the clone AS-ART and the one from the clone AS-ATN, the other clones and parasite lines were fully sequenced; those were AS-SENS, AS-15CQ and AS-30CQ.

The full *ubp-1* gene sequence and alignments can be seen found in Appendix 4. For a question of simplicity and interest for the discussion, the portion of the alignments where mutations are to be found is presented in Figure 37. A summary of gene/protein analysis of the changes found in the *ubp-1* gene is depicted in Table 16.

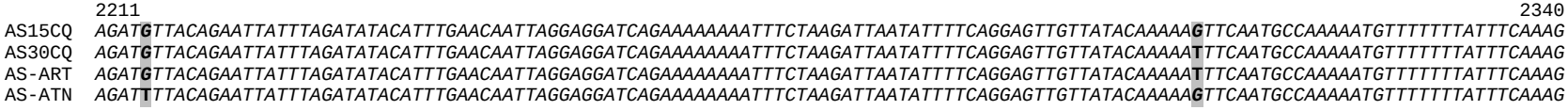


Figure 37 – Part of the *ubp-1* gene alignments where the different mutations are highlight.

The rest of the gene alignment is represented on Appendix 4.

Just to resume the gene/protein analysis of the changes found on the *ubp-1* gene Table 16 was built.

Table 16 – A summary of the *ubp-1* gene mutations and their correspondence on ubp-1 transcript for each of the *P. chabaudi* clones and parasite lines analysed.

***P. chabaudi* clone or parasite line**	**DNA**		**Protein**	
	2215 bp	**2308 bp**	**739 aa**	**770 aa**
AS-15CQ	G	G	V	V
AS-30CQ	G	T	V	F
AS-ART	G	T	V	F
AS-ATN	T	G	F	V

From Table 16 and Figure 37 it can be seen that during the process of artesunate resistance a mutation base 2215 of the *ubp-1* gene had occurred. This was a transversion where a G was changed into a T. This change generates a non-synonymous substitution in the ubp-1 protein that results in a swap from a valine in codon 739 to a phenylalanine (V739F).

Also, in base position 2308 of the *ubp-1* gene, a difference was found between the parasite line AS-15CQ and the clone AS-30CQ (obtained from AS-15CQ after chloroquine pressure), meaning that during the chloroquine selection this gene suffered a transversion of a G to a T in this base. This gene change corresponds to a non-synonymous substitution in the ubp-1 protein from a valine to a phenylalanine at residue 770 (V770F).

As a summary from the results obtained for the mutational analysis of the *ubp-1* gene:

a) A G to T transversion occurred in DNA base 2308 from AS-15CQ, intermediate chloroquine resistant, to AS-30CQ, high chloroquine resistant, under chloroquine pressure.

b) A G to T transversion occurred in DNA base 2215, from AS-15CQ to AS-ATN under artesunate pressure.

c) The G2215T mutation above does not exist in the AS-30CQ nor in the AS-ART clone. Thus whilst this mutation may be responsible for the appearance of artesunate resistance in the AS-ATN clone, the gene changes responsible for the appearance of artemisinin resistance after artemisinin pressure on clone AS-30CQ are yet to be found.

At this stage it seemed important to evaluate the biological and biochemical significance of the mutations found in the *P. chabaudi* gene homologue of the *P. falciparum ubp-1* gene. A profound analysis of this significance was done by Hunt P and colleagues (Hunt P *et al.* in press at the Molecular Microbiology, 2007) and it is out of the scope of this thesis.

For this thesis only very preliminary comparisons were made between the ubp-1 predicted protein of *P. chabaudi* and for *P. falciparum.* The two proteins, ubp-1 from *P. chabaudi* and ubp-1 from *P. falciparum* are only moderated similar, possessing a very high degree of identity only in a particular area of the protein, near the C-terminus of the protein. Because both mutations V739F and V770F are mapped to the C-end portion of the ubp-1 protein it is expected that these mutations occurred in parts of the protein/enzyme that are very important, maybe even fundamental for its biological activity.

5.7 Discussion

Most of this chapter was devoted to present results from the use of LGS in the analysis of pooled genetic crosses called AS-ART x AJ and AS-ATN x AJ. LGS was chosen as a faster approach to identify the genetic loci involved in artemisinin and artesunate resistance.

For the artemisinin resistant line, AS-ART, three independent genetic crosses were obtained, these three were not analysed individually and were pooled together in a super cross named AS-ART x AJ. For the artesunate resistant line, AS-ATN, two independent genetic crosses were obtained, theses two independent crosses were not analysed individually and were also pooled together in a super cross named AS-ATN x AJ.

Four loci were found to be under selection in the uncloned progeny of the treated pooled crosses: *P. chabaudi* chromosomes 1, 2, 8 and 14.

A note to the fact that the selection observe in the AS-ATN x AJ was much less pronounced than the one that we were allowed to observe in the AS-ART x AJ cross. One possible explanation to this fact is that in the AS-ATN x AJ pooled cross there was a limited number of genetic recombinants. An alternative explanation can also be that the artesunate dose given to the cross was higher that the one that allows a more correct discrimination between resistant and sensitive progeny.

Due to the fact that all markers on *P. chabaudi* chromosome 2 were under strong selection, further analysis was only done on *P. chabaudi* chromosome 2. Within this chromosome 2 a gene encoding a deubiquitinating enzyme (ubp-1 protein) was found to be mutated twice. A transversion of a G to a T was found in DNA base 2215 of the *ubp-1* gene, in the clone AS-ATN in relation to its parasite line progenitor AS-15CQ, corresponding to a protein change of V739F. The same gene was also found mutated in base 2308 in the clone AS-30CQ when compared to its progenitor AS-15CQ. Thus the V739F change occurred from AS-15CQ to AS-ATN under artesunate pressure, independently from the V770F change that occurred from AS-15CQ to AS-30CQ under chloroquine pressure. From the very preliminary alignments done between the *P. falciparum* and *P. chabaudi* proteins it was possible to observe that the changes occurred in the most conversed area of the protein thus for that reason supposedly important maybe even fundamental for the enzyme function.

The analysis of this gene and these two mutations is most incomplete and the physiological significance of these mutations is still to clarify. Plus the fact that there were no mutation found in any of the genes analysed so far that could explain or justify the artemisinin resistant phenotype.

The failure to find a new mutation in the AS-ART clone can be explained by the fact that there may still be a gene, lets call it gene A, different from *ubp-1* gene but still on *P. chabaudi* chromosome 2, which is indeed mutated and which mutation arose during artemisinin drug selection. If on future further analysis of this chromosome 2 other gene is found to be mutated, then the selection observed after artemisinin and artesunate treatment on the AS-ART x AJ and AS-ATN x AJ pooled crosses can be explained. This gene A should also be found to be mutated on the AS-ATN clone.

But a pendent question will then remain, if in the future another gene either on chromosome 2 or any other chromosome, either found under selection like *P. chabaudi* chromosome 1, 8 and 14 or not found under selection, is found mutated and the mutation found then could indeed explain or justify the resistant phenotype. The question will then be: what would be the importance of the mutations already found in the *ubp-1* gene? One explanation is that mutations on the *ubp-1* gene may be compensatory mutations. By compensatory mutations we mean a mutation that is not directly responsible for the resistant phenotype *per si* but on one hand is responsible for a change in a protein that might be involved in increasing the parasite general fitness, giving him proliferative and surviving advantages in relation to the ones that do not posses the same mutation.

But an important note has to be made again because if another gene on another *P. chabaudi* chromosome either 1, 8 or 14 or any other not under selection on these LGS experiments is indeed found mutated there is still unanswered the question, why is there a selection valley on chromosome 2?

A very curious finding was that two different, but at the same time similar mutations were found in the *ubp-1* gene, that occurred during chloroquine and artesunate selection. Why this particular gene? Maybe the ubp-1 protein is involved in a particular cellular function involved in drug metabolism like for example the regulation of oxidative stress and drug detoxification [Krungkrai SR *et al.* 1987, Tilley GJ *et al.* 2001].

Artemisinin and its derivatives resistance is most likely to be a multigenic phenomenon and many reasons can be pointed to support this statement: first the fact that artemisinin resistance in the field is yet to be reported though its enormous use makes it likely, even in vitro stable resistance (besides this study) an though many researchers have tried is yet to be reported. One can even suggest evidences for the phenomenon of multigenic resistance when in our project four loci were found to be under drug selection (though *P. chabaudi* chromosome 2 was under greater selection), might each loci correspond to a different gene involved in the resistant phenotype? Future studies on these four loci are necessary but before engaging in further analysis on *P. chabaudi* chromosome 1, 8 and 14 for example the recommended

procedure is to narrow down the selection valleys and to do that the pooled genetic crosses used in this project should be submitted again to another genetic cross (so called back-crossing) and another round of LGS.

Another future question that should be answer is what effect might be expected for *ubp-1* gene mutations on malarial parasites. This discussion is largely done in Hunt P *et al.* in press at Molecular Microbiology of which I am a co-author (this paper is in Appendix to this thesis). Thus I would not like to repeat Hunt P and colleague's arguments. *Ubp-1* gene allelic exchange transfection on *P. falciparum* parasites in culture and evaluation of the importance of the mutations found on this gene.

CHAPTER VI

GENERAL CONCLUSIONS

A - Chapter III

Stable resistance to artemisinin and one of its derivatives, artesunate, was described for the first time to our knowledge in a rodent malaria model, *P chabaudi*.

A line was selected that was 6-fold more resistant to artesunate than the initial progenitor, AS-ATN and other was selected that was 15-fold more resistant to artemisinin than the initial progenitor, AS-ART. Importantly, these results showed that malaria parasites are capable of developing resistance to these drugs and that this resistance can be passed on through generations, because it is genetically stable.

There was full cross resistance between ART and ATN selected parasites clone lines. This is a strong indication that genetic mechanisms in common might be involved in drug resistant to ART and ATN.

The resistant clones obtained were used downstream to investigate the putative mutations involved in these phenotypes.

B - Chapter IV

The previously described putative genetic modulators for artemisinin and its derivatives, *Plasmodium chabaudi* homologues of the genes *pfmdr*, *pfcrt*, *pftctp* and *pfatp6* were sequenced and their gene copy number calculated in relation to their progenitors.

No changes were found, between resistant parasites and related sensitive ones allowing the conclusion that, at least in this model, resistance is not determined by these genes.

C – Chapter V

Genetic crosses between AS-ART and AJ and AS-ATN and AJ were done. These were submitted to LGS and four loci were considered to be under selection. These correspond to areas in *P. chabaudi* chromosomes 1, 2, 8 and 14.

This complex picture clearly points towards a multigenic mechanism of resistance to artemisinin and its derivatives.

Detailed analyses of chromosome 2 allowed to uncover mutations in a gene denoted *ubp-1* whuich may be implicated in resistance to both artesunate and chloroquine. No novel mutations could be detected in artemisinin-resistant parasites however implying that understanding the genetic basis of resistance to this drug requires further efforts.

There may be many hypothetical roles for the involvement of this gene in drug resistance, whose study lie outside the time frame of this work. Evidence of a particular role for the mutations found in the *ubp-1* gene in the rodent malaria parasite *P. chabaudi* or, eventually, in the human malaria parasite, *P. falciparum* is not yet demonstrated. In this trend, further investigations on the role of the mutations detected on *ubp-1* gene are necessary. Allelic replacement experiments represent an attractive strategy to elucidade functional properties of the *ubp-1* mutations disclosed.

SUPPLEMENT

APPENDIX 1

SOLUTIONS AND BUFFERS

1:1 FCS: Ringer's Solution
50% (v/v) heat inactivated foetal calf serum
50% (v/v) mammalian Ringer's solution
20 units heparin/ml mouse blood

Buffer A
150 mM NaCl
25 mM EDTA

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

Deep-freeze solution
28% (v/v) glycerol
3.0% (v/v) sorbitol
0.65% (v/v) NaCl- sterilise by filtration.

Mammalian ringer solution
27mM KCl
27mM $CaCl_2$
0.15M NaCl

TBE buffer (sterilised by filtration)

100nM Tris

100 mM Boric Acid

2 mM EDTA (Ethylenediaminetetraacetic Acid)

TE buffer (Tris/EDTA)

10mM Tris-HCl

1mM EDTA

APPENDIX 2

GENE AND PRIMER SEQUENCE FOR THE GENES *pcmdr1, pccg10, pctctp* and *pcatp6*

A-*pcmdr1* gene

Note: Primers are underlined in the sequence.

TATATTATTACAAAAAGGTCTATAGATGTTGTAAAATTTGTTGTCTTAAATATATTCAGTTATTTTATAAGTATGCATAATTTGTGTCGTTATC
CACAC(45)TATAAAATTACAGAATAAAGTAATCACATATTTTTAACGATAGACATATTTATTTATGATTGCGCTTGATAATATATACCTCATT
ATATAATTTCTTAATACCTGATTTTTCAAAATGGCGGACAAAAGAAGTAATAACAATAGTATCAAAGATGAAGTTGAGAAAG(34)AGTTAAAT
AAGCAATCCACCGTTGATCTGTTTAAAAAG(37)ATAAAGAAACAAAATATCCCACTGTTTTTGCCATTTCATTCACTACCATCCAAACACAAG
AGATTATTAGCTATATCTTTTATATGTGCGACAATATCAGGAGCTGCGTTACCCATTTTTATTTCGGTGTTTGGTCTTACTATGGCAAATATGA
ATATGGGAGAGAGCGTAAATGACATAGTTTTAAAATTAGTAATAGTTGGTATATGCCAATTTGTGATATCATCGATTTCATGTTTATGCATGGG
TACTGTTAC(29)TACAGAGATTATAAGAATGTTAAAATTAAAATATTTAAAAAGTGTTTTTCATCAAGATGGAGAATTTCATGATAATAACCC
AGGTTCTAAATTAACATCCG(30)ATTTAGATTTTTATTTAGAACAAGTAAATTCAGGAATAGGAACAAAATTTACTACAATATTTACATATAG
CAGTTCATTCTTAGGTTTGTATTTCTGGTCACTATATAAAAATGTAAGATTAACATTATGTGTTACATGTGTTTTTCCAGTAATATATATAATC
AGTACGATATGTAATAAAAGAGTTCGATTAAATAAAAAAACATCATTATTATATAATAACAATTCAATGTCTATAATTGAAGAAGCATTAGTTG
GTATTAAAACTGTTGTAAGTTATTGTGGAGAAAGTACAATATTAAAAAAATTTAAATTATCAGAACAATTTTACAGTAAATACATGTTAAAGGC
(44)AAATTTTATGGAATCGCTACATGTTGG(39)TTTTATTAATGGATTTATATTAGCTTCATATGCTATGGGATTTTGGTATGG(23)TACT
AGAATTATGATACATGATATTAAAAATCTGAGTTCACCCAGCGATTTC(25)AATGGAGGATCAGTTATATCAATTTTATTAGGAGTTCTTATA
AGTATGTTTATGCTTACTATTATATTACCAAATGTTACTGAATATATGAAAGCGTTAGAGGCAACGAACAATATACATGAAGTTATTAACAGAA
AACCAGCTGTTGACAGAAATCAAAATAAAGGTAGAAAATTAGATGATATTAAAAAAATAGAATTTAAAAATGTCAAATTTCATTATGGTACTAG
(40)AAAAGATGTTGAAATTTATAAGGATTTAAATTTTACATTAAAAGAAGGAAATACTTATGCATTTGTTGGAGAATCTGGATGTGGTAAATC
AACAATTTTAAAATTGCTTGAGCGATTTTATGATCCAACAGAAGGAGATATTGTTATTAATGGTGCACACAATTTGAAAGACGTTGACTTAAAA
TGGTGGAGATCTAAAATTGG(17)AGTAGTTAGTCAAGATCCTTTATTATTTAGCAATTCGATTAAAAATAATATTAAATATAGTTTAATAAGT
CCAAATACTTTAGAAGCAATGGAAAATGGATTCACCATGAACGGAAGTAGCGATTCTTCATTAAATAATAACAAAAATGGAAAGTCCACTAGTA
TTTTGGATGAAATATCTAAGAGAAATACAACTAATGATTTATTAGAAGTAATATCATCTATTAATTCAGTTGAAGATTCAAAAGTAGTTGATGT
ATCTAAGAAAGTGCTAATCCACGATTTTGTTGCAGCATTACCAGACAAATATGACACTTTAGTAGGTTCTAGCGCATCTAAGTTGTCAGGTGGA
CAAAAACAAAGAATATCGATAGGTAGAGCTGTTATTAGAAACCCTAAGATTTTAATACTTGATGAAGCTACATCATATCTTGATAATAAATCAG
AATATTTAGTTCAGAAAACAATTAACAATTTAAAAGGAAATGAAAATCGTATCACCATTATTATTGCTCATAGATTGAGTACTATTCGATATGC
(26)TAACCAAATTTTTGTCTTATCAAATAGAGATCAAGAATCAACAGGAATTGATGAAAAAAAACAAGGTGCTATAAATAGCAATAACGGAAG
TGTAATAGTTGAACAAGGTACTCATGATAGTTTGATGAAAAATAAAAATGGTATTTACTATTCTATGATTCAAAACCAGAAAGTATCCTCAAGT
GGAAATGGTGAAAATGGTGATGACAATAATAGTAGCGTATATAAAGACTCTAATCCAGGTGATGCTAAATCTGTTACTG(27)ATACAAATATG
GACATTGGTACAAATAAAAATCTTAATACTAAAAAAGAAAAAGAGATTGCTGATGCTGATAAACAAACTAAACCATCAATCTTTAAAAGAATGT
TTGGAAAGAAAAAGAAGAAACCTAACAATTTGAATATGGTGTATAAAGAAATATTTTCTCACAGAAAAGAGGTTGCTATTATGCTTTTAAGTAC
TATAGTAGCAGGTGGTTTATATCCATTGTTTGCTGTATTATATGCAGAATACGTTGTGACATTATTTGATATCCCGAACTTAGAATATAATTCA
AATAAATATTCTATATTCATATTGTTTATTGCTTTAGCTATGTTTATTTCTGAAACATTAAAAAATTATTACAATAATAAAATTGGAGAAAAGG
TTGAAAGCAAAA(14)TTAAATATTTATTATTCGAGAATATAATACACCAAGAAATTGCCTTTTTTGATAAAGATGCACATGCCCCTGGATTTT
TATCATCATATATTAACAGAGATGTACATTTATTAAAAACTGGTTTAGTAAATAATATTGTAATATTTACGCATTTTATTATTTTGTTTATTGT
TAGTATGATTTTGTCATTTTATTTTTGCCCAATAATAGCAGCAGCTTTAACATTAACATATACCTTTATAATGCGAGCTGTTACTGCAAGAGTA
CGAATGGAAAAATCG(18)AATAAAATAGAGAAAATCGGAGATAAAAAAGATGGATGCCTTTCATATACTACTGATGACGAAATATTTAAAGAT
CCTAACTTTTTAATTCAAGAAGCATTTTATAACATGCAGACAATTATTACATATGGATTAGAAGATTATTATTGTAAACTGATAGAAAATGCAA
TAGATCATTACAATAAAGAGCAAAGAAAATCAATTATAGTAAATTCAATATTATGGGGATTTAGTCAATGTACACAATTATTTATTAATGCATT
TGCTTATTGGTTAGGTTCCATTTTGATAAAACACAAAATTATAGTTGTTGATGATTTTATGAAATCTTTATTTACATTTATATTTACTGGTAGT
TATGGTGGTAAGTTAATGTCCTTCAAAGGAGACTCAGATAATGCTAAATTAACATATGAGAAATATTATCCTATAATGGTTAGAAAATCAAATA
TCGATGTAAGAGATGAAGGTGGTATAAGAATAAAGAATCCACATCAAATAGACGGAAAAGTAGAAGTGAAGGATGTTAACTTTAGATATTTATC
TCGACCAAATGTACCAATATATAAAGATTTATCATTTAGTTGTGATAGCAAAAAAACTACTGCTATAGTTGGAGAAACTGGATGTGGTAAATCC
ACAATTATGCATTTATTGATGCGATTTTATGATTTGAAAGATGACCACGTTTTATTAGATAATCAACATGTTGAAAAAGACAATAAAGATAAAT
CAAATGATATAGAAATGACTAATGCAACCTCTATGAACGAATTGAATGAATTGCATAAGAAAAACGCAACTGAAGAATATACTCTTTACAAAAA
TAGTGGTAAAATTTTACTTGATGGTGTAGACATTTGCGATTATAACTTAAAAGATCTAAGAAAATTATTTGCGATAGTTAACCAAGAACCAATG
TTGTTTAATATGTCTATTTATGAAAATATAAAATTCGGTAAAGAAGATGCAACATTAGAAGATGTAAAAAGGGCTTGTAGATTGGCTGCTATTG
ACGAATTTATTGAATCATTACCAAATAAATATGATACTAATGTAGGACCTTATGGAAAAAGC(5)TTATCAGGTGGTCAAAAACAACGTGTTGC
TATTGCTAGAGCCC(3)TATTAAGAGAACCTAAAATATTGTTGTTAGACGAAGCTACATCAGCTCTTGATTCACACTCAGAAAAATTAATCGAA
AAAACTATTGTTGATATTAAAGATAGAGCTGACAAAACCATCATTACTATTGCTCACAGAATTGCATCTATCAAAAGATCAAATAAAATTGTAG
TTTTTAACAACAATGATAAAAATGGATCTTTTGTTCAAGCCC(53)AAAAAACACACGACGAATTGATTCGTGATAAAGATAGTGTTTATACAA
AATATGTCAAATTAACTAAATAAACGATTGAAAGTTGGGCAAATACATAACCAGATAATAGGAATGGAAAAAAATTGTGTTGAAATTTATTATT
AGCTCACTCTTGATCTATGAC(31)TATAGAAAGAGTTCATTTTACCTTTTAATCGGCTATTGAAAAAGAACTAAAAAAAATGAATTTGCTAAT
ACAAGATGATGAGTTGATATTTGGTCATGATTTTTATATAACATATAATTATTATGAACAAATATGCTATTAAAATTATATTTTGATGTATGCT
TTTTGAAAATGTTTTATATGTTCTCTCTTTATATCTTCATATATTTTATTTTTGCATATTTTTTTGTATTCACAATATACATATTGTTTTTTTT
TTTCATTTTTCCTATAAGAGAGGGAATTTTTGTGCATAT(50)TTAGCACCCGCTAAACCCTAATAATGCGCAAAATACATATTTTGTATACAT
ACATTTCTCATTAAATTTTGTTCTTATTATCGTTTGTGTTACTGTATTTTTTTTCATATGTAATATAGTGTATGTAAAATAACACCGTTTTTTA
CCGAATATTATATTTTTATGATTATCTAAATTTTAAACTATTTTTATAAAAAAAAAATGCGATTTTTATATATAGTAAAGATAAGTAGGGATGT
TATTCCCAGGAAAATA(51)

B-*pccg10* gene

Note: Primers are underlined in the sequence.

GTACTATAATATTATTATTATAGGGTGAATAATAAAATTGTTTGCTTTATTAGGAGTCGACCAAAAATAAATGTATTATGGAAATCACCGCATA
C(37)CAATTGATTATAAAAAAATATATATATAATATTTATTGCATTTAAAAAAAAAAAAAAAAAAAATATCCCTATATAAATAATAATTCCTA
TGTATTAGAAAATAAGACTCACCTATATCGAAATAGTGCTGATATGAATGGGAAAAAAAATTTTATGGATAATAAAATTCTAATATATGCATAT
AATAACTACTATAAGCATTCATATTGATTTCCTTATCATTTTTAAAACTATAAAAAAATCAAGGTTTATATTATTTTTATATCTTTTTTTTTA
AACTATTATATATAGTAAAATAATAAGTTTTTATGGAAAATATTAATATTCCAGTTTGAATATCATCATATTATCCATTAATATATATATAACC
AATTATATTAATACATTATAATATTCTAATTTTTTTAATATTTATTTTAATAGTATAAGAGTTTGTGCTAATTG(35)AAATAAATATATTATA
TATATTTTCTTTAACATTAGGTTATATATATATATAATTGATAATATGAATTATTTTCATTACTTAGTTAATGATATACATTGAGAATAAAACA
ATATATTGAAAAATAATATTATATATTTAATATAAATTATACAATTATATGTAAAAAAACCAAGACATATTATTTATATATCAACTTTTTAAGA
TATACTAAATCATTGTATCAATAATGACAGGAATGAAAAAAGGAAAAAACAAAAAAAAAAATGTAAAAAATGATGAACGCTATAAAGAATTGGA
TAGTCTAATAAGCAATGATAG(15)TAATAATCAAATAAAATTAAAAATGAAAATATAAAAACAAAGGAAACAAAATAAATATGAAAATATTAA
ATAATTAAGCTCGAAGCCAAACAATAAGGGGGGATATAAGAGAAACTAATTGAAATGATAATTAGCTACTAATTTTGGAAGACAGAATAAACTA
TGATGAACTGATTATGTATTTTCACACATGATTACTGATTGTATTATAGTTTTTTTTGTCTTATTTTTCTTATAACCATTCCAAATACT(36)T
ATGTTTGAGACTTAATCGTGGCTTAATTATATTTGACAAAAGTTTCGCAAACTTAATAAAAAAAGTTAAGGGTGTAGTGATATGG(27)TAGTA
GTATTACGACTTATATAGCACTAGTTACTCTAAACTTATTATTATGTTAACGATAAAATATATAGTAATATCACACATGCCATGTATAGAAAAC
ATACATATATTTTTCCCTTATTATAATAAATATATTTGTAGGCGAAATAGGAAATAACTCAC(18)GATGGGGTGGTGCCAAAAGAATTTGCAA
ACTAATTGGAAATGAAATG(20)AGAAATAACATTTATGTTTATTTACTAAGTATATTATATTTATGCGTCAGTGTAATGAATAAAGTTTTTTC
AAAAAGAACCCTGAATAAGATTGGAAATTATAGTTTTGTTACTTCAGAAGTACATAACATGATTTGTAC(17)TATAGTTTTCCAATTATTATA
TTTTATTTACCGTAAAACATCAAATCCTGCATCTAGAAATGAGAGCCAAAAAAATTTTGGATGGCAGTTTTTCCTTATCTCATTATTAGATGCC
TCCACAGTTATAATTACTATGATTGGTATGTCAATTTTATCCCGAAAATTATATATTAAGCTTTAAAACGGCGCATGCTCGCTACTCATATGTA
TATATATGCACACACACACACACTTACGACTCTTCTCCTTTTTAAGGTCTCACAAGAACAACGGGGAACATCCAATCATTTATTATGCAACTGA
TTATTCCAGTTAACATGTATTTTTGTTTCATCTTCCTTGGATATAGGTAAAAAATCGAAATTAAAAAATGGAAACCATTACACGTATATTTATA
GCACCAAATTTTATGCATACTAAAATTTATACATTTTACTATTATCCTTTTTTTGCTACTCTTAGATATCACTTATTTAACTATTTGGGAGCTT
TCATTATACTTATTAC(32)TATAGCCGCCGTAGAAACTGTTTTATCTTATGAAACCCAAAGTGACAACTCAATC(33)ATTTTTAACTTAATT
ATGATTTTCGCCTTAATTGTAAGTTTATTAATATAATCGTCAACGAACAGTTTGCAAATTTATATGCGCCCCTTTTCC(12)TTTTTATTAATA
TTTTTCAATTTTCATTTTTCAATTTTCATTTTTCAATTTTCATTTACAGCCTTTAAGCTTTTCAAATATGACC(13)AGAGAAGTTGTCTTCAA
AAAACACAAAATAAATATCATAAGATTGAATGTAAGAAATATGTTAAAATTGCGTTTGAAAAAGATGGCAAAAAATCGTAGAATTGCAATAGGG
CAATTTATCAAACTGATTGCACCACTAATAATCACACTATGATAAAATTTTTCCAACAATTTTTCCAACAATTTTTTATTTTTATTAGGCTATG
GTCGCTTTGTTCCAATTTTTTACTTCCCTTTTAGTTTTACCAGTCTATAATATCTCATTTTTGAAAGAAAGTAATGAAAATAAGATTTCAGTTT
ATTTGAAAAAGAATAGTACCAGTTTTTAGCTATATTACTG(21)TCTGCAAAATTGTTATTTTTCATTTTTCATTTTTTCACTTTTTAGTTTAT
ATGCCATTCTCTGAAATGGGCACAAACATTAATGACGGTTT(22)AAGATGCTTATTTTATGGACAAAGCACAATCGTTGAGGTAGGACATAAT
TTTACATTCTCAAGATGAAATTCACATATTTTTATTCAGCTAATGTTTTATAACATACTTATACAATCATGTTTATTACTAATTTTGTGTATTA
TTTTTTTTACAGAATTGTGGTGTTGGTATGGTCAAAATGTGTGACCAATGTGAAGGAGCATGGGTAATTAATTTTTATAAAAAAAAAAAAAAA
AAAATACAGAATAAAATGCATTATTCAAGTTGATATATTCAACAAAATTATCTAACTGTTTTTTATCTCTTTTTCAGAAAACCTTTATAACATA
TTCCTTTTTCAAC(11)ATATGTGACAACTTACTTGTTTGCT(26)ATGTAAGATAAAAAAAATGCACATAATATATCATTATTTAGATCAAAA
AACATATAAAATTATTATTATTTTTTTTTTCGTTTTAGATAATTGATAAATTTTCAACAATGACATATACCATTGTTAGTTGT(16)ATACAAG
GACCAGCCATAACAATAGCTTATTACTTTAAATTTCTTGCGGTAAAATATTTTATAACAGAACAAAATTCGAAATATTTTCACCTTTTTTTTAC
TTTATTTTGACAATATATGTTTTTTTTTTGTGTCTTTAGGGTGATGTTGTAAGACAACCAAGACTATTGGATTTTCTCACTTTG(24)GTAAGG
AATATGAAATAAAAATTTTTAACAATTTTGTACATAGCTAGTAATGATTTC(28)TTTTTTAATATAATTTTTTTGTTTTCTTTCTTACAGTTT
GGATACTTATTGGGAAC(29)AATAATTTACAGAATTGGAAATATCATATTAGAAAGTTAGTTTTAAAAATAGTTAAATAAAACTGGAAAATTA
TATTTATACATATATTATGTGTCTACATATG(30)TGTACAACATATTGATATTTTTTTATTCTTTTTTAATNCAGAAAAAAAAAATGTTAAAGG
CACTAAACACCGATGGATCCGAAGCAGAATTAACTTCTATAGAAACATCAACAGCATAATAGATATGACAAGTTTATATGTTAAATATTTTTTT
TAAATGCCACATAAAATTGTAAAGAAG(34)AGTATGCTAATAATTAAATATATGTATTTTTTTTTGAATCAAACTACTTTATTTTGATTCATA
AATATTTATATTTTATAAAATTTGTGAATTTTTTTTTTTATGAAAATTATGTGTTACATTTTGAATATTAAATATATGACTTTTTAATACATATT
TTGTAAATCTTAAAATTTGCTGATATTCTGCTATTATATATTAAT

C-*pctctp* gene

Note: Primers are underlined in the sequence.

AATGATGAAGTATGCTCTGACTCA(1F)TACGCTCAAGAAGATCCATTTGGAAATCCAGAATTCCGTGAAATTGCTTTTGAAGTCAAATCAAAT
AAAAGAATAAAAGGAAATGATGACTATGGTATAGCCGATAATAGTGAAGATGCAGTAGAAGGAATGGGAGCTGATGTTGAACACGTCATTGATA
TTGTTGACTCTTTTCAATTAACATCCACTAGTTTAAGCAAAAAGGAATACAGTGCTTATGTTAAAAACTTTATGCAAAGGATTCTTAAGCATTT
AGAAGAAAAGAAACCAGATCGTGTAGAGATTTTTAAAACAAAGGCACAACCCTTAATTAAACATATTTTAACAAATTTCGATGACTTTGAATTT
TATATGGGAGAATCACTTGATATGGAAGCTGGTTTAATTTATTCATATTATAAAGGTGAAGAAATTACTCCTAGATTTGTTTACATATCA(1R)
A

D-*pcatp6* gene

Note: Primers are underlined in the gene sequence, initiating and stop codon are presented in bold, the introns are highlighted in grey.

AAGGATCTTTATATAGTTTATATTATATATGAAATAAAAAAATACACATGTATATATAATTATAAAAAACAAATTATCCTAATAATAAAAACAT
TTCAAACAAAAAATAGAAGAAAAGAAAAAAGAAACAATTGCTAAATATGTTTATGCTTATATGTTGATACTCAAAATTTTCTTATAATCTTAGA
AATATTCACAACTCGTTTGGTC(1F)ATTTTTATTTTTTTTTTAACCAAAGATGAAATAAATCATTTGTAAAAAAAATATATATTAGCGATTTTT
TCATATAGCTAGAATTTCCCTTTAAAAAAAATATGATAAATATAGCTATTTGCAATAAGAAGTATATAAATTATAAAAAAAAACATGAACAGTT
CAGATTATTTTATCCGTTATGTATGATTTGTACATGTTTTTTTCCCCTTTTACTGAATATTTCAAAATGGTACAAAATAATTTGATATAACAAA
TGTTCATTATTCTTGTTGCTAGTGATATGTTTAATAGACAAAAGTAATTTTTATCACAGGAAAAAAAATATGTGTATATATTTGACTTATTCTG
CATACACATATGTATAGGAAATATTTAGGTTTTTCAATGAGCTTTATTTATTGAATGTCTTCTGATTCATATGAATCCGAATATGCTTGATTGG
AATCGTCCTCATCTGAATATTCGCTAGCTGAATCATCTTCTTCATCTATATCCGCTTCTTGTATTAAATTATGTTCTCTACAAGAGTTTAAATC
GGATTC(2F)TTGTATATTTCTAATTTTTTCAATCATTTCTAAATTATTGTTTTTTAAATCTTTGTACAAATCTAATATTAAATCAGAAACTTC
GATCTACAAATTGG(1R)AAAAAAAATAAAGCGTATAATTGTTAATAAATTAATATTTTTCATAGTTTTGCTTATATTTCTATTATTTATTCGT
TTGTTTGTTTATCTTACATCACTATCATCATCAAGATCCACATTAAAAAGCGTATTAATTTCATCTCTTAAGTAGTCACAAAGTTTATCTTTGG
GTCCCGTGTCTTGAGAATAGTATATGAAAAAAATAAAAAAATGCATATATATATTTTGATTATCTAAGTAATAAGAAATGGGAATGCATTTTAG

CGGCTAATTCGGTATATGCATACACGTTAACATTTTTTGTGTGCTTACTTGATAAAACATAATTATGGACATATTCAATTAACTTTTTTTTCTT
TTCCTCTGAGGATGTTCCACCCCAATTATTTGTCACAGCTAAACGGAGCACTGTCCTATATC(3F)AATGGAAAACGAAATTTTATATTTTTTT
TATTTATTCAATCGTATGTAAATAAATATAAATTGTAGATATGCATGATGGAAGGTATAGCAATAATCCTTGTAGATGACAAATGCAGG(2R)T
TTTTGCCAAATACATAGAATATTTACATATATTAAATTGCTTTTTTTATATAATACCATTTATCAAATATTAAGTTAATCCCTTCAAGTAATAA
AGTAGATAAATTTTCACTATTCATTATTATTATTATTTTATTTAAAGTAAAATTAAAATATGTTTTTCAAAAAATAACCCGTTATATATTTTAT
AGAAACCCTGTTTCATTAATTTTTAAGCAATCCGTTTTTTTATATTTTTTTTGTCGTTGTTTTTTTTCCACTCGTTTTTTAATTACAACGATTA
CATTTTATTGTGCCCAAAAAAGGGGAAAAAGTATAAAGATACTTTTATTTTATAAAAAAAAAATGTCTATATATTCCTGATTTACTATAAAATAA
ATATTATATCCAATTATATATTGCTACAAGTATTGACGTTTGCCATTCTCGTG(4F)ATGCTATTTTTATTTTTTTTATTTTTTACTAGCCAAA
TATTGTTACATATTAGGAAATAACATATAAAAAATATAATAAAAGAAAAAAATTAAAAAATTTACAAACGTAGAAAATGATAGCACGG(3R)TA
AATAAGAATAATAAAACAAGTGAACTTTGAACAACTTTAGAAAAAAGTTAAATGAAAAATATAAATTTCCCAAATAATATATTATTTCTTATTA
TTGCCCCCACCCCAATTTGTAAAATATTACTGTATATTTTTTACATGAACTGTTAATAATTAAATGGGATAATTCATAAATTTTAATTTTATTA
TTCCAAAATGAACAGGTTGAAATCGTAAGTTATCAAAAATAATGCTAATTTTTTCTTTTATTTGTCATATTTTTTATAATTATAAATGAGCATA
GATAAAATATGTGCATATGTAAGTTGGTGTTATACGTTTTTATGGATTAAACTTATAAATGGGCGTATATATTTCTCCTCTATACTTTCGGATA
GCCATATCAATATGCTAATGTTTGTATAACCTTGTTCAGTCTCATAAGGTATAGAAATGGTTATATAACAATGAGGTATAATTTAGCTTATCCA
ATTTCTTTAC(5F)ATAAATTTATTTATTTAAAGACAGAATAATATTATATATATACAAAACAATATTTTTGTATGTAGGGGTATTTTATAAAA
AATATTTTTTCCCCTTTTCTCACACACATAAAG(4R)TAAATAATTATATCCAATGTAATATAAAAAGGGTAACAAAATTAATAAGAGATATAC
TTATCATATCATCTTATGAATAATTTAACACAATGCTTACGCATATGTATGTGCATATTTATTGGGGGTAATATTTATTATTAACATTACAAAG
CAATAATTTTTTTTTCCTAATAAGACTTTTTTTATGACTGTTCATAAATATTTATCTAAAAAAATTATTAGTATAAATTTACTTTTATATCATT
ATGTGTGTTTAGTAAATGTGCTTATGAATAATTTTACACGCATAATATAGTAATACTAAATAAAAGAAAAAAATGTGTTAGTTGCCAAATATCA
TTGTCATTAATTTTATTATTCTTATGAAAGAAATAATATATCATAAAGATAAATTAGCTAAAGATATTTATAAAATAAGCGAATGGAAATATGA
GCATATTTAAAAAAGTATAATATATATTGTTGTTAATTTGCTTTTGTATATTATAAACACTATATGGTATTTACGC(6F)TTTTTCTTATACAC
ATTTTTTATTTAATTTATAATAATTTAGTATGGAATAATTAATTTTTTTTTTCTTAATTGAGATATATATTTTATTTTGTGTCAGTTTTCTACAA
TGC(5R)AAATAGAAAAAAAATATATTTTTATGCATACATAACATATTATTTTAAAAATAATTGAAAATTAAGAAATAATAGCAATTTTCATGA
TATATTTTTTTCATTTATTAATCATTTCATTTATGCTTAACATGATCTCGTAACAATAAAAAATAAAAAAATAAATCGCTAAAAAGATACAAAA
ATATATAAAATGTATGGTGATTTATTTTTATAAGTGTACATATATATATATACATACACGGTATTATAAGTGCTCATTACTTAATAGCTTTAAA
ATTTATAAAATTTTTTACCATATTTGTTATGTATGTAAAATAGATACGCTTATTTATGTATACATTTTTTTTTCCTGTTTCAATAAATAAAAAT
ATATATATATATTTTATCCCTTGTTTTATATTTCTATTATTTTTATTTATTTTTCTTTTTTTTTTTAATGTGCTTTTCTTTTTGTTTTACATAA
TGCCAAAAAATGCGTAG(7F)TAAAATTAGGATTTATATATAATATATACACATATATTATTATAAGTATGCACATGAAAAGGATTATAAATAT
ATTGATTTAGGTATGTACATATAGTATAATAAAGTACGCTAATTAAGATAAAACAAACTTAAAAAAAAAATTATATAAAAAATAATTCTGTGGATT
TACAAAATAATTGATC(6R)CCCCAAAAGAAACAAGAGAAACACATCACGTATGTACATGTATGTATTTATGCGTATTATGTGTTAAATAAACA
TTAGAGGTGATTATATTTGTTTTTTATTTTTTTCATAATATTTTAAAAAAATAATATACAAAATTTGGAGTCACAATTATTTTAATCTATGATA
AAAGTTTATATTGAAGTGTTTGCTTTTCGTCGCAGCATATGCCCGTGAGTATATGCATATATGTATATGTGCCTATATGCATATAGATAAATAG
AAATATAATTTATGAGTAGGTTAGCCCTTTGAT(7R)TTAAAAATAAATTTTGATTTAAGATCACATTACAATTATCAAATAAAAAGATATATA
TATCGAGAGACATTTCCAGTAGATATCATTCGATGTATTTTGCCATATTGTCAATAAAATAAGCATACAAAATAATAATTGATACTGCGAAAAT
ATA**ATG**GAAGATATTTTGAAATATGCACATGTATATAATGTAGAAGATGTTTTGAGAGCAG(8F)TAAAAGTAGATGAAAATCGGGGTTTATCC
GAAAATGAAATAAGAAAGAGAATAATGCAATATGGGTTCAACGAATTGGAAGTAGAAAAAAAAAAAGGAATTTTGAATTGATATTGAATCAGT
TTGATGACTTATTAGTAAAGATATTGCTTTTAGCTGCTTTTGTTAGTTTTGCACTAACATTACTAGATATGAAAGATAATGAAGTAGCTTTATG
TGATTTTATAGAACCCGTTGTTATATTATTGATACTTATATTAAATGCAGCTGTTGGAGTATGGCAAGAATGTAATGCAGAAAAATCGTTAGAG
GCACTAAAACAGTTGCAGCCAACAAAGGCAAAAGTATTGAGAGAAGGTAAATGGGAAGTTATAGATAGTAAATATTTAACAGTTGGTGATATAA
TTGAATTAAGTGTTGGTAATAAAACACCAGCAGATGTTCGAATTATTAAAATATTTTCAACAAGTATTAAAGCAGAACAAAGTATGTTAAC(9F
)TGGTGAATCATGCTCTGTTGATAAATATGCAG(8R)AAAAATTAGATGAATCATTAAAAAATTGTGAAATTCAATTAAAAAAAAATATATTAT
TTTCTTCTACAGCTATTGTAGCAGGTAGATGTATAGCTGTTGTAATTAAAATAGGTATGAAAACTGAAATAGGTAATATACAACATGCAGTTAT
AGAATCAAATAATGAAGAAACAGATACACCATTACAAATAAAAATAGATTCATTTGGAAAACAATTATCAAAAATTATATTTATTATTTGTGTA
ACCGTTTGGATTATTAATTTTAAACATTTTCAGATCCAGTACATGAATCATTTTTATATTGGATGTTTATATTATTCAAAATCAGTGTGGCAT
TAGCTGTTGCTGCAATTCCTGAAGGATTACCAGCAGTTATTACTACATGTTTAGCTTTAGGAACCAGACGAATGGTCAAAAAAAATGCTATTGT
TAGAAAGTTACAAAGTGTTGAAACATTAGGTTGTACCACTGTTATATGTTCTGATAAAACCGGAACCTTAACAACAAATCAAATGACAGCTACT
GTTTTTC(10F)ATATATTTAGAGAATCAAATACATTAAAAGAATATCAACTATGTCAAAGAGGAG(9R)AAACCTATTTCTTTTATGAAACTA
ATACTAATCAAGATGGTGAAGAAGATTCATTTTTTAAAAAATTACAAGAAGAGGAAAATAATGAATCTAATTATAAAAGACAAATAAGTAAAAA
TATAATACATGATGAAGAAGATTCAGATGATGAAAGAGCACCGTTAATGAATATGAAATCAAATGTAAATACAATTATAAGTAGAGGTAGTAGA
ATTATTGATGATAAAATAAATAAATATAGTTATTCCGATCTTGATTATCATTTTTATATGTGTTTATGTAATTGTAATGAAGCTAGTATTTTAT
GTAATAGGAATAATAAGATTATTAAAACATTTGGAGATAGTACCGAATTAGCTTTACTTCATTTCGTTCATAATTTTAATATAACTCCTAATAG
TGCAAAAAATAATAAAATGACATCTGAATATGAAAAATTAAATAGTGGAAGCAGAAAAAATAGTGACCTGGATACCGATTGTGATTCTTTATAT
AGTAGTGAAAAAAAAACAAAAGTTTCAGATAAAAAATCGGAACCATCATTCCCAAGTGAATGTATAACTGCATGGAGAAACGAATGTACAACAT
TGCGAATTATTGAATTTACTCGTGAAAGAAAACTAATGAGTGTCATTGTAGAAAATAATAAGAATGAATATATATTATATTGTAAAGGTGCACC
AGAA(11F)AATATTATTAATAGATGTAAATATTACATGTCTAAAAATGATGTACGGTCATTAACTGATTC(10R)TATGAAAAATGAAATTTT
AAATAAAATTAAAAATATGGGAAAAAGAGCTTTGAGAACTTTAAGTTTTGCATATAAAAAAGTTAAAGCCAATGATATTAATATAAAAAATGCT
GAAGATTATTATAAATTAGAATATGATTTAATATATATAGGAGGATTAGGTATAATTGATCCCCCAAGGAAGAATGTAGGAAAAGCTATTAGTT
TATGTCACTTAGCAGGCATTCGTGTTTTTATGATTACTGGTGATAATATTGATACTGCAAAAGCTATTGCTAAAGAAATCCATATATTGAATAA
TGATGATACTGATAAATATAGTTGTTGCTTTAATGGACGTGAATTTGAAGAACTACCTTTAGAAAAACAAAAATATATTTTAAAAAATTATCAA
CAAATAGTATTCTGTAGAACTGAACCAAAACATAAAAAAAATATTGTTAAAATTTTAAAAGATTTAGGAGAAACTGTTGCTATGACAGGTGATG
GTGTAAATGATGCACCTGCTCTTAAATCTGCTGATATAGGTATTGCTATGGGTATAAATGGAACTCAAGTTGCTAAGGAAGCTTCCGATATTGT
TTTAGCTGATGATAATTTTAATACTATTGTTGAAGCTATTAAAGAAGGACGATG(12F)TATATATAACAACATGAAAGCTTTTATACGATATC
TTATAAGTAGTAATATTGGAGAAGTTGC(11R)TTCGATTTTCATTACTGCTATTTTGGGTATCCCCGATAGTTTGGCTCCCGTCCAGTTACTA
TGGGTTAACTTAGTAACTGATGGTTTACCTGCCACTGCTCTCGGTAAGTTTGTGAATCTTCACATGTTTCCATTTTCTCACCTTTTCCATTTCT
TACTTTGTTTCGCTTATTTTGTCATTTCTTTACCATCGCAGGATTCAACCCCCCCGAGCACGACGTGATGAAATGCAAACCCAGACACAGGAAC
GACAACTTAATAAACGGGCTGACATTGCTTAGATACATAATTATAGGTACATATGTTGGAATAGCTACAGTGTCAATCTTTGTATATTGGTTTG
TGTTTTATCCAGATATGGATAATCACACTCTAATAAATTTTTATCAACTTTCTCACTACAATCAATGTAAAACGTGGGAAAATTTTAAAGTGAA
TAAAGTATATGGTATGTCCGAGGATCTATGCTCTTATTTTTCTGCTGGCAAAGTCAAGGTAATAAAATCATTCCGTTTTTATCATAAATTTTGT
ATGCATGCAACAATATAGTTATTCATTTGACGTTTGTTTACTTCTTTTTTTTAGGCAAGTACCTTATCATTATCC(13F)GTTCTAGTCTTGAT
CGAAATGTTCAATGCGCTTAATG(12R)CACTTAGTGAGTATAACTCATTATTTGTGCTTCCACCATGGCGAAATATGTACCTAGTATTTGCAA
CAATAGGATCTTTACTTCTTCACTGTATGATCATATATTTCCCACCACTAGCTAGAATATTTGGGGTTGTGCCACTTAATTTACACGATTGGTT
TTTAGTATTCTTGTGGTCCTTCCCTGTAATAATAATTGATGAAATTATCAAATTTTATGCAAAAAAACAATTAAACAAAGAACTCGGGTATGGT
CAAAAATTGAAGACGC(13R/15F)AC**TAA**TGCACACATTTACTTCGCATCAAATAATTATAAACATGTTGTACATGGAAGTTTATGTCTTCTT
TCGTTTCTTTTCTTTTCTGTTTTTTTTTTTTTTTTTTTATTGTGATACTTTTCTAAATTAAACCCCCAAATTATGAATATGCATAATGTATACTTA
TCTCTTAATTATTTTACTTCGCTTATTTTTATTTTATTTTCTTCTTCCTATTTTATATTGTATTTAAATTAGTATAACATTATTACTATCCCAT
TTTTATTTTTTTAAATATATTATAAAAGTACAATAAAAAGAAAAATAACGAACATCAATTTTTTATATATTCCTTATTTATAAATATTTACAAT
TTTTTTTTTAAATATATATTGATGTCTTTTTCGAATAATTCTAATTTTTTTTCGACAATTTTTTTAAGTATACACATATTTGGGTATTGAAAAA
GGTGTGCGTAAAATGAGG(14F)AAAATTATACACATTCTTCCTTTTCATTATTTTTAATTTTTATAAAAAAAAATGAAAATAACAAGATATAT
AATTGGTAATTAAAAATATAAACTACGAATTTACTACTGTTTTATGTATTTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAACTTTATT
ATTTGTTACATCCCTATGCAAATGGCC(15R)ACAATGATACTACTTGACAAATAAAGAAGGGAACATAACGAAATACAGTTATTTTAAAAATT

TTTTTATTTTATACATTTTGAGAAAAAATGGAACTAGCATACGAACATGAAGTTACAAAAAAAATAGTTTATAAAATAATAAACAATATGAACA
GCAAAAATGATGTAACATTAGAAAGGATGCAACACACCAGGTACAGTATTCATTTTGTTACCATCTTACTACTGCCTTACATGTGTATTTACTT
GAAAATATAAGAATGGTCATGAAGTAATGGCACACTTTCAAAAAGGCACAAAGCCAAAACACGTTCATACAATATGCCATATGTCATAGCACAA
AGAATAATTCATTTTGTAATATACTTTATAATTATACTTTATTTTGTACATTTTCTTGCCCTTTTTAGAAATAAGATAGTGAAATACTTTTTCG
AAGCAGAATTGTTATTTAAATACTATGACATAGGAAAGACTGGTGAG(14R)ATAGATGTAAGTCTATTCCCACAAATGGCAAGAACATTAAAA
CAAATATATGATGCAGAAGATATTAAACGGT

APPENDIX 3

PRIMER SEQUENCE AND PCR AMPLIFICATION CONDITIONS FOR DETERMINING GENE COPY NUMBER OF *pcmdr1, pctctp* and *pcatp6* GENES

Table A3-1 - Polymerase Chain Reactions for gene quantification of *pcmdr1, pctctp* and *pcatp6* genes in comparison to *pcmsp1*.

Gene	Primer sequence 5' - 3'	[$MgCl_2$] (mM) / [Primer] (μM)	PCR amplification programme
pcmdr1	**sense** - TCT CGA CCA AAT GTA CCA ATA TA **antisense** - GCA TTA GTC ATT TCT ATA TCA TTT G	3 mM / 0.8 μM	40 cycles 95ºC for 600'', 95ºC with a 0' hold, cooling at 20ºC/s to 63ºC with a 7' hold, heating at 20ºC/s to 72ºC with a 7' hold. heating at 20ºC/s to 95ºC with 0' hold, cooling at 20ºC/s at 65ºC and heating at 0.2ºC/s to 95ºC in a continuous acquisition mode produced the melting curve
pctctp	**sense** - AAT GAT GAA GTA TGC TCT GAC TCA **antisense** - CAT TCC TTC TAC TGC ATC TTC AC	4.5 mM / 0.8 μM	40 cycles 95ºC for 600'', 95ºC with a 0' hold, cooling at 20ºC/s to 60ºC with a 7' hold, heating at 20ºC/s to 72ºC with a 7' hold. heating at 20ºC/s to 95ºC with 0' hold, cooling at 20ºC/s at 65ºC and heating at 0.2ºC/s to 95ºC in a continuous acquisition mode produced the melting curve
pcatp6	**sense** - AGG CAA GTA CCT TAT CAT TAT CC **antisense** - GTG AAG AAG TAA AGA TCC TAT TG	4.5 mM / 0.8 μM	40 cycles 95ºC for 600'', 95ºC with a 0' hold, cooling at 20ºC/s to 58ºC with a 7' hold, heating at 20ºC/s to 72ºC with a 7' hold. heating at 20ºC/s to 95ºC with 0' hold, cooling at 20ºC/s at 65ºC and heating at 0.2ºC/s to 95ºC in a continuous acquisition mode produced the melting curve
pcmsp1	**sense** – ACA GTA ACA CAA GAA GGA AC **antisense** – GAT ACT TGT GTT GAT GCT GG	3.5 mM / 0.4 μM	40 cycles 95ºC for 600'', 95ºC with a 0' hold, cooling at 20ºC/s to 59ºC with a 6' hold, heating at 20ºC/s to 72ºC with a 7' hold. heating at 20ºC/s to 95ºC with 0' hold, cooling at 20ºC/s at 65ºC and heating at 0.2ºC/s to 95ºC in a continuous acquisition mode produced the melting curve

APPENDIX 4

GENE AND PRIMER SEQUENCE FOR THE GENE *ubp-1*

A - *Pcubiquitin carboxyl-terminal hydrolase* gene summary (*ubp-1* gene)

Note: Primers are underlined in the gene sequence, initiating and stop codon are presented in bold.

TTATCAAAGAGTTCAATTTTAATGATATTGTTAATGCTGAGGTGAGTTCAAAAAACCTAATGCACTCCCCTCGCGATCACAATCACGATTCCCA
CGGAACCCCCCCTCCGAATCCATTGAATCAGGTATGTTCGGCGCCCGATGCCACGCCCACCATTTGCTTGTCATGCTTGCCATGCATCGGTTTA
GGCCCACTTGTTTAGTTTCATTGCATTTTCTTTGTTTCACTTGCATTTTCTTTGTTGCACTCGTTTCGTTCTTCGCCCACTCTCACATTGACA(
1F)TTCCCATTCTCTTTGCAGCAGAAAACCGAGAGGAAATCGAACAGGCCAAGGAAAACACGATCGGAATAAAAAATATGCTTAAGTTCATAAT
CCAAAAAAGCAAAAAGGAAAAAAAT**ATG**ATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTC
ATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCATAAATCATAAAATATTGGGTTTAAAAATATTTC
GACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAAT
TATTAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCA
TACACTCGTAGTAATACATCATTACCTGAAGTAGATCATTTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTA
CCCAATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATC(2F)AATAGAAAATAACAATTCAAAACAAAACTTTTTTAAACATA
AACAGAATGATTTATCATTAGATTCTGATTTGCCAACACCAG(1R)ATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAAATTCTGAAA
AATTTGAAGATTCACATAATAAAAATCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAA
TATTAATAATATTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGATCACGGTATTACTTATGATGACATGTTTAGAGAT
AATGACAATAGCAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATT
TACCGGACTATCCTACTTCTTTG(3F)AAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAA
ATCAAAATAGTAAAGAAGAACAAGATAATGGG(2R)GAAGAAGAAAATCATAGTAATAAAACTCAACATAAAGAAAATAATAATATTTCAAAAG
TCAATGAGCAAAATGAAAATATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAAATATCCAAAAATTCTAACACTACTCGAAA
ATATATGATTGATGAAAACCTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTT
GAAGAAGATAAGTATAGACATAATAATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATA
ATTCAAATAATATGGTGGATAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAGAGAGAAAAAAGAAAATTTTGGATCCTCAAAAAA
GTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACC
AATGG(4F)ACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACT(3R)TTGTAAAAGATAAAAAAAACGA
TTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAACAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAA
AAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAAAAAAATGCAAA
AAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTC
ATTTGTTGTTAATTTATACATTTTTAATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAA(
5F)AAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACTTACTTTCAAAACGATTTTTATATGAGTTG
AAAATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAA
ATCAACAAGATGTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGT
TATACAAAAAGTTCAATGCCAAAAATGTTTTTTTATTTCAAAGAAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAG(
4R)TAAAAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCTCGAAATGCAAC
AAAAGGCGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGGTAAACCAAAAAATGCAAAAAAA
TCCAAAAAAAATCCAAAAAAAATCCAAAAAAATCCAAAAAAAAAGCAAAAAAAAATATTTTTCACTTCGTCAATTTTTCATTTTTCAATTTTCCATT
TCGTCAATTTTTCACTTCGTCAATTTTTCACTTCGTCATTTTTCCATTTTCCACTCCTTCCCACTTTCAGGTACAACTGGTCGTTTAGCTCCAA
CGAAAAGAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGT
GGCGTGTCAGCATC(6F)ATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGAC
GACTCAGCTATTACTA(5R)AAGTTAGTTCCAAATCCATAAACCGAATTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTAT
CGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTT**TAA**TCATATTTCAAAATTGCAACAATTTTGCTAACGCTTTATTATTTTATATACG
TGCACCTTTGAACGAAAAC(6R)CCTTTTTAAGTTATATTTTTTGTTTAGTTTTTCCGATTATGTTTGTATATATATATATGTATATATAATGT
GTGCGATCTTTTTTGTCGCCTAATTAATTAATGAGTATTTGCTCTATGAAATCTAGTTTTTTTATAGTTTTCATTTCTTTGTTATTGTATATTA
CATATA

Note: The gene is composed by:
3 exons,

197855 – 197933bp	79 b. p.
197998 – 200899bp	2902 b. p.
201129 – 201468bp	340 b. p.

2 short introns
* 63 b. p.
* 229 b. p.

Total genomic = 3321 + 63 + 229 = 3613b. p.

Protein =1106 a. a.

B - *Pc*ubiquitin carboxyl-terminal hydrolase predicted translation

MICCSDANELKLLVFLGICIKIVICRISNLLNAKACLEQFYYFINHKILGLKIFRHSHIVLVYFIPFFKKYYFLWKFIEHEIDPEIIKLINAII
NNLENLQNNKNINNIGSNPMLHPYTRSNTSLPEVDHFSFPSKDKNLNHTFNNFSTQYNPHDYKTSFSHSISIENNNSKQNFFKHKQNDLSLDSD
LPTPDLFNPKNKKKKKKNSEKFEDSHNKNQQHPHDYSEFDSHNNFHKNIINNINNINYFRPSSLFNTNENCDHGITYDDMFRDNDNSSDDNYFD
KGKNKICNVKEYITNLHFNNLPDYPTSLKNSDENKKSEDKKKKKKKIKNENQNSKEEQDNGEEENHSNKTQHKENNNISKVNEQNENILLYNIQ

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DNENAENKISKNSNTTRKYMIDENLNEYKIENKKDVSNNYNNKEGSPTFEEDKYRHNNRSSTPQNNGIKKFPTVSYEDADNSNNMVDKKSQKNY
INLELDREKKENFGSSKKLTNIESAKGMNNDDNYNSNNEILNNRRTIQTNGHTVNYNRNNNNMRPDEYENFVKDKKNDLDIIQRKGISLVNATT
NNNYEEINIVNNPIEKNKHVSSDKLIQKRPKYLMLPIDTTELKKMQKGKLRHPPVGLINLGNTCYLNSLLQALYSTVSFVVNLYIFNIDDNKEL
KHINNKNISNEMPIKNKLSFNLNNTNMNNNNNNNANLLSKRFLYELKILFKLMTTTNKKYVSPDNILGILPQELNNRNQQDVTELFRYTFEQLGG
SEKKFLRLIFSGVVIQKVQCQKCFFISKKEEIIHDLSFHVPAKSSKKQSIQKFFDTYIQKEKIYGNNKYKCSKCNKRRNALKWNEIISPPCHLI
LILNRYNWSFSSNEKKKIKTHVKINKKIVVNNFDYRLYGGIIHSGVSASSGHYYFIGKKSEKGDNSKNEWYQMDDSAITKVSSKSINRISKDPS
NDHTPYVLFYRCKQAPDSPSLYF*
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Note: In grey the codons where mutations were found
Effect of mutations:G2215T gives V739F **G**TT to **T**TT
G2308T gives V770F **G**TT to **T**TT

C – Sequence of the *ubp-1* gene of the different *P. chabaudi* clones

Note: non-coding sequence in small caps
Length: unspliced 3079 bp; spliced 2942 bp

C.1 - AS-15CQ (Intermediate chloroquine-resistant, progenitor of AS-ATN)

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ttcccattctctttgcagcagaaaaccgagaggaaatcgaacaggccaaggaaaacacgatcggaataaaaaatatgcttaagttcataatcca
aaaaagcaaaaaggaaaaaaat**ATG**ATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTCATA
TGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCATAAATCATAAAATATTGGGTTTAAAAATATTTCGAC
ATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAATTAT
TAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCATAC
ACTCGTAGTAATACATCATTACCTGAAGTAGATCATTTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTACCC
AATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCAAAACAAAACTTTTTTAAACATAAACAGAA
TGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAAAAATTCTGAAAAATTTGAAGAT
TCACATAATAAAAATCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAATATTAATAATA
TTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGATCACGGTATTACTTATGATGACATGTTTAGAGATAATGACAATAG
CAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATTTACCGGACTAT
CCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAAATCAAAATAGTAAAG
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TATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAAATATCCAAAAATTCTAACACTACTCGAAAATATATGATTGATGAAAAC
CTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTTGAAGAAGATAAGTATAGAC
ATAATAATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATAATTCAAATAATATGGTGGA
TAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAGAGAGAAAAAAGAAAATTTTGGATCCTCAAAAAAGTTAACTAACATTGAAAGT
GCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACCAATGGACATACTGTTAACT
ACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAACGATTTAGATATTATACAAAGAAAAGGAAT
ATCTCTTGTTAATGCTACAACAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAAAAAAATAAACATGTTAGTTCAGATAAG
TTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAAAAAAATGCAAAAAGGAAAATTACGACACCCACCTGTTG
GTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTCATTTGTTGTTAATTTATACATTTTTAA
TATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAAAAATAAATTATCATTTAACCTGAACAAT
ACAAACATGAATAATAATAATAATAATGCAAACTTACTTTCAAAACGATTTTTATATGAGTTGAAAATATTATTTAAATTAATGACTACAACAA
ATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAATCAACAAGATGTTACAGAATTATTTAGATA
TACATTTGAACAATTAGGAGGATCAGAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTATACAAAAAGTTCAATGCCAAAAATGTTTT
TTTATTTCAAAGAAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAAAAAACAGTCTATCCAAAAATTCTTTGATA
CATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCTCGAAATGCAACAAAAGGCGAAATGCCCTCAAGTGGAACGAAATCAT
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ACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGCGTGTCAGCATCATCAGGTCATTATTATTTTAT
CGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTATTACTAAAGTTAGTTCCAAATCCATAAAC
CGAATTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTT**TAA**t
catatttcaaaattgcaacaattttgctaacgctttattattttatata
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C.2 - AS-30CQ (High chloroquine-resistant; derived from AS15CQ, progenitor from AS-ART)

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ttcccattctctttgcagcagaaaaccgagaggaaatcgaacaggccaaggaaaacacgatcggaataaaaaatatgcttaagttcataatcca
aaaaagcaaaaaggaaaaaaat**ATG**ATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTCATA
TGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCATAAATCATAAAATATTGGGTTTAAAAATATTTCGAC
ATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAATTAT
TAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCATAC
ACTCGTAGTAATACATCATTACCTGAAGTAGATCATTTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTACCC
AATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCAAAACAAAACTTTTTTAAACATAAACAGAA
TGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAAAAATTCTGAAAAATTTGAAGAT
TCACATAATAAAAATCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAATATTAATAATA
TTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGATCACGGTATTACTTATGATGACATGTTTAGAGATAATGACAATAG
CAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATTTACCGGACTAT
CCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAAATCAAAATAGTAAAG
AAGAACAAGATAATGGGGAAGAAGAAAATCATAGTAATAAAACTCAACATAAAGAAAATAATAATATTTCAAAAGTCAATGAGCAAAATGAAAA
TATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAAATATCCAAAAATTCTAACACTACTCGAAAATATATGATTGATGAAAAC
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CTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTTGAAGAAGATAAGTATAGAC
ATAATAATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATAATTCAAATAATATGGTGGA
TAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAGAGAGAAAAAAGAAAATTTTGGATCCTCAAAAAAGTTAACTAACATTGAAAGT
GCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACCAATGGACATACTGTTAACT
ACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAACGATTTAGATATTATACAAAGAAAAGGAAT
ATCTCTTGTTAATGCTACAACAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAAAAAAATAAACATGTTAGTTCAGATAAG
TTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAAAAAAATGCAAAAAGGAAAATTACGACACCCACCTGTTG
GTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTCATTTGTTGTTAATTTATACATTTTTAA
TATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAAAAATAAATTATCATTTAACCTGAACAAT
ACAAACATGAATAATAATAATAATAATGCAAACTTACTTTCAAAACGATTTTTATATGAGTTGAAAATATTATTTAAATTAATGACTACAACAA
ATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAATCAACAAGATGTTACAGAATTATTTAGATA
TACATTTGAACAATTAGGAGGATCAGAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTATACAAAAATTTCAATGCCAAAAATGTTTT
TTTATTTCAAAGAAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAAAAAACAGTCTATCCAAAAATTCTTTGATA
CATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCTCGAAATGCAACAAAAGGCGAAATGCCCTCAAGTGGAACGAAATCAT
ATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGgtaaaccaaaaaatgcaaaaaaatccaaaaaaatccaaaaaaatccaaaaaaatccaa
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tcatttttccattttccactccttcccactttcagGTACAACTGGTCGTTTAGCTCCAACGAAAAGAAGAAAATAAAAACGCACGTCAAGATCA
ACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGCGTGTCAGCATCATCAGGTCATTATTATTTTAT
CGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTATTACTAAAGTTAGTTCCAAATCCATAAAC
CGAATTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTT**TAA**t
catatttcaaaattgcaacaattttgctaacgctttattattttatata

Note: A mutation G2308T detected between 15CQ and 30CQ

C.3 - AS-ATN (Artesunate-resistant; derived from AS15CQ)

tttcccaaacagaaattataataaactttcatccaaagcttcttcaacatatgctgaatcacaaaacaaaggtaattacaatgatgaatctgat
gatttcaatctttttgatgttgaaaatattatcaaagagttcaattttaatgatattgttaatgctgaggtgagttcaaaaaacctaatgcact
cccctcgcgatcacaatcacgattcccacggaacccacccctccgaatccattgaatcaggtatgttcggcgcccgatgccacgcccaccattt
gcttgtcatgcttgccatgcatcggtttaggcccacttgtttagtttcacttgcatttttctttgtttcacttgcattttctttgttgcactcgt
ttcgttcttcgcccactctcacattgacattcccattctctttgcagcagaaaaccgagaggaaatcgaacaggccaaggaaaacacgatcgga
ataaaaaatatgcttaagttcataatccaaaaaagcaaaaaggaaaaaaat**ATG**ATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTAT
TTTTAGGCATATGTATAAAAATTGTCATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCATAAATCA
TAAAATATTGGGTTTAAAAATATTTCGACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTT
ATTGAACATGAAATTGATCCAGAAATTATTAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATA
TTGGAAGTAATCCTATGTTACATCCATACACTCGTAGTAATACATCATTACCTGAAGTAGATCATTTCTCTTTCCCTTCAAAAGATAAGAATCT
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ACTTACATTTTAATAATTTACCGGACTATCCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAA
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CCTCAAAAAAGTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAAC
AATTCAAACCAATGGACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAAC
GATTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAACAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAG
AAAAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAAAAAAATGCA
AAAAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTT
TCATTTGTTGTTAATTTATACATTTTTAATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCA
AAAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACTTACTTTCAAAACGATTTTTATATGAGTTGAA
AATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAAT
CAACAAGATTTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTA
TACAAAAAGTTCAATGCCAAAAATGTTTTTTTATTTCAAAGAAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAA
AAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCTCGAAATGCAACAAAAGG
CGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGgtaaaccaaaaaatgcaaaaaaatccaaa
aaaatccaaaaaaatccaaaaaaatccaaaaaaaagcaaaaaaaaatattttcacttcgtcaatttttcatttttcaatttttccatttcgtca
atttttcacttcgtcaatttttcacttcgtcatttttccatttttccactccttcccactttcagGTACAACTGGTCGTTTAGCTCCAACGAAAA
GAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGCGTG
TCAGCATCATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTA
TTACTAAAGTTAGTTCCAAATCCATAAACCGAATTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGC
TCCCGATTCCCCAAGCTTATACTTT**TAA**tcatatttcaaaattgcaacaattttgctaacgctttattattttatatacgtgcacctttgaacg
aaaaccctttttaagttatatttttgtttagtttttccgattatgtttgtatatatatatatgtatatataatgtgtgcgatctttttgtcg
cctaattaattaatgagtatttgctctatgaaatctagttttttatagttttcatttctttgttattgtatattacatatatttgataactta
tttttatttaattcatttaaagtttgtaataatttcaagcaaaattatgggcacaataaatcctatacttttctatgcataggtaaacctaaac
gggtttcacatcgacaatttttttttttcttattttccaactctacaaagtta

Note: There is one difference between this sequence and 15CQ - G2736T

C.4 - AS-ART (Artemisinin-resistant; derived from AS30CQ)

tttcccaaacagaaattataataaactttcatccaaagcttcttcaacatatgctgaatcacaaaacaaaggtaattacaatgatgaatctgat
gatttcaatctttttgatgttgaaaatattatcaaagagttcaattttaatgatattgttaatgctgaggtgagttcaaaaaacctaatgcact
cccctcgcgatcacaatcacgattcccacggaacccacccctccgaatccattgaatcaggtatgttcggcgcccgatgccacgcccaccattt
gcttgtcatgcttgccatgcatcggtttaggcccacttgtttagtttcacttgcattttctttgtttcacttgcattttctttgttgcactcgt
ttcgttcttcgcccactctcacattgacattcccattctctttgcagcagaaaaccgagaggaaatcgaacaggccaaggaaaacacgatcgga
ataaaaaatatgcttaagttcataatccaaaaaagcaaaaaggaaaaaaat**ATG**ATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTAT
TTTTAGGCATATGTATAAAAATTGTCATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCATAAATCA
TAAAATATTGGGTTTAAAAATATTTCGACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTT
ATTGAACATGAAATTGATCCAGAAATTATTAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATA
TTGGAAGTAATCCTATGTTACATCCATACACTCGTAGTAATACATCATTACCTGAAGTAGATCATTTCTCTTTCCCTTCAAAAGATAAGAATCT
AAATCATACTTTTAATAATTTTTCTACCCAATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCA
AAACAAAACTTTTTTAAACATAAACAGAATGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAA
AAAAAAAAAATTCTGAAAAATTTGAAGATTCACATAATAAAAATCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCA
CAAAAATATAATTAATAATATTAATAATATTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGATCACGGTATTACTTAT
GATGACATGTTTAGAGATAATGACAATAGCAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAA
ACTTACATTTTAATAATTTACCGGACTATCCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAA
AATAAAAAATGAAAATCAAAATAGTAAAGAAGAACAAGATAATGGGGAAGAAGAAAATCATAGTAATAAAACTCAACATAAAGAAAATAATAAT
ATTTCAAAAGTCAATGAGCAAAATGAAAATATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAAATATCCAAAAATTCTAACA
CTACTCGAAAATATATGATTGATGAAAACCTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATC
ACCAACTTTTGAAGAAGATAAGTATAGACATAATAATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAA
GATGCTGATAATTCAAATAATATGGTGGATAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAGAGAGAAAAAAGAAAATTTTGGAT
CCTCAAAAAAGTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAAC
AATTCAAACCAATGGACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAAC
GATTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAACAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAG
AAAAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAAAAAAATGCA
AAAAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTT
TCATTTGTTGTTAATTTATACATTTTTAATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCA
AAAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACTTACTTTCAAAACGATTTTTATATGAGTTGAA
AATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAAT
CAACAAGATGTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTA
TACAAAAATTTCAATGCCAAAAATGTTTTTTTATTTCAAAGAAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAA
AAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCTCGAAATGCAACAAAAGG
CGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGgtaaaccaaaaaatgcaaaaaaatccaaa
aaaatccaaaaaaatccaaaaaaatccaaaaaaaagcaaaaaaaaatatttttcacttcgtcaatttttcatttttcaattttccatttcgtca
atttttcacttcgtcaatttttcacttcgtcatttttccatttttccactccttcccactttcagGTACAACTGGTCGTTTAGCTCCAACGAAAA
GAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGCGTG
TCAGCATCATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTA
TTACTAAAGTTAGTTCCAAATCCATAAACCGAATTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGC
TCCCGATTCCCCAAGCTTATACTTT**TAA**tcatatttcaaaattgcaacaattttgctaacgctttattatttatatacgtgcacctttgaacg
aaaaccctttttaagttatatttttgtttagtttttccgattatgtttgtatatatatatatgtatatataatgtgtgcgatctttttgtcg
cctaattaattaatgagtatttgctctatgaaatctagttttttttatagttttcatttctttgttattgtatattacatatatttgataactta
tttttatttaattcatttaaagtttgtaataatttcaagcaaaattatgggcacaataaatcctatacttttctatgcataggtaaacctaaac
gggtttcacatcgacaatttttttttttcttattttccaactctacaaagtta

Note: This has a mutation G2308T that occurred during the chloroquine selection going from AS-SENS to AS-15CQ. AS30CQ contains the same mutation. Therefore confirms that ART is a direct descendant of AS30CQ (as expected because AS-ART was selected by ART pressure from AS-30CQ), AS-ATN has a different mutation.

D – Alignment of the *ubp-1* gene sequence for AS-15CQ, AS-30CQ, AS-ART and AS-ATN

Note: Mutations indicated in the sequence in highlighted grey, bold and underlined.

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       1                                                                                                                             130
AS15CQ ATGATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTCATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCA
AS30CQ ATGATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTCATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCA
AS-ART ATGATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTCATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCA
AS-ATN ATGATATGTTGTAGTGATGCTAATGAACTGAAACTGTTGGTATTTTTAGGCATATGTATAAAAATTGTCATATGTAGGATATCAAATTTATTAAATGCAAAAGCATGCTTAGAACAATTTTACTATTTCA

       131                                                                                                                             260
AS15CQ TAAATCATAAAATATTGGGTTTAAAAATATTTCGACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAATTAT
AS30CQ TAAATCATAAAATATTGGGTTTAAAAATATTTCGACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAATTAT
AS-ART TAAATCATAAAATATTGGGTTTAAAAATATTTCGACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAATTAT
AS-ATN TAAATCATAAAATATTGGGTTTAAAAATATTTCGACATTCTCATATCGTTTTAGTTTATTTTATTCCGTTTTTCAAAAAATATTATTTTTTATGGAAGTTTATTGAACATGAAATTGATCCAGAAATTAT

       261                                                                                                                             390
AS15CQ TAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCATACACTCGTAGTAATACATCATTACCTGAAGTAGATCAT
AS30CQ TAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCATACACTCGTAGTAATACATCATTACCTGAAGTAGATCAT
AS-ART TAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCATACACTCGTAGTAATACATCATTACCTGAAGTAGATCAT
AS-ATN TAAACTCATAAATGCAATTATAAATAATTTAGAAAACTTACAAAATAATAAAAATATAAATAATATTGGAAGTAATCCTATGTTACATCCATACACTCGTAGTAATACATCATTACCTGAAGTAGATCAT

       391                                                                                                                             520
AS15CQ TTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTACCCAATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCAAAACAAA
AS30CQ TTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTACCCAATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCAAAACAAA
AS-ART TTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTACCCAATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCAAAACAAA
AS-ATN TTCTCTTTCCCTTCAAAAGATAAGAATCTAAATCATACTTTTAATAATTTTTCTACCCAATATAACCCACATGATTATAAAACAAGCTTCTCTCACAGCATATCAATAGAAAATAACAATTCAAAACAAA

       521                                                                                                                             650
AS15CQ ACTTTTTTAAACATAAACAGAATGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAATTCTGAAAAATTTGAAGATTCACATAATAAAAA
AS30CQ ACTTTTTTAAACATAAACAGAATGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAATTCTGAAAAATTTGAAGATTCACATAATAAAAA
AS-ART ACTTTTTTAAACATAAACAGAATGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAATTCTGAAAAATTTGAAGATTCACATAATAAAAA
AS-ATN ACTTTTTTAAACATAAACAGAATGATTTATCATTAGATTCTGATTTGCCAACACCAGATTTATTTAACCCAAAAAATAAAAAAAAAAAAAAAAAAAATTCTGAAAAATTTGAAGATTCACATAATAAAAA

       651                                                                                                                             780
AS15CQ TCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAATATTAATAATATTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGAT
AS30CQ TCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAATATTAATAATATTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGAT
AS-ART TCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAATATTAATAATATTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGAT
AS-ATN TCAGCAACATCCTCATGATTATTCCGAATTTGATTCACATAATAATTTTCACAAAAATATAATTAATAATATTAATAATATTAATTATTTTAGACCTAGCTCTTTATTTAATACAAATGAAAATTGTGAT

       781                                                                                                                             910
AS15CQ CACGGTATTACTTATGATGACATGTTTAGAGATAATGACAATAGCAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATTTAC
AS30CQ CACGGTATTACTTATGATGACATGTTTAGAGATAATGACAATAGCAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATTTAC
AS-ART CACGGTATTACTTATGATGACATGTTTAGAGATAATGACAATAGCAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATTTAC
AS-ATN CACGGTATTACTTATGATGACATGTTTAGAGATAATGACAATAGCAGTGATGATAATTATTTTGATAAAGGTAAAAATAAAATTTGTAATGTTAAAGAATATATTACAAACTTACATTTTAATAATTTAC
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       911                                                                                                                              1040
AS15CQ CGGACTATCCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAAATCAAAATAGTAAAGAAGAACAAGATAATGGGGAAGAAGAAAA
AS30CQ CGGACTATCCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAAATCAAAATAGTAAAGAAGAACAAGATAATGGGGAAGAAGAAAA
AS-ART CGGACTATCCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAAATCAAAATAGTAAAGAAGAACAAGATAATGGGGAAGAAGAAAA
AS-ATN CGGACTATCCTACTTCTTTGAAAAATAGTGATGAAAATAAAAAATCGGAAGATAAAAAAAAAAAAAAAAAAAAAATAAAAAATGAAAATCAAAATAGTAAAGAAGAACAAGATAATGGGGAAGAAGAAAA

       1041                                                                                                                             1170
AS15CQ TCATAGTAATAAAACTCAACATAAAGAAAATAATAATATTTCAAAAGTCAATGAGCAAAATGAAAATATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAATATCCAAAAATTCTAAC
AS30CQ TCATAGTAATAAAACTCAACATAAAGAAAATAATAATATTTCAAAAGTCAATGAGCAAAATGAAAATATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAATATCCAAAAATTCTAAC
AS-ART TCATAGTAATAAAACTCAACATAAAGAAAATAATAATATTTCAAAAGTCAATGAGCAAAATGAAAATATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAATATCCAAAAATTCTAAC
AS-ATN TCATAGTAATAAAACTCAACATAAAGAAAATAATAATATTTCAAAAGTCAATGAGCAAAATGAAAATATTTTACTTTATAATATACAAGATAATGAAAATGCAGAAAACAAATATCCAAAAATTCTAAC

       1171                                                                                                                             1300
AS15CQ ACTACTCGAAAATATATGATTGATGAAAACCTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTTGAAGAAGATAAGTATAGACATAATA
AS30CQ ACTACTCGAAAATATATGATTGATGAAAACCTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTTGAAGAAGATAAGTATAGACATAATA
AS-ART ACTACTCGAAAATATATGATTGATGAAAACCTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTTGAAGAAGATAAGTATAGACATAATA
AS-ATN ACTACTCGAAAATATATGATTGATGAAAACCTTAATGAATATAAAATTGAAAACAAAAAAGATGTATCAAACAATTATAATAATAAAGAAGGATCACCAACTTTTGAAGAAGATAAGTATAGACATAATA

       1301                                                                                                                             1430
AS15CQ ATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATAATTCAAATAATATGGTGGATAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAG
AS30CQ ATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATAATTCAAATAATATGGTGGATAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAG
AS-ART ATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATAATTCAAATAATATGGTGGATAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAG
AS-ATN ATCGATCAAGCACTCCTCAAAACAATGGAATTAAAAAATTTCCTACTGTTTCCTATGAAGATGCTGATAATTCAAATAATATGGTGGATAAAAAATCACAAAAGAATTATATAAATTTAGAATTAGATAG

       1431                                                                                                                             1560
AS15CQ AGAGAAAAAAGAAAATTTTGGATCCTCAAAAAAGTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACCAAT
AS30CQ AGAGAAAAAAGAAAATTTTGGATCCTCAAAAAAGTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACCAAT
AS-ART AGAGAAAAAAGAAAATTTTGGATCCTCAAAAAAGTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACCAAT
AS-ATN AGAGAAAAAAGAAAATTTTGGATCCTCAAAAAAGTTAACTAACATTGAAAGTGCTAAAGGAATGAATAATGATGACAATTATAATAGCAATAATGAAATATTAAATAATCGTAGAACAATTCAAACCAAT

       1561                                                                                                                             1690
AS15CQ GGACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAACGATTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAA
AS30CQ GGACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAACGATTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAA
AS-ART GGACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAACGATTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAA
AS-ATN GGACATACTGTTAACTACAACAGAAATAATAACAATATGAGACCAGATGAATATGAAAACTTTGTAAAAGATAAAAAAAACGATTTAGATATTATACAAAGAAAAGGAATATCTCTTGTTAATGCTACAA

       1691                                                                                                                             1820
AS15CQ CAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAAAAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAA
AS30CQ CAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAAAAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAA
AS-ART CAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAAAAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAA
AS-ATN CAAATAATAATTATGAAGAAATAAATATAGTTAATAATCCTATAGAAAAAAATAAACATGTTAGTTCAGATAAGTTAATACAAAAACGACCCAAATATTTAATGTTACCAATTGATACGACTGAATTAAA

       1821                                                                                                                             1950
AS15CQ AAAAATGCAAAAAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTCATTTGTTGTTAATTTATACATTTTT
AS30CQ AAAAATGCAAAAAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTCATTTGTTGTTAATTTATACATTTTT
AS-ART AAAAATGCAAAAAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTCATTTGTTGTTAATTTATACATTTTT
AS-ATN AAAAATGCAAAAAGGAAAATTACGACACCCACCTGTTGGTTTGATAAATTTAGGAAATACTTGCTACTTAAATAGTTTATTACAAGCATTATATAGTACTGTTTCATTTGTTGTTAATTTATACATTTTT
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      1951                                                                                                                 2080
AS15CQ AATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAAAAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACT
AS30CQ AATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAAAAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACT
AS-ART AATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAAAAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACT
AS-ATN AATATTGATGATAATAAAGAATTAAAACACATAAATAATAAAAATATCTCTAACGAAATGCCCATCAAAAATAAATTATCATTTAACCTGAACAATACAAACATGAATAATAATAATAATAATGCAAACT

      2081                                                                                                                 2210
AS15CQ TACTTTCAAAACGATTTTTATATGAGTTGAAAATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAATCAACA
AS30CQ TACTTTCAAAACGATTTTTATATGAGTTGAAAATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAATCAACA
AS-ART TACTTTCAAAACGATTTTTATATGAGTTGAAAATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAATCAACA
AS-ATN TACTTTCAAAACGATTTTTATATGAGTTGAAAATATTATTTAAATTAATGACTACAACAAATAAAAAATATGTTTCACCAGATAATATTTTAGGTATACTACCTCAAGAACTTAACAATAGAAATCAACA

      2211                                                                                                                 2340
AS15CQ AGATGTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTATACAAAAAGTTCAATGCCAAAAATGTTTTTTTATTTCAAAG
AS30CQ AGATGTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTATACAAAAATTTCAATGCCAAAAATGTTTTTTTATTTCAAAG
AS-ART AGATGTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTATACAAAAATTTCAATGCCAAAAATGTTTTTTTATTTCAAAG
AS-ATN AGATTTTACAGAATTATTTAGATATACATTTGAACAATTAGGAGGATCAGAAAAAAAAATTTCTAAGATTAATATTTTCAGGAGTTGTTATACAAAAAGTTCAATGCCAAAAATGTTTTTTTATTTCAAAG

      2341                                                                                                                 2470
AS15CQ AAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAAAAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCT
AS30CQ AAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAAAAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCT
AS-ART AAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAAAAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCT
AS-ATN AAAGAAGAAATTATCCACGATTTATCATTTCATGTTCCTGCAAAGTCAAGTAAAAAACAGTCTATCCAAAAATTCTTTGATACATATATTCAAAAAGAAAAAATTTATGGAAACAATAAATACAAATGCT

      2471                                                                                                                 2600
AS15CQ CGAAATGCAACAAAAGGCGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGGTAAACCAAAAAATGCAAAAAAATCCAAAAAAATCCAAAAAAATCCAA
AS30CQ CGAAATGCAACAAAAGGCGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGGTAAACCAAAAAATGCAAAAAAATCCAAAAAAATCCAAAAAAATCCAA
AS-ART CGAAATGCAACAAAAGGCGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGGTAAACCAAAAAATGCAAAAAAATCCAAAAAAATCCAAAAAAATCCAA
AS-ATN CGAAATGCAACAAAAGGCGAAATGCCCTCAAGTGGAACGAAATCATATCCCCCCCCTGCCACCTCATACTAATTCTGAACAGGTAAACCAAAAAATGCAAAAAAATCCAAAAAAATCCAAAAAAATCCAA

      2601                                                                                                                 2730
AS15CQ AAAAATCCAAAAAAAAGCAAAAAAAAATATTTTTCACTTCGTCAATTTTTCATTTTTCAATTTTCCATTTCGTCAATTTTTCACTTCGTCAATTTTTCACTTCGTCATTTTTCCATTTTCCACTCCTTCC
AS30CQ AAAAATCCAAAAAAAAGCAAAAAAAAATATTTTTCACTTCGTCAATTTTTCATTTTTCAATTTTCCATTTCGTCAATTTTTCACTTCGTCAATTTTTCACTTCGTCATTTTTCCATTTTCCACTCCTTCC
AS-ART AAAAATCCAAAAAAAAGCAAAAAAAAATATTTTTCACTTCGTCAATTTTTCATTTTTCAATTTTCCATTTCGTCAATTTTTCACTTCGTCAATTTTTCACTTCGTCATTTTTCCATTTTCCACTCCTTCC
AS-ATN AAAAATCCAAAAAAAAGCAAAAAAAAATATTTTTCACTTCGTCAATTTTTCATTTTTCAATTTTCCATTTCGTCAATTTTTCACTTCGTCAATTTTTCACTTCGTCATTTTTCCATTTTCCACTCCTTCC

      2731                                                                                                                 2860
AS15CQ CACTTTCAGGTACAACTGGTCGTTTAGCTCCAACGAAAAGAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGC
AS30CQ CACTTTCAGGTACAACTGGTCGTTTAGCTCCAACGAAAAGAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGC
AS-ART CACTTTCAGGTACAACTGGTCGTTTAGCTCCAACGAAAAGAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGC
AS-ATN CACTTTCAGGTACAACTGGTCGTTTAGCTCCAACGAAAAGAAGAAAATAAAAACGCACGTCAAGATCAACAAAAAGATAGTTGTAAACAATTTTGATTACCGATTGTATGGAGGAATAATTCACAGTGGC

      2861                                                                                                                 2990
AS15CQ GTGTCAGCATCATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTATTACTAAAGTTAGTTCCAAATCCATAAACCGAA
AS30CQ GTGTCAGCATCATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTATTACTAAAGTTAGTTCCAAATCCATAAACCGAA
AS-ART GTGTCAGCATCATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTATTACTAAAGTTAGTTCCAAATCCATAAACCGAA
AS-ATN GTGTCAGCATCATCAGGTCATTATTATTTTATCGGAAAGAAATCCGAAAAAGGTGACAATTCAAAAAATGAATGGTATCAAATGGACGACTCAGCTATTACTAAAGTTAGTTCCAAATCCATAAACCGAA
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        2991                                                                                                  3079
AS15CQ  TTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTTTAA
AS30CQ  TTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTTTAA
AS-ART  TTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTTTAA
AS-ATN  TTTCTAAAGATCCATCAAATGATCACACTCCTTACGTTTTATTTTATCGTTGCAAACAAGCTCCCGATTCCCCAAGCTTATACTTTTAA
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APPENDIX 5

THE RELATIVE INTENSITY AND COMPARATIVE INTENSITES OF ALL AFLP MARKERS ANALYSED IN THE LGS EXPERIMENTS DESCRIBED IN CHAPTER V

A - AS-ART x AJ – AJ specific AFLP markers

Marker Name		Relative Intensity			
AJ Marker	CM distance along chromosome	Non passaged	Untreated	ART treated	Comparative Intensity ART treated/Untreated
Chromosome 1					
AJTG01GT	12	1,31	1,26	1,09	0,8651
AJAC03TA	16,2	1,19	1,23	0,59	0,4797
AJAG02TC	16,2	0,5	0,3	0,2	0,6667
AJTA03AT	16,2	0,5	0,42	0,34	0,8095
AJAG01GT	25,4	1,8	2,3	1,2	0,5217
AJTG01AA	43,4	0,64	0,9	0,57	0,6333
Chromosome 5					
AJAA04AC	70	0,5	0,7	0,6	0,8571
AJAT07AT	70	0,4	0,41	0,23	0,5610
AJAT01AT	73,9	1,16	2,51	1,28	0,5100
AJTA01AC	77,5	1,97	1,02	0,56	0,5490
AJAG01TT	82,7	0,4	0,3	0,3	1,0000
AJAA03TA	82,9	0,6	0,6	0,3	0,5000
Chromosome 6					
AJAG02AG	11,3	0,1	0,3	0,3	1,0000
AJAG02CT	11,3	0,6	0,4	0,2	0,5000
AJAC01GT	11,3	1,06	0,55	0,8	1,4545
AJAT02TA	11,3	0,93	0,88	0,8	0,9091
AJTC02CT	11,3	1,08	0,81	0,64	0,7901
AJTC01TC	11,3	0,45	0,45	0,45	1,0000
AJTG02AA	11,3	0,84	0,82	0,75	0,9146
AJTT05AT	11,3	7,03	10,78	1,35	0,1252
AJAG01CT	16,1	0,5	0,7	0,5	0,7143
AJAT03TG	29,3	0,82	0,8	0,6	0,7500
AJTA02GA	37,1	2,07	2,3	2,22	0,9652
AJTT01CA	44,3	1,44	0,45	0,38	0,8444
AJTA02AC	62,7	0,34	0,31	0,22	0,7097
Chromosome 7					
AJAA02TG	24,1	2	2	1	0,5000
AJAG01AG	37,2	0,5	0,5	0,5	1,0000
AJAA01GA	37,3	2,9	1,7	1,2	0,7059
AJAT01GT	37,3	0,61	0,54	0,31	0,5741
AJTT06AT	48,5	0,49	2,55	2,66	1,0431
AJTA01CA	62,3	0,73	0,58	0,26	0,4483
AJGA03AC	77,5	0,87	1,29	0,58	0,4496
AJAT02TC	77,5	1,05	0,73	0,45	0,6164
AJTG02AT	77,5	2,54	1,51	0,71	0,4702
AJTG01TC	77,5	1,28	1,07	0,69	0,6449
AJAT02AC	77,7	1,82	1,41	0,81	0,5745
AJTT03TG	81,4	0,1	0,54	0,88	1,6296
AJAA05CA	96,3	0,68	0,47	0,29	0,6170
AJAG02CA	100,3	0,8	0,7	0,6	0,8571

AJTG01AG	104,4	0,05	0,15	0,12	0,8000
AJTG01GA	104,4	0,68	0,96	0,7	0,7292
AJAC02TT	123,8	3,39	2,44	1,23	0,5041
AJTC01AG	135,1	0,06	0,03	0,03	1,0000
AJAA03AT	135,3	0,3	0,4	0,3	0,7500
		Chromosome 8			
AJAG03AG	0	0,2	0,2	0,06	0,3000
AJTT02AT	17,3	0,8	0,4	0,06	0,1500
AJAT01TA	21,3	2,99	3,22	1,04	0,3230
AJTT07AT	25,9	0,86	0,82	1,04	1,2683
AJAC02AG	43	1,2	1	0,7	0,7000
AJAC02AT	46,2	0,37	0,42	0,21	0,5000
AJTG03AA	55	0,69	1,2	0,56	0,4667
AJTC01TG	58,5	2,13	2,15	1,33	0,6186
AJAC03AT	73,2	1,96	1,57	0,5	0,3185
		Chromosome 9			
AJAT05TG	40,2	2,26	1,45	1,35	0,9310
AJAT01TC	40,2	1,86	1,31	0,84	0,6412
AJTA01GT	40,2	5,96	6,75	4,09	0,6059
AJAC01AA	51,7	0,83	0,9	0,5	0,5556
AJAC01CT	51,7	1,45	0,64	0,53	0,8281
AJAG01TC	63,5	0,2	0,4	0,3	0,7500
AJAT04TA	67,2	0,57	0,64	0,47	0,7344
		Chromosome 10			
AJGA01TT	7,2	0,65	0,45	0,12	0,2667
AJAG03AC	10,9	0,8	0,8	0,2	0,2500
AJAC02AC	10,9	3,7	2,6	0,8	0,3077
AJAC02AA	18,4	0,75	0,6	0,1	0,1667
AJGA03AG	18,4	0,67	0,82	0,18	0,2195
AJGA01CA	18,4	0,75	0,74	0,15	0,2027
AJGA02CA	18,4	1,46	1,18	0,33	0,2797
		Chromosome 11			
AJAT03CT	5,3	0,64	0,83	0,41	0,4940
AJAC01CA	9,6	1,26	1,52	0,93	0,6118
AJTT03AT	9,7	0,7	0,39	0,06	0,1538
AJTT03CA	9,7	14,2	0,8	0,58	0,7250
AJTT02GA	9,7	0,26	0,4	0,38	0,9500
AJTT03AA	13,6	0,99	0,9	0,66	0,7333
AJAT03AA	17,6	1,67	1,75	1	0,5714
AJAT01AA	21,6	0,71	0,99	0,46	0,4646
AJGA02TT	31,2	0,53	0,23	0,12	0,5217
AJTA01TT	39,3	0,74	0,71	0,55	0,7746
AJTG05AT	39,3	0,5	0,61	0,38	0,6230
AJAT06CT	44,6	3,08	2,15	1,72	0,8000
AJAG01TA	48,7	0,5	0,5	0,4	0,8000
AJAC01TA	53	1,71	1,11	0,82	0,7387
		Chromosome 12			
AJTT04AT	13,3	1,05	0,44	0,06	0,1364
AJAC01GA	20,8	0,42	0,38	0,24	0,6316
AJTT01CT	24,6	0,04	0,19	0,69	3,6316
AJAA02TC	35,9	2	0,8	0,5	0,6250
AJTC01CT	50,9	1,29	0,8	0,46	0,5750
AJAT04CT	79,9	0,53	0,8	0,59	0,7375
AJAG01TG	106,8	0,3	0,5	0,4	0,8000

AJTC01TA	111,4	0,56	0,46	0,29	0,6304
AJTT02AA	123,5	0,79	0,47	0,34	0,7234
AJAA03TC	123,6	0,5	0,4	0,3	0,7500
		Chromosome 13			
AJTT01AG	15,2	1,02	0,77	0,69	0,8961
AJAA03TG	28,5	1	1,5	1	0,6667
AJAC04.0AT	28,5	0,51	0,65	0,46	0,7077
AJTA01GA	28,5	1,51	2,38	1,35	0,5672
AJTT04AA	32,4	0,31	0,29	0,42	1,4483
AJTT02AC	36,7	0,71	0,97	1,03	1,0619
AJAA01AT	44,5	0,8	0,8	0,8	1,0000
AJAT02TG	64	1,01	0,53	0,51	0,9623
AJTA01AG	72,9	15,39	18,47	6,69	0,3622
AJGA02AA	76,9	1,02	0,98	0,71	0,7245
AJAA03AC	136,1	0,7	0,8	0,5	0,6250
AJAA01TG	151,2	0,3	0,1	0,1	1,0000
AJAG04AG	177,9	0,9	0,3	0,2	0,6667
AJTG02AG	183,4	0,57	0,76	0,83	1,0921
AJTT02TG	183,4	1,4	1,47	1,16	0,7891
AJTT02TT	183,4	0,95	1,38	1,35	0,9783
AJTT02CA	183,4	1,28	0,32	0,2	0,6250
AJGA02AG	183,5	1,88	1,96	1,47	0,7500
AJGA02CT	183,5	0,3	0,29	0,23	0,7931
AJTA01TC	187,9	0,3	0,32	0,17	0,5313
AJAA03CA	200	1,09	1,23	0,73	0,5935
AJAC03TT	200	5,47	4,07	2,94	0,7224
AJAT02AT	200	1,49	1,09	0,97	0,8899
AJAA01AG	208,2	0,5	0,3	0,3	1,0000
AJTG01TG	217,8	0,04	0,02	0,03	1,5000
		Group 31			
AJTT02CT	0	58,5	1,33	1,11	0,8346
		Group 12			
AJAG02AC	28,3	0,6	0,4	0,3	0,7500
AJAG02TA	0	0,2	0,3	0,2	0,6667
AJGA01AG	4	0,35	0,35	0,5	1,4286
AJAC04TA	4	0,89	1,11	0,67	0,6036
AJTG03AT	12,8	0,73	0,54	0,37	0,6852
AJAC04AA	20,3	0,5	0,6	0,6	1,0000
AJGA01AC	20,3	1,78	1,44	1,06	0,7361
AJAT04AT	20,3	2,1	1,35	1,15	0,8519
AJTT03CT	28,3	57,19	0,49	0,42	0,8571
AJGA02AC	28,3	1,64	1,91	0,84	0,4398
		Group 14			
AJTG02CT	4	1,5	1	1,14	1,1400
AJTT01TT	8,2	0,31	0,49	0,45	0,9184
		Group 15			
AJAT03AC	0	1,03	0,88	0,5	0,5682
AJTC01GT	4,4	0,9	0,87	0,64	0,7356
AJTG04AT	24,5	0,94	1,19	0,56	0,4706
AJTT01AA	48,4	O,73	0,41	0,28	0,6829
		Group 2			
AJAA02TA	0	0,6	0,5	0,3	0,6000
AJAG02TT	19,8	0,8	0,9	0,5	0,5556
AJAC05AT	24,4	2,44	1,49	0,75	0,5034

AJAA01TA	28,1	0,4	0,5	0,2	0,4000
AJGA02TA	33,1	0,67	0,69	0,37	0,5362
AJAG02AT	36,8	0,7	0,6	0,2	0,3333
AJTT01TC	44,6	2,25	2,43	1,5	0,6173
AJGA01GA	71,9	1,37	1,22	0,46	0,3770
AJTT01GT	71,9	4,82	0,67	1,03	1,5373
AJTA03AC	79,6	0,55	0,47	0,17	0,3617
AJAC01TT	88,4	3,9	2,9	1,27	0,4379
AJGA01AT	88,4	0,42	0,59	0,53	0,8983
AJAT05AT	120,6	1,13	0,83	0,52	0,6265
		Group 20			
AJAT03TA	4,6	5,01	4,35	3,43	0,7885
AJAA01GT	8,7	0,5	0,5	0,5	1,0000
AJAT02AA	8,7	0,36	0,41	0,28	0,6829
AJAT01AC	8,7	1,26	1,4	0,96	0,6857
AJTC02TA	8,7	2,36	2,12	1,47	0,6934
AJAA05AC	16,8	1,5	1,3	1	0,7692
AJAT02GT	16,8	1,13	0,83	0,61	0,7349
AJAT04TG	25,5	1	0,65	0,57	0,8769
AJTT05AA	43,7	0,15	0,11	0,13	1,1818
AJAT01TG	43,7	0,95	0,5	0,42	0,8400
AJAT04AA	47,5	0,31	0,45	0,34	0,7556
AJAT02CT	71,9	1,12	0,99	0,41	0,4141
AJAT01CT	84,2	1,98	0,9	0,8	0,8889
AJTG01CT	84,2	0,98	1,08	1	0,9259
AJTT06AA	84,2	0,16	0,15	0,13	0,8667
AJAG03AT	92,7	1,5	1	0,6	0,6000
AJGA02AT	92,7	0,85	0,81	0,74	0,9136
		Group 21			
AJAA01AC	0	1,2	1,3	1,2	0,9231
		Group 29			
AJAT01CA	0	0,33	0,44	0,29	0,6591
		Group 3			
AJTT07AA	40,7	1,63	0,91	0,87	0,9560
AJAC01AG	44,2	1,44	1,42	1,4	0,9859
AJTT01AC	49,5	0,49	0,53	0,45	0,8491
AJTA02AT	51,4	0,29	0,3	0,21	0,7000
AJAC01AT	63,8	0,88	0,97	0,87	0,8969
AJAA02TT	75,5	1,9	1,1	0,6	0,5455
AJTA01AT	86,4	0,58	0,48	0,42	0,8750
		Group 33			
AJTT01AT	8,1	0,52	1,21	0,18	0,1488
AJGA01AA	22,5	1,28	0,83	0,44	0,5301
		Group 38			
AJTA02AG	7,5	1,24	0,91	0,26	0,2857
AJAC01TG	11	0,65	0,53	0,24	0,4528
AJGA01TA	15,8	0,64	0,66	0,21	0,3182
		Group 6			
AJGA01TG	0	1,03	0,86	0,59	0,6860
AJGA01CT	0	0,64	0,6	0,32	0,5333
AJTT01TG	0	2,08	1,69	1,51	0,8935
AJAA02AT	8,5	1	1	0,8	0,8000
AJAG01AT	19,4	0,4	0,6	0,7	1,1667
		No data			

AJAG02TG	.....	0,7	0,7	0,4	0,5714
AJAA01TC	.....	1	0,9	0,9	1,0000
AJAC04.5AT	.....	0,45	0,25	0,09	0,3600
AJGA04AT	.....	0,56	0,8	0,49	0,6125
AJGA05AT	.....	0,37	0,39	0,25	0,6410
AJAC02TA	.....	0,92	0,75	0,46	0,6133
AJGA03AA	.....	0,86	0,78	0,61	0,7821
AJTA01TG	.....	3,91	3,46	2,86	0,8266
AJTA03GA	.....	0,72	1,39	1,37	0,9856
AJTC02AG	.....	0,43	0,53	0,24	0,4528
AJTC02TG	.....	0,12	0,3	0,42	1,4000
AJTG04AG	.....	0,88	0,97	0,76	0,7835
AJTG05AG	.....	1,16	1,75	2,95	1,6857
AJTG02TG	.....	0,04	0,03	0,04	1,3333
AJTG03TG	.....	2,41	0,98	0,65	0,6633
AJAG03CA	.....	0,3	0,4	0,3	0,7500
AJAG05CT	.....	0,6	0,5	0,3	0,6000
AJAG01AC	.....	0,09	0,09	0,15	1,6667
AJAT01GA	.....	1,69	1,68	0,85	0,5060
		Unlinked			
AJAG01AA	.....	0,5	0,8	1	1,2500
AJAA02AC	.....	1	1	1	1,0000
AJAC03AG	.....	3,74	3,11	2,23	0,7170
AJAA01CA	.....	1,39	1,08	0,7	0,6481
AJAC02CT	.....	3,33	2,07	1,57	0,7585
AJGA03AT	.....	1,41	0,91	0,41	0,4505
AJAT06AT	.....	0,66	0,5	0,31	0,6200
AJAT02CA	.....	0,8	0,53	0,3	0,5660
AJTA01CT	.....	0,73	0,61	0,64	1,0492
AJTC01AT	.....	0,86	0,3	0,5	1,6667
AJTT03GA	.....	10,92	1,57	1,32	0,8408

B - AS-ART x AJ – AS specific AFLP markers

Marker Name		**Relative Intensity**			
AS Markers	**CM distance along chromosome**	**Non passaged**	**Untreated**	**ART treated**	**Comparative Intensity ART treated/Untreated**
		Chromosome 1			
ASAA01TC	16,2	0,56	0,09	0,28	3,1111
ASAA02CA	16,2	1,39	0,25	0,9	3,6000
ASTA01TT	16,2	1,08	0,42	0,65	1,5476
ASTA02AT	16,2	0,29	0,07	0,22	3,1429
ASTA02GT	16,2	1,47	0,24	0,57	2,3750
ASTG01AA	43,4	0,7	0,13	0,3	2,3077
		Chromosome 5			
ASAA03TG	26,7	0,81	0,24	0,46	1,9167
ASAA02GA	54,4	0,85	0,18	0,38	2,1111
ASAC03AT	69,9	1,24	0,24	0,68	2,8333
ASAA04AC	69,9	0,31	0,09	0,19	2,1111
ASAC01AG	74,6	0,67	0,09	0,32	3,5556
ASTA01AC	74,6	1,3	0,36	0,63	1,7500
		Chromosome 6			
ASAC02AT	11,3	0,75	0,08	0,2	2,5000
ASAT01AA	11,3	0,31	0,13	0,24	1,8462
ASTC01TC	11,3	0,17	0,09	0,18	2,0000

ASTG01AT	11,3	1,94	0,3	0,69	2,3000
ASTT01CA	44,2	0	1,54	0,09	0,0584
ASAG01TG	62,6	1,6	0,25	0,46	1,8400
		Chromosome 7			
ASAA02GT	31,6	1,08	0,29	0,42	1,4483
ASAC01GT	31,6	0,68	0,13	0,18	1,3846
ASAA01CA	48,4	1,21	0,28	1,03	3,6786
ASAA05CA	62,2	1	0,35	0,97	2,7714
ASTT03CA	62,2	1,09	0,72	0,8	1,1111
ASAC05AA	69,7	0,85	0,58	0,6	1,0345
ASAT02AT	69,7	0,55	0,11	0,37	3,3636
ASTG01TC	77,5	0,17	0,11	0,31	2,8182
ASAG02CA	100,3	1,7	0,2	0,6	3,0000
ASTT01AG	123,6	0,07	0,13	0,14	1,0769
ASAC01TA	127,6	1,23	0,19	0,61	3,2105
		Chromosome 8			
ASAC01TG	30,8	1,19	0,3	0,85	2,8333
ASTA01AT	34,3	0,38	0,16	0,25	1,5625
ASAG01CA	38,2	1,4	0,1	0,5	5,0000
ASGA02AC	43	1,36	0,41	1,23	3,0000
ASTG02AA	50,8	0,12	0,59	0,43	0,7288
ASTC01CT	58,5	44,6	12,7	31,35	2,4646
ASAC01AT	73,2	0,44	0,05	0,28	5,6000
ASAT03AC	73,2	1,46	0,39	1,22	3,1282
		Chromosome 9			
ASTA01GT	40,2	4,04	2,18	3,43	1,5734
ASAA04CA	40,2	1,02	0,24	0,68	2,8333
ASAC03CT	51,7	0,91	0,18	0,64	3,5556
ASTT05CA	51,7	0,85	0,8	0,6	0,7500
ASAT04AA	59,2	0,41	0,16	0,33	2,0625
ASTT02TT	71,1	389	0,55	0,04	0,0727
		Chromosome 10			
ASGA01TC	7,2	0,46	0,11	0,39	3,5455
ASTG01CA	7,2	20,8	1,03	0,98	0,9515
ASTT01TT	10,9	0,01	18,6	2,93	0,1573
ASGA01CA	18,4	0,99	0,22	0,75	3,4091
ASAT05AA	18,4	0,31	0,11	0,28	2,5455
ASTA02AG	18,4	0,37	0,15	0,47	3,1333
		Chromosome 11			
ASAG03CA	5,3	2,4	0,1	0,9	9,0000
ASAT03AA	5,3	0,95	0,27	0,78	2,8889
ASTC02AC	5,3	0,25	0,12	0,28	2,3333
ASTT02AA	5,3	0,09	0,07	0,07	1,0000
ASTA03GA	21,5	1,17	0,17	0,59	3,4706
ASTC01CA	21,5	1,21	0,28	0,8	2,8571
ASTA01TA	39,2	0,29	0,12	0,21	1,7500
ASTC01AC	39,2	0,07	0,04	0,06	1,5000
ASTG01TA	39,2	0,59	0,15	0,34	2,2667
ASAG01TA	48,7	0,6	0,3	0,4	1,3333
ASAA01TG	52,9	1,32	0,82	0,47	0,5732
ASAC02TA	52,9	1,65	0,14	0,35	2,5000
		Chromosome 12			
ASAC02CT	20,8	0,83	0,06	0,31	5,1667
ASAA01AT	54,6	0,82	0,38	0,86	2,2632

ASAC03AA	54,6	0,99	0,25	0,66	2,6400
ASGA02AG	66,4	1,17	0,39	1,09	2,7949
ASTC01TA	111,4	0,61	0,07	0,33	4,7143
		Chromosome 13			
ASAA05AT	0	1,67	0,56	0,55	0,9821
ASTG02TT	3,9	0,8	0,1	0,19	1,9000
ASTA01AA	15,2	1,08	0,41	0,71	1,7317
ASTT02AG	15,2	0,14	0,1	0,24	2,4000
ASTT01GA	28,5	1,23	1,84	1,47	0,7989
ASAA01TA	36,1	1,57	0,14	0,39	2,7857
ASAA04TA	36,1	0,73	0,09	0,26	2,8889
ASTA02CA	36,1	0,42	0,14	0,29	2,0714
ASTA02CA	36,1	1,22	0,29	0,42	1,4483
ASAA03AT	44,6	0,31	0,15	0,23	1,5333
ASTA01AG	73	11,2	7,84	5,9	0,7526
ASTT02TC	132,5	0,01	0,01	0,02	2,0000
ASGA02TA	177,9	0,14	0,02	0,06	3,0000
ASAT01AC	177,9	0,94	0,4	0,62	1,5500
ASGA03CA	177,9	0,58	0,08	0,19	2,3750
ASTA03TA	183,5	1,65	0,45	1,23	2,7333
ASTG03AA	183,5	0,43	0,09	0,27	3,0000
ASTT02CA	183,5	0	2,65	0,93	0,3509
ASTA01CT	200,1	0,36	0,11	0,16	1,4545
ASTA01CT	200,1	0,79	0,29	0,42	1,4483
ASTT04AT	200,1	0,96	1,88	0,98	0,5213
ASAT01AT	209,2	1,33	0,3	0,65	2,1667
ASGA02AT	221,9	0,85	0,14	0,34	2,4286
		Group 12			
ASAC01GA	4	1,15	0,24	0,67	2,7917
ASGA01AG	4	0,82	0,12	0,3	2,5000
ASAT09AA	4	0,44	0,2	0,25	1,2500
ASAT08AA	12,8	0,58	0,14	0,29	2,0714
ASAA01GT	20,3	0,73	0,15	0,4	2,6667
ASGA03AC	20,3	0,46	0,24	0,23	0,9583
ASGA01AC	24,4	1,35	0,25	0,93	3,7200
ASTA02TA	28,3	3,24	0,92	1,74	1,8913
		Group 14			
ASTA03AA	0	2,65	0,26	0,69	2,6538
ASTA02AA	4	0,62	0,15	0,31	2,0667
		Group 16			
ASAT04AC	0	0,98	0,26	0,64	2,4615
ASTG01TT	0	0,51	0,1	0,18	1,8000
ASTT01CT	4,4	0	0,25	0,28	1,1200
ASAG01CT	12,4	1,6	0,2	1	5,0000
ASAA03TA	32,2	0,61	0,21	0,35	1,6667
ASTT01AC	40,3	8,43	2,25	0,93	0,4133
ASTT03AA	59,2	0,08	0,07	0,08	1,1429
ASTT01AA	74,5	0,16	2,4	0,65	0,2708
		Group 2			
ASAA02AT	0	0,8	0,17	0,59	3,4706
ASAG01TT	7,8	1,5	0,3	0,8	2,6667
ASAC02AG	24,4	0,91	0,16	0,55	3,4375
ASTC01GA	24,4	3,59	1,04	3,31	3,1827
ASTT01AT	71,9	0,41	0,28	0,41	1,4643

ASTT04CA	71,9	46,8	0,71	1,11	1,5634
		Group 20			
ASGA02CA	4,6	0,59	0,06	0,15	2,5000
ASAA04TC	8,7	0,77	0,11	0,21	1,9091
ASAA01GA	8,7	1,34	0,27	0,66	2,4444
ASTC02TC	8,7	0,04	0,03	0,03	1,0000
ASAA06AC	16,8	0,17	0,68	0,52	0,7647
ASAA03TC	43,7	1,06	0,25	0,35	1,4000
ASAA02TG	55	0,99	0,52	0,65	1,2500
ASAG01GA	64,2	2,3	0,48	1,4	2,9167
ASAC01CT	84,2	0,42	0,14	0,36	2,5714
ASTG02CT	84,2	1,09	1,19	1,07	0,8992
		Group 21			
ASAA01AC	0	0,09	0,05	0,08	1,6000
		Group 3			
ASGA01AT	40	0,81	0,06	0,28	4,6667
ASAA02TA	44,2	1,11	0,57	0,34	0,5965
ASAA03CT	44,2	2,55	0,54	0,95	1,7593
ASAT11AA	44,2	0,94	0,25	0,3	1,2000
ASAT02AA	63,8	0,21	0,12	0,07	0,5833
ASTA01CA	75,5	1,24	0,13	0,66	5,0769
ASTA01CA	75,5	0,8	0,13	0,27	2,0769
		Group 30			
ASTT01TC	11,9	1,97	1,16	1,22	1,0517
		Group 33			
ASTT02AT	0	0,09	0,04	0,02	0,5000
ASAT02AC	8,1	1,73	0,4	0,62	1,5500
		Group 6			
ASGA01TA	0	1,92	0,49	1,05	2,1429
ASGA01GA	0	1,7	1,22	0,46	0,3770
ASTG01GA	0	0,13	1,4	1,18	0,8429
ASAT06AA	23,4	0,33	0,13	0,25	1,9231
		No data			
ASTA04GA	.....	4,25	0,54	2,05	3,7963
ASTA04CA	.....	0,27	0,2	0,18	0,9000
ASTA02TG	.....	0,55	0,76	0,64	0,8421
ASTA02GA	.....	0,71	0,16	0,4	2,5000
ASTA03TG	.....	0,7	0,23	0,33	1,4348
ASTA04TC	.....	2,68	1,39	4,56	3,2806
ASTA02TC	.....	0,96	0,12	0,48	4,0000
ASTA03TC	.....	1,78	0,12	0,58	4,8333
ASGA01AA	.....	1,27	0,12	0,29	2,4167
ASGA02AA	.....	1,26	0,14	0,66	4,7143
ASGA03AA	.....	0,04	0,03	0,07	2,3333
ASGA04AA	.....	1,56	0,94	0,63	0,6702
ASGA05AA	.....	2,68	0,48	1,28	2,6667
ASGA06AA	.....	1,35	0,31	0,68	2,1935
ASGA07AA	.....	1,78	0,37	1,09	2,9459
ASGA08AA	.....	0,78	0,16	0,31	1,9375
ASTA01GA	.....	1,37	0,36	0,84	2,3333
ASTA04TG	.....	1,7	1,33	1,29	0,9699
ASTA02TT	.....	0,83	0,3	0,3	1,0000
ASTA01TC	.....	1,77	0,05	0,04	0,8000
ASTC02GT	.....	1,35	2,06	1,66	0,8058

ASTA03CA	.....	0,35	0,09	0,1	1,1111
ASTA01TC	.....	1,27	0,13	0,43	3,3077
ASTC03AA	.....	0,51	0,11	0,33	3,0000
ASTC03TC	.....	1,41	0,28	1,19	4,2500
ASTG04AA	.....	0,36	0,15	0,16	1,0667
ASTG02TC	.....	0,58	0,06	0,49	8,1667
ASTG02GA	.....	0,13	1,35	1,28	0,9481
ASTG0AT	.....	0,84	0,08	0,18	2,2500
ASTG0GT	.....	11,6	1,57	1,14	0,7261
ASTT0TC	.....	0,14	0,44	0,44	1,0000
ASTT00TC	.....	0,01	1,12	1,23	1,0982
ASTT0TT	.....	58,1	0,82	0,9	1,0976
ASTT00TT	.....	0,89	0,97	1	1,0309
ASTT000TT	.....	0	1,02	1,15	1,1275
ASTT0CT	.....	0,06	114	5,53	0,0483
ASTT00CT	.....	1,61	1,46	1,14	0,7808
ASTC02TT	.....	0,19	0,02	0,07	3,5000
ASTG01CT	.....	0,03	1	0,84	0,8400
ASAA05AC	.....	0,48	0,12	0,37	3,0833
ASGA01TT	.....	0,38	0,24	0,28	1,1667
ASTC01TT	.....	1,38	0,57	0,05	0,0877
ASTT03AT	.....	1,29	1,04	1,24	1,1923
		Unlinked			
ASAA04AT	.....	1,86	0,47	0,72	1,5319
ASAC06AA	.....	5,25	3,33	4,21	1,2643
ASAA01CT	.....	1,28	0,23	0,6	2,6087
ASAT07AA	.....	0,45	0,07	0,17	2,4286
ASTC01AA	.....	0,95	0,31	0,88	2,8387
ASTC02AA	.....	1,04	0,22	0,54	2,4545

C - AS-ATN x AJ – AJ specific AFLP markers

Marker Name		**Relative Intensity**			
AJ Marker	**CM distance along chromosome**	**Non passaged**	**Untreated**	**ATN treated**	**Comparative Intensity ATN treated/Untreated**
			Chromosome 1		
AJTG01GT	12	1,31	1	1,05	1,0500
AJAC03TA	16,2	1,19	0,91	0,62	0,6813
AJAG02TC	16,2	0,5	0,3	0,3	1,0000
AJTA03AT	16,2	0,5	0,38	0,36	0,9474
AJAG01GT	25,4	1,8	2,2	2	0,9091
AJTG01AA	43,4	0,64	1,08	0,79	0,7315
			Chromosome 5		
AJAA04AC	70	0,5	0,8	0,5	0,6250
AJAT07AT	70	0,4	0,39	0,33	0,8462
AJAT01AT	73,9	1,16	2,59	1,57	0,6062
AJTA01AC	77,5	1,97	0,8	0,7	0,8750
AJAG01TT	82,7	0,4	0,2	0,2	1,0000
AJAA03TA	82,9	0,6	0,5	0,4	0,8000
			Chromosome 6		
AJAG02AG	11,3	0,1	0,2	0,2	1,0000
AJAG02CT	11,3	0,6	0,4	0,3	0,7500
AJAC01GT	11,3	1,06	1,63	2,05	1,2577
AJAT02TA	11,3	0,93	0,66	0,58	0,8788

AJTC02CT	11,3	1,08	0,59	0,56	0,9492
AJTC01TC	11,3	0,45	0,34	0,35	1,0294
AJTG02AA	11,3	0,84	0,65	0,63	0,9692
AJTT05AT	11,3	7,03	1,09	1,28	1,1743
AJAG01CT	16,1	0,5	0,3	0,4	1,3333
AJAT03TG	29,3	0,82	0,49	0,44	0,8980
AJTA02GA	37,1	2,07	1,75	1,09	0,6229
AJTT01CA	44,3	1,44	1,84	2,6	1,4130
AJTA02AC	62,7	0,34	0,14	0,1	0,7143
			Chromosome 7		
AJAA02TG	24,1	2	1	1	1,0000
AJAG01AG	37,2	0,5	0,4	0,7	1,7500
AJAA01GA	37,3	2,9	1,5	1,8	1,2000
AJAT01GT	37,3	0,61	0,45	0,45	1,0000
AJTT06AT	48,5	0,49	2,1	1,75	0,8333
AJTA01CA	62,3	0,73	0,69	0,39	0,5652
AJGA03AC	77,5	0,87	0,92	0,84	0,9130
AJAT02TC	77,5	1,05	0,61	0,69	1,1311
AJTG02AT	77,5	2,54	1,19	1,28	1,0756
AJTG01TC	77,5	1,28	0,93	0,74	0,7957
AJAT02AC	77,7	1,82	1,19	0,98	0,8235
AJTT03TG	81,4	0,1	32	214	6,6875
AJAA05CA	96,3	0,68	0,52	0,56	1,0769
AJAG02CA	100,3	0,8	0,5	0,8	1,6000
AJTG01AG	104,4	0,05	0,3	0,26	0,8667
AJTG01GA	104,4	0,68	0,78	1	1,2821
AJAC02TT	123,8	3,39	2,09	2,02	0,9665
AJTC01AG	135,1	0,06	0,03	0,02	0,6667
AJAA03AT	135,3	0,3	0,4	0,6	1,5000
			Chromosome 8		
AJAG03AG	0	0,2	0,14	0,1	0,7143
AJTT02AT	17,3	0,8	0,5	0,3	0,6000
AJAT01TA	21,3	2,99	2,37	1,62	0,6835
AJTT07AT	25,9	0,86	0,83	0,73	0,8795
AJAC02AG	43	1,2	1,5	0,8	0,5333
AJAC02AT	46,2	0,37	0,45	0,25	0,5556
AJTG03AA	55	0,69	1,25	0,73	0,5840
AJTC01TG	58,5	2,13	2,33	1,06	0,4549
AJAC03AT	73,2	1,96	1,54	0,91	0,5909
			Chromosome 9		
AJAT05TG	40,2	2,26	2,06	1,76	0,8544
AJAT01TC	40,2	1,86	0,94	1,05	1,1170
AJTA01GT	40,2	5,96	2,93	4,98	1,6997
AJAC01AA	51,7	0,83	0,7	0,7	1,0000
AJAC01CT	51,7	1,45	0,88	0,94	1,0682
AJAG01TC	63,5	0,2	0,4	0,4	1,0000
AJAT04TA	67,2	0,57	0,58	0,55	0,9483
			Chromosome 10		
AJGA01TT	7,2	0,65	0,48	0,32	0,6667
AJAG03AC	10,9	0,8	0,9	0,5	0,5556
AJAC02AC	10,9	3,7	2,8	1	0,3571
AJAC02AA	18,4	0,75	0,6	0,4	0,6667

AJGA03AG	18,4	0,67	0,75	0,5	0,6667
AJGA01CA	18,4	0,75	0,79	0,42	0,5316
AJGA02CA	18,4	1,46	1,34	0,92	0,6866
			Chromosome 11		
AJAT03CT	5,3	0,64	0,57	0,6	1,0526
AJAC01CA	9,6	1,26	0,89	1,16	1,3034
AJTT03AT	9,7	0,7	0,6	0,9	1,5000
AJTT03CA	9,7	14,2	0,74	1,58	2,1351
AJTT02GA	9,7	0,26	0,11	0,05	0,4545
AJTT03AA	13,6	0,99	1	1,05	1,0500
AJAT03AA	17,6	1,67	1,58	1,41	0,8924
AJAT01AA	21,6	0,71	0,87	0,95	1,0920
AJGA02TT	31,2	0,53	0,22	0,24	1,0909
AJTA01TT	39,3	0,74	0,62	0,75	1,2097
AJTG05AT	39,3	0,5	0,52	0,44	0,8462
AJAT06CT	44,6	3,08	2,13	1,49	0,6995
AJAG01TA	48,7	0,5	0,5	0,5	1,0000
AJAC01TA	53	1,71	1,21	0,78	0,6446
			Chromosome 12		
AJTT04AT	13,3	1,05	0,47	0,61	1,2979
AJAC01GA	20,8	0,42	0,32	0,4	1,2500
AJTT01CT	24,6	0,04	30,7	0,96	0,0313
AJAA02TC	35,9	2	0,7	0,5	0,7143
AJTC01CT	50,9	1,29	0,65	0,73	1,1231
AJAT04CT	79,9	0,53	0,76	0,92	1,2105
AJAG01TG	106,8	0,3	0,3	0,2	0,6667
AJTC01TA	111,4	0,56	0,35	0,23	0,6571
AJTT02AA	123,5	0,79	0,48	0,5	1,0417
AJAA03TC	123,6	0,5	0,3	0,4	1,3333
			Chromosome 13		
AJTT01AG	15,2	1,02	0,89	1,21	1,3596
AJAA03TG	28,5	1	1,3	1,4	1,0769
AJAC04.0AT	28,5	0,51	0,68	0,87	1,2794
AJTA01GA	28,5	1,51	1,97	2,19	1,1117
AJTT04AA	32,4	0,31	0,32	0,49	1,5313
AJTT02AC	36,7	0,71	1,35	1,29	0,9556
AJAA01AT	44,5	0,8	0,7	0,6	0,8571
AJAT02TG	64	1,01	0,68	0,67	0,9853
AJTA01AG	72,9	15,39	13,84	5,67	0,4097
AJGA02AA	76,9	1,02	0,95	0,94	0,9895
AJAA03AC	136,1	0,7	0,8	0,5	0,6250
AJAA01TG	151,2	0,3	0,2	0,2	1,0000
AJAG04AG	177,9	0,9	0,4	0,3	0,7500
AJTG02AG	183,4	0,57	0,77	0,76	0,9870
AJTT02TG	183,4	1,4	1,51	1,29	0,8543
AJTT02TT	183,4	0,95	0,04	0,04	1,0000
AJTT02CA	183,4	1,28	0,7	0,5	0,7143
AJGA02AG	183,5	1,88	2	1,38	0,6900
AJGA02CT	183,5	0,3	0,26	0,34	1,3077
AJTA01TC	187,9	0,3	0,32	0,35	1,0938
AJAA03CA	200	1,09	1,29	0,78	0,6047
AJAC03TT	200	5,47	3,89	3,18	0,8175

AJAT02AT	200	1,49	1,15	0,78	0,6783
AJAA01AG	208,2	0,5	0,4	0,4	1,0000
AJTG01TG	217,8	0,04	0,02	0,07	3,5000
			Group 31		
AJTT02CT	0	58,5	1,35	2,07	1,5333
			Group 12		
AJAG02AC	28,3	0,6	0,5	0,3	0,6000
AJAG02TA	0	0,2	0,2	0,2	1,0000
AJGA01AG	4	0,35	0,44	0,33	0,7500
AJAC04TA	4	0,89	1,17	0,94	0,8034
AJTG03AT	12,8	0,73	0,39	0,44	1,1282
AJAC04AA	20,3	0,5	0,6	0,7	1,1667
AJGA01AC	20,3	1,78	1,48	1,2	0,8108
AJAT04AT	20,3	2,1	1,09	1,07	0,9817
AJTT03CT	28,3	57,19	0,28	0,62	2,2143
AJGA02AC	28,3	1,64	1,6	1,11	0,6938
			Group 14		
AJTG02CT	4	1,5	0,81	1	1,2346
AJTT01TT	8,2	0,31	0,04	0,03	0,7500
			Group 16		
AJAT03AC	0	1,03	0,71	0,82	1,1549
AJTC01GT	4,4	0,9	0,62	0,94	1,5161
AJTG04AT	24,5	0,94	0,89	0,94	1,0562
AJTT01AA	48,4	O,73	0,45	0,45	1,0000
			Group 2		
AJAA02TA	0	0,6	0,5	0,3	0,6000
AJAG02TT	19,8	0,8	0,9	0,4	0,4444
AJAC05AT	24,4	2,44	1,72	0,76	0,4419
AJAA01TA	28,1	0,4	0,4	0,3	0,7500
AJGA02TA	33,1	0,67	0,57	0,48	0,8421
AJAG02AT	36,8	0,7	0,5	0,4	0,8000
AJTT01TC	44,6	2,25	2	1,58	0,7900
AJGA01GA	71,9	1,37	1,14	0,42	0,3684
AJTT01GT	71,9	4,82	1,47	0,85	0,5782
AJTA03AC	79,6	0,55	0,65	0,6	0,9231
AJAC01TT	88,4	3,9	2,95	1,64	0,5559
AJGA01AT	88,4	0,42	0,63	0,61	0,9683
AJAT05AT	120,6	1,13	0,86	0,64	0,7442
			Group 20		
AJAT03TA	4,6	5,01	3,73	2,58	0,6917
AJAA01GT	8,7	0,5	0,5	0,4	0,8000
AJAT02AA	8,7	0,36	0,3	0,34	1,1333
AJAT01AC	8,7	1,26	0,97	1,38	1,4227
AJTC02TA	8,7	2,36	1,74	0,85	0,4885
AJAA05AC	16,8	1,5	1	0,9	0,9000
AJAT02GT	16,8	1,13	0,68	0,43	0,6324
AJAT04TG	25,5	1	0,79	0,54	0,6835
AJTT05AA	43,7	0,15	0,13	0,13	1,0000
AJAT01TG	43,7	0,95	0,55	0,36	0,6545
AJAT04AA	47,5	0,31	0,36	0,29	0,8056
AJAT02CT	71,9	1,12	0,61	0,27	0,4426

AJAT01CT	84,2	1,98	0,96	0,84	0,8750
AJTG01CT	84,2	0,98	1,09	0,87	0,7982
AJTT06AA	84,2	0,16	0,16	0,1	0,6250
AJAG03AT	92,7	1,5	0,7	0,7	1,0000
AJGA02AT	92,7	0,85	0,64	0,58	0,9063
Group 21					
AJAA01AC	0	1,2	1,6	0,8	0,5000
Group 29					
AJAT01CA	0	0,33	0,35	0,23	0,6571
Group 3					
AJTT07AA	40,7	1,63	0,88	0,71	0,8068
AJAC01AG	44,2	1,44	1,6	1,1	0,6875
AJTT01AC	49,5	0,49	0,36	0,4	1,1111
AJTA02AT	51,4	0,29	0,34	0,29	0,8529
AJAC01AT	63,8	0,88	0,84	0,79	0,9405
AJAA02TT	75,5	1,9	0,9	0,6	0,6667
AJTA01AT	86,4	0,58	0,36	0,45	1,2500
Group 33					
AJTT01AT	8,1	0,52	1,65	1,21	0,7333
AJGA01AA	22,5	1,28	0,86	0,62	0,7209
Group 38					
AJTA02AG	7,5	1,24	0,65	0,54	0,8308
AJAC01TG	11	0,65	0,75	0,66	0,8800
AJGA01TA	15,8	0,64	0,63	0,64	1,0159
Group 6					
AJGA01TG	0	1,03	0,71	0,57	0,8028
AJGA01CT	0	0,64	0,33	0,35	1,0606
AJTT01TG	0	2,08	2,48	0,46	0,1855
AJAA02AT	8,5	1	0,9	0,9	1,0000
AJAG01AT	19,4	0,4	0,5	0,8	1,6000
No data					
AJAG02TG	.....	0,7	0,6	0,4	0,6667
AJAA01TC	.....	1	0,9	1	1,1111
AJAC04.5AT	.....	0,45	0,27	0,21	0,7778
AJGA04AT	.....	0,56	0,76	0,5	0,6579
AJGA05AT	.....	0,37	0,35	0,32	0,9143
AJAC02TA	.....	0,92	0,67	0,83	1,2388
AJGA03AA	.....	0,86	0,7	0,61	0,8714
AJTA01TG	.....	3,91	2,62	2,38	0,9084
AJTA03GA	.....	0,72	1,69	1,18	0,6982
AJTC02AG	.....	0,43	0,33	0,44	1,3333
AJTC02TG	.....	0,12	0,51	0,43	0,8431
AJTG04AG	.....	0,88	0,77	0,76	0,9870
AJTG05AG	.....	1,16	2,48	0,49	0,1976
AJTG02TG	.....	0,04	0,15	0,1	0,6667
AJTG03TG	.....	2,41	1,11	0,83	0,7477
AJAG03CA	.....	0,3	0,3	0,3	1,0000
AJAG05CT	.....	0,6	0,3	0,4	1,3333
AJAG01AC	.....	0,09	0,16	0,11	0,6875
AJAT01GA	.....	1,69	1,22	1,04	0,8525
Unlinked					
AJAG01AA	.....	0,5	0,8	1	1,2500

AJAA02AC	.....	1	1	0,6	0,6000
AJAC03AG	.....	3,74	2,89	2,71	0,9377
AJAA01CA	.....	1,39	0,89	1,01	1,1348
AJAC02CT	.....	3,33	2,04	2,29	1,1225
AJGA03AT	.....	1,41	1,11	0,54	0,4865
AJAT06AT	.....	0,66	0,47	0,51	1,0851
AJAT02CA	.....	0,8	0,62	0,34	0,5484
AJTA01CT	.....	0,73	0,69	0,77	1,1159
AJTC01AT	.....	0,86	0,8	0,38	0,4750
AJTT03GA	.....	10,92	2,82	1,23	0,4362

D - AS-ATN x AJ – AS specific AFLP markers

Marker Name		Relative Intensity			
AS Markers	CM distance along chromosome	Non passaged	Untreated	ATN treated	Comparative Intensity ATN treated/Untreated
		Chromosome 1			
ASAA01TC	16,2	0,6	0,2	0,3	2,1
ASAA02CA	16,2	1,4	0,3	0,4	1,3
ASTA01TT	16,2	1,1	0,3	0,6	2,3
ASTA02AT	16,2	0,3	0,1	0,1	1,2
ASTA02GT	16,2	1,5	0,9	0,7	0,8
ASTG01AA	43,4	0,7	0,3	0,6	1,8
		Chromosome 5			
ASAA03TG	26,7	0,8	0,4	0,6	1,6
ASAA02GA	54,4	0,9	0,2	0,4	1,8
ASAC03AT	69,9	1,2	0,3	0,6	2,4
ASAA04AC	69,9	0,3	0,1	0,2	2,3
ASAC01AG	74,6	0,7	0,1	0,3	3,1
ASTA01AC	74,6	1,3	0,4	0,2	0,5
		Chromosome 6			
ASAC02AT	11,3	0,8	0,2	0,4	1,5
ASAT01AA	11,3	0,3	0,2	0,4	1,6
ASTC01TC	11,3	0,2	0,2	0,2	1,1
ASTG01AT	11,3	1,9	0,7	1,5	2,1
ASTT01CA	44,2	0,0	3,5	1,5	0,4
ASAG01TG	62,6	1,6	0,7	0,6	0,8
		Chromosome 7			
ASAA02GT	31,6	1,1	0,5	0,5	1,1
ASAC01GT	31,6	0,7	0,4	0,8	1,9
ASAA01CA	48,4	1,2	0,3	0,4	1,3
ASAA05CA	62,2	1,0	0,4	0,7	1,7
ASTT03CA	62,2	1,1	1,0	1,0	1,1
ASAC05AA	69,7	0,9	0,5	0,6	1,2
ASAT02AT	69,7	0,6	0,1	0,2	1,3
ASTG01TC	77,5	0,2	0,3	0,3	1,2
ASAG02CA	100,3	1,7	0,3	0,3	1,0

ASTT01AG	123,6	0,1	0,1	0,5	3,7
ASAC01TA	127,6	1,2	0,4	0,3	0,8
		Chromosome 8			
ASAC01TG	30,8	1,2	0,3	0,9	3,3
ASTA01AT	34,3	0,4	0,3	0,4	1,3
ASAG01CA	38,2	1,4	0,1	0,5	6,6
ASGA02AC	43,0	1,4	0,3	0,9	3,3
ASTG02AA	50,8	0,1	0,6	0,7	1,1
ASTC01CT	58,5	44,6	13,8	20,5	1,5
ASAC01AT	73,2	0,4	0,1	0,3	5,4
ASAT03AC	73,2	1,5	0,3	1,1	4,1
		Chromosome 9			
ASTA01GT	40,2	4,0	1,9	3,7	2,0
ASAA04CA	40,2	1,0	0,5	0,8	1,7
ASAC03CT	51,7	0,9	0,4	0,3	0,7
ASTT05CA	51,7	0,9	0,7	1,6	2,3
ASAT04AA	59,2	0,4	0,2	0,2	1,3
ASTT02TT	71,1	388,8	0,1	0,5	8,8
		Chromosome 10			
ASGA01TC	7,2	0,5	0,1	0,3	3,1
ASTG01CA	7,2	20,8	1,0	1,1	1,1
ASTT01TT	10,9	0,0	3,1	0,5	0,2
ASGA01CA	18,4	1,0	0,1	0,4	3,3
ASAT05AA	18,4	0,3	0,2	0,2	1,5
ASTA02AG	18,4	0,4	0,1	0,3	4,1
		Chromosome 11			
ASAG03CA	5,3	2,4	0,3	0,1	0,3
ASAT03AA	5,3	1,0	0,5	0,4	0,8
ASTC02AC	5,3	0,3	0,2	0,1	0,7
ASTT02AA	5,3	0,1	0,1	0,1	1,4
ASTA03GA	21,5	1,2	0,4	0,3	0,8
ASTC01CA	21,5	1,2	0,6	0,5	0,9
ASTA01TA	39,2	0,3	0,1	0,3	2,3
ASTC01AC	39,2	0,1	0,0	0,1	3,5
ASTG01TA	39,2	0,6	0,2	0,4	1,8
ASAG01TA	48,7	0,6	0,3	0,4	1,3
ASAA01TG	52,9	1,3	0,5	0,3	0,6
ASAC02TA	52,9	1,7	0,1	0,6	4,6
		Chromosome 12			
ASAC02CT	20,8	0,8	0,2	0,3	1,6
ASAA01AT	54,6	0,8	0,6	0,5	0,9
ASAC03AA	54,6	1,0	0,4	0,6	1,8
ASGA02AG	66,4	1,2	0,6	0,8	1,3
ASTC01TA	111,4	0,6	0,1	0,4	2,6

		Chromosome 13			
ASAA05AT	0,0	1,7	0,6	0,6	1,1
ASTG02TT	3,9	0,8	0,1	0,1	1,4
ASTA01AA	15,2	1,1	0,4	0,3	0,7
ASTT02AG	15,2	0,1	1,6	1,5	0,9
ASTT01GA	28,5	1,2	2,1	1,5	0,7
ASAA01TA	36,1	1,6	0,1	0,3	2,9
ASAA04TA	36,1	0,7	0,1	0,2	2,3
ASTA02CA	36,1	0,4	0,2	0,4	1,7
ASTA02CA	36,1	1,2	0,2	0,2	0,9
ASAA03AT	44,6	0,3	0,2	0,2	1,3
ASTA01AG	73,0	11,2	4,9	4,1	0,8
ASTT02TC	132,5	0,0	0,0	0,0	2,0
ASGA02TA	177,9	0,1	0,0	0,1	3,0
ASAT01AC	177,9	0,9	0,3	0,4	1,5
ASGA03CA	177,9	0,6	0,1	0,2	2,1
ASTA03TA	183,5	1,7	0,4	1,0	2,7
ASTG03AA	183,5	0,4	0,1	0,3	2,2
ASTT02CA	183,5	0,0	0,9	0,9	1,0
ASTA01CT	200,1	0,4	0,1	0,1	1,1
ASTA01CT	200,1	0,8	0,3	0,5	1,9
ASTT04AT	200,1	1,0	2,1	1,6	0,8
ASAT01AT	209,2	1,3	0,3	0,6	1,9
ASGA02AT	221,9	0,9	0,4	0,8	1,8
		Group 12			
ASAC01GA	4,0	1,2	0,4	0,5	1,1
ASGA01AG	4,0	0,8	0,1	0,6	3,9
ASAT09AA	4,0	0,4	1,0	0,3	0,2
ASAT08AA	12,8	0,6	0,2	0,4	1,8
ASAA01GT	20,3	0,7	0,3	0,4	1,5
ASGA03AC	20,3	0,5	0,4	0,2	0,6
ASGA01AC	24,4	1,4	0,4	0,7	1,8
ASTA02TA	28,3	3,2	1,3	1,1	0,8
		Group 14			
ASTA03AA	0,0	2,7	0,3	0,9	2,8
ASTA02AA	4,0	0,6	0,2	0,6	2,5
		Group 16			
ASAT04AC	0,0	1,0	0,5	0,4	0,8
ASTG01TT	0,0	0,5	0,2	0,2	1,1
ASTT01CT	4,4	0,0	0,9	0,7	0,8
ASAG01CT	12,4	1,6	0,5	0,7	1,4
ASAA03TA	32,2	0,6	0,2	0,3	1,2
ASTT01AC	40,3	8,4	0,9	0,2	0,2
ASTT03AA	59,2	0,1	0,1	0,1	1,6

ASTT01AA	74,5	0,2	2,3	1,6	0,7
		Group 2			
ASAA02AT	0,0	0,8	0,3	0,6	2,6
ASAG01TT	7,8	1,5	0,4	1,0	2,5
ASAC02AG	24,4	0,9	0,2	0,5	3,5
ASTC01GA	24,4	3,6	1,3	2,8	2,2
ASTT01AT	71,9	0,4	0,3	0,4	1,3
ASTT04CA	71,9	46,8	2,0	1,9	1,0
		Group 20			
ASGA02CA	4,6	0,6	0,2	0,2	1,1
ASAA04TC	8,7	0,8	0,2	0,4	1,8
ASAA01GA	8,7	1,3	0,6	1,0	1,8
ASTC02TC	8,7	0,0	0,0	0,0	1,5
ASAA06AC	16,8	0,2	0,6	0,6	1,0
ASAA03TC	43,7	1,1	0,3	0,5	1,7
ASAA02TG	55,0	1,0	0,7	1,0	1,4
ASAG01GA	64,2	2,3	1,0	1,8	1,9
ASAC01CT	84,2	0,4	0,3	0,5	1,4
ASTG02CT	84,2	1,1	1,2	0,9	0,8
		Group 21			
ASAA01AC	0,0	0,1	0,1	0,1	1,1
		Group 3			
ASGA01AT	40,0	0,8	0,1	0,3	2,1
ASAA02TA	44,2	1,1	0,5	0,4	0,9
ASAA03CT	44,2	2,6	0,6	1,4	2,3
ASAT11AA	44,2	0,9	0,2	0,5	2,4
ASAT02AA	63,8	0,2	0,1	0,2	3,0
ASTA01CA	75,5	1,2	0,1	0,4	2,8
ASTA01CA	75,5	0,8	0,3	0,4	1,4
		Group 30			
ASTT01TC	11,9	2,0	42,0	0,8	0,0
		Group 33			
ASTT02AT	0,0	0,1	0,0	0,0	0,5
ASAT02AC	8,1	1,7	0,3	0,4	1,5
		Group 6			
ASGA01TA	0,0	1,9	0,6	1,2	2,0
ASGA01GA	0,0	1,7	1,1	0,4	0,4
ASTG01GA	0,0	0,1	2,5	1,1	0,4
ASAT06AA	23,4	0,3	0,2	0,5	3,2
		No data			
ASTA04GA	.....	4,3	1,2	2,8	2,4
ASTA04CA	.....	0,3	0,2	0,2	1,0
ASTA02TG	.....	0,6	0,4	0,4	0,8
ASTA02GA	.....	0,7	0,4	0,5	1,3

ASTA03TG	.....	0,7	0,3	0,5	1,9
ASTA04TC	.....	2,7	3,2	3,0	0,9
ASTA02TC	.....	1,0	0,3	0,3	1,3
ASTA03TC	.....	1,8	0,4	0,5	1,4
ASGA01AA	.....	1,3	0,3	0,5	2,1
ASGA02AA	.....	1,3	0,4	0,8	2,0
ASGA03AA	.....	0,0	0,0	0,1	4,0
ASGA04AA	.....	1,6	0,7	1,0	1,4
ASGA05AA	.....	2,7	0,5	0,8	1,5
ASGA06AA	.....	1,4	0,5	0,7	1,6
ASGA07AA	.....	1,8	0,3	0,8	2,9
ASGA08AA	.....	0,8	0,4	0,6	1,4
ASTA01GA	.....	1,4	0,3	0,3	0,9
ASTA04TG	.....	1,7	0,2	0,2	1,2
ASTA02TT	.....	0,8	0,5	0,4	0,7
ASTA01TC	.....	1,8	1,0	1,2	1,2
ASTC02GT	.....	1,4	1,9	2,0	1,0
ASTA03CA	.....	0,4	0,1	0,1	1,4
ASTA01TC	.....	1,3	0,3	0,5	1,6
ASTC03AA	.....	0,5	0,3	0,2	0,7
ASTC03TC	.....	1,4	0,7	1,3	1,9
ASTG04AA	.....	0,4	0,2	0,2	1,0
ASTG02TC	.....	0,6	0,1	0,2	1,2
ASTG02GA	.....	0,1	1,3	0,7	0,6
		Unlinked			
ASAA04AT	.....	1,9	0,6	0,5	0,9
ASAC06AA	.....	5,3	3,4	3,2	0,9
ASAA01CT	.....	1,3	0,2	0,7	3,4
ASAT07AA	.....	0,5	0,1	0,3	3,0
ASTC01AA	.....	1,0	0,5	0,5	1,1
ASTC02AA	.....	1,0	0,7	1,2	1,9

GLOSSARY

Artemisinin combination therapy (ACT) -

As a response to increasing levels of resistance to antimalarial medicines, WHO recommends that all countries experiencing resistance to conventional monotherapies, such as chloroquine, amodiaquine or sulfadoxine–pyrimethamine, should use combination therapies, preferably those containing artemisinin derivatives (ACTs) for *falciparum* malaria.
WHO currently recommends the following combination therapies (in alphabetical order):

1. Artemether/lumefantrine
2. Artesunate plus amodiaquine (In areas where the cure rate of amodiaquine monotherapy is greater than 80%)
3. Artesunate plus mefloquine (Insufficient safety data to recommend its use in Africa)
4. Artesunate plus sulfadoxine / pyrimethamine (In areas where the cure rate of sulfadoxine / pyrimethamine is greater than 80%)

Note: Amodiaquine plus sulfadoxine / pyrimethamine may be considered as an interim option where ACTs cannot be made available, provided that efficacy of both is high.

Clone -

A collection of parasites in which all individuals have been derived from a single cell by asexual reproduction.
Therefore, a particular clone should represent a population of genetically identical parasites, assuming that no spontaneous mutations had taken place during parasite growth.

Isolate -

A population of parasites that have been collected from mosquitoes, humans or rodents living in the wild on a single occasion and subsequently kept as deep-frozen material.
An isolate does not necessarily represent a homogeneous parasite population and may contain more than one representative of the same species or different species.

Line -

A population of parasites that have undergone one or more *in vitro* or *in vivo* laboratory passages.
Usually, a line is produced when the parasites being passaged in the laboratory have been subjected to some kind of induced pressure, such as drug treatment. As for the isolate, parasites constituting a line may not be genetically identical.

Linkage Group Selection (LGS) -

Was devised for application to malaria parasites in order to locate genes that control selectable phenotypes such as drug sensitivity, growth rate and strain-specific immunity without the disadvantages of classical linkage analysis.
LGS uses a genetic cross between two unrelated (genetically distinct) parasites of the same species, one of which is sensitive and the other resistant to the relevant selection pressure (such us drug treatment). Following cross-fertilisation between gametes of each parasite and zygote formation in the mosquito, there is recombination between the parental genomes during meiosis, producing haploid recombinant progeny.

Minimum Curative Dose (MCD) -

The MCD of each drug was first assessed in drug-selected parasites and untreated control lines. MCD was defined as the minimum dose of each drug that would prevent re-appearance of parasites in all five mice within each treated group at any time during the first 10 days of the follow-up period.

N-fold resistance -

A resistance index was determined using the following equation:
N-fold resistance = MCD drug selected parasites/ MCD drug unselected parasites

Polymerase chain reaction (PCR) -

Is a biochemistry and molecular biology technique for exponentially amplifying DNA, via enzymatic replication, without using a living organism (such as *E. coli* or yeast). As PCR is an *in vitro* technique, it can be performed without restrictions on the form of DNA, and it can be extensively modified to perform a wide array of genetic manipulations.
PCR is commonly used in medical and biological research labs for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic

fingerprints, and the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing.

Proportional Sequencing - Technique for analysing parasite mixtures. The method is rapid, and in principle can be applied to any single nucleotide polymorphism (SNP) at any locus, with only minimal requirements for optimisation and assay development. Proportional sequencing exploits the fact that during a PCR reaction, DNA is amplified in proportion to the initial template.

Quantitative Polymerase Chain Reaction (RTQ-PCR) - Is a modification of the polymerase chain reaction used to rapidly measure the quantity of DNA, complementary DNA or ribonucleic acid present in a sample. Like other forms of polymerase chain reaction, the process is used to amplify DNA samples, via the temperature-mediated enzyme DNA polymerase.
PCR theoretically amplifies DNA exponentially, doubling the number of molecules present with each amplification cycle. The number of amplification cycles and the amount of PCR end-product should allow one to calculate the initial quantity of genetic material, but numerous factors complicate this calculation. The ethidium bromide staining typically used to assess a successful PCR prevents further amplification, and is only semi-quantitative. The polymerase chain reaction may not be exponential for the first several cycles, and furthermore, plateaus eventually, so care must be taken to measure the final amount of DNA while the reaction is still in the exponential growth phase. To overcome these difficulties, several different quantitative methods have been developed.
The most sensitive quantification methods are done by the real-time polymerase chain reaction, where the amount of DNA is measured after each cycle of PCR by use of fluorescent markers. Other end-point methods measure DNA after PCR is completed. These methods depend on addition of a competitor RNA (for reverse-transcriptase PCR) or DNA in serial dilutions or co-amplification of an internal control to ensure that the amplification is stopped while in the exponential growth phase.
Although real-time quantitative polymerase chain reaction is often marketed as RT-PCR, it should not to be confused with reverse transcription polymerase chain reaction, which is also referred to as RT-PCR, but is used to amplify RNA samples. The two methods may be used in concert to reverse transcribe RNA and then quantitate the resulting cDNA using real-time PCR (often referred to as real-time RT-PCR).

RI - Delayed Recrudescence - The WHO 1973 definition of the level of parasite drug resistance remains in use. According to the WHO the asexual parasitaemia reduces to < 25% of pre-treatment level in 48 hours, but reappears between 2-4 weeks.

RI - Early Recrudescence - The WHO 1973 definition of the level of parasite drug resistance remains in use. According to the WHO the asexual parasitaemia reduces to < 25% of pre-treatment level in 48 hours, but reappears within 2 weeks.

RII – Resistance - The WHO 1973 definition of the level of parasite drug resistance remains in use. According to the WHO there is a marked reduction in asexual parasitaemia (decrease >25% but <75%) in 48 hours, without complete clearance in 7 days.

RIII – Resistance - The WHO 1973 definition of the level of parasite drug resistance remains in use. According to the WHO there is a minimal reduction in asexual parasitaemia, (decrease <25%) or an increase in parasitaemia after 48 hours

Sensitive Parasite (S) - The WHO 1973 definition of the level of parasite drug resistance remains in use. According to the WHO the asexual parasite count reduces to 25% of the pre-treatment level in 48 hours after starting the treatment, and complete clearance after 7 days, without subsequent recrudescence - Complete Recovery.

Strain - All parasites of a single subspecies present in a single isolate.
For example, *Plasmodium chabaudi chabaudi* strain AS-sens is composed of all the parasites belonging to the *P. c. chabaudi* sub-species in blood isolated from the thicket rat *Thamnomys rutilans*.

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PUBLICATIONS THAT AROSE FROM THIS PROJECT

Hunt P, **Afonso A**, Creasey A, Culleton R, Sidhu A, Logan J, Valderramos S, McNae I, Cheesman S, do Rosario V, Carter R, Fidock D and Cravo P. Gene encoding a de-ubiquitinating enzyme is mutated in artemisinin- and chloroquine-resistant rodent malaria parasites. In press at the *Molecular Microbiology*

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