SUMMARY

INTRODUCTION

Malaria is still responsible for almost half a million deaths every year. Currently, campaigns for the control or elimination of malaria, depending on the intensity of transmission, are settled in malaria endemic areas. However, drug resistance is one of the major impediments to achieve malaria elimination. We and others have described how parasites develop resistance to some toxic compounds by functional variation linked to epigenetic changes, contrasting with the previously described mechanisms based on genetic changes. This newly described mechanism involves *clag3* genes (*clag3.1* and *clag3.2*), which are necessary for the formation of a transmembrane channel placed in the host erythrocyte membrane.

clag3 genes are part of the *clag* multigene family, formed by 5 members. *clag3.1* and *clag3.2* share 95% sequence identity and are neighbour genes localized at chromosome 3, in a region between 110 and 140 kb from the chromosome end in the left subtelomeric region, separated by only 10kb with a *var*-pseudogene located between them (Fig. 11B) (Bowman et al, 1999; Otto et al, 2010). The most variable domain in *clag3*, located at the C terminus of the protein and containing around 120 nucleotides (Alexandre et al, 2011; Nguitragool et al, 2011), will be referred to as the **hypervariable region (HVR)** (Fig. 10). Besides, chromosome-level recombination can derive to reduction to a single *clag3* gene (Chung et al, 2007; Iriko et al, 2008), such that some parasite isolates carry a single *clag3* gene instead of two paralogues (Fig. 12C). This recombinant gene carries the promoter of *clag3.2* and the 3' UTR of *clag3.1*.

clag3 genes expression is regulated by epigenetic mechanisms, which involve changes at the chromatin level, not affecting the primary DNA structure, and determines the active or repressed state of the genes. *clag3* genes show **clonally variant expression,** which implies that individual parasites of the same genetic background and at the same stage of the life cycle use these genes differently. Due to this property, parasites present different combinations of repressed or active genes among a population of identical parasites, presenting a variety of phenotypes. Those parasites presenting the best fitness in a given situation will be naturally selected and the transcriptional state of their genes will be transmitted to the next generations, through a process known as epigenetic memory. This way, parasites adapt to fluctuating conditions in the environment, a strategy so-called bet-hedging. Additionally, *clag3* genes present **mutually exclusive expression** (Cortes et al, 2007). This property denotes that only one of the members of a multigenic family will be expressed at a

time. Thus, in a population of genetically identical parasites we find that most individual parasites express one of the two genes, either *clag3.1* or *clag3.2*, and keep the other epigenetically silenced.

clag3 genes have been proposed to play a role in different biological processes. However, the only one that has been clearly demonstrated is the formation of the *Plasmodium* surface anion channel (**PSAC**). PSAC is a parasite encoded channel placed at the membrane of infected erythrocytes and is responsible for the uptake of nutrients and some compounds that are toxic for the parasite, such as sorbitol, leupeptin (LEUP) and blasticidine (BS) (Hill et al, 2007; Lisk et al, 2008; Wagner et al, 2003)or the antibiotic blasticidine (BS). This channel is poorly characterized: it has been observed that *clag3* genes play a key role in transport activities through PSAC (Nguitragool et al, 2011), but little is known about its structure, its regulation and its significance in natural infections. Interestingly, PSAC mediates permeability to a broad range of solutes, but it also presents solute transport specificity, being able to discern between structurally similar solutes.

Before the start of this thesis and as part of ongoing investigations on the rules that govern the mutually exclusive expression of *clag3* genes in our lab in Barcelona, switches in *clag3* genes expression had been observed in transfected parasites selected with BS. Because of the association between *clag3* genes and PSAC firstly described by then (Nguitragool et al, 2011), we hypothesized that parasites could modify the erythrocyte membrane permeability to BS and become resistant to this drug through modifications in *clag3* genes expression.

RESULTS

ARTICLE 1. EPIGENETIC SWITCHES IN *CLAG3* GENES MEDIATE BLASTICIDIN S RESISTANCE IN MALARIA PARASITES.

In order to investigate the expression dynamics of *clag3* genes in parasites under BS pressure, 10G parasites (a lab-adapted strain that under standard culture conditions predominantly expresses *clag3.2*) were selected under different concentrations of BS. After three weeks, low concentrations of the drug selected for parasites expressing *clag3.1*, whereas parasites exposed to higher concentrations of BS repressed the expression of both *clag3* genes. Parasites kept in culture under standard conditions continued to express *clag3.2*. We concluded that parasites develop resistance to BS through changes in their *clag3* gene expression pattern: those expressing *clag3.1* presented higher values of IC_{50} than parasites expressing *clag3.2*; parasites not expressing any *clag3* presented high levels of resistance to the drug. Therefore, expression of *clag3.1* determines reduced erythrocyte permeability, at least to BS and in the 3D7 genetic background, and silencing of both

clag3 genes could imply a non-functional channel, preventing the entrance of the drug into the cell. Indeed, the 10G-0.6-2 parasite line, which does not express any of the *clag3* genes, presented also resistance to other compounds such as sorbitol. With these experiments, we also demonstrated that the mutually exclusive expression property can be disrupted. However, we observed that when 10G-0.6-2 line is cultured in the absence of BS, parasites recover expression of *clag3* genes, suggesting that simultaneous silencing of the two *clag3* genes poses a fitness cost for the parasite. We did not find any mutation in the genome of these parasites that could explain the change in the phenotype. Thus, we concluded that this mechanism of drug resistance is regulated at the epigenetic level, being the first one of this kind to be described in *Plasmodium* parasites.

ARTICLE 2. EXPRESSION OF THE *PLASMODIUM FALCIPARUM* CLONALLY VARIANT *CLAG3* GENES IN HUMAN INFECTIONS.

After the results obtained with lab-adapted strains, which provided a proof of principle that *P. falciparum* can develop drug resistance by epigenetic mechanisms, we decided to study the expression of the genes that mediate this type of resistance in human infections. To fulfil this aim, we started investigating the dynamics of *clag3* expression in natural human infections, which had been never explored before. We found that parasites collected from peripheral blood in patients with uncomplicated malaria predominantly express one of the two paralogues, consistent with the property of mutually exclusive expression previously observed in culture-adapted parasite lines. Interestingly, parasites from all the isolates analyzed expressed the same paralogue: *clag3.2*, contrary to most lab-adapted parasites, which in culture conditions express *clag3.1*. We also studied *clag3* expression in parasites collected from experimentally infected patients and we also observed preferential expression of *clag3.2* in parasites coming from this kind of samples. In contrast, NF54, the parental line used for the creation of the infective sporozoites, expresses *clag3.1* in culture conditions. Samples collected at day 9 post-infection (when parasites had been in the peripheral blood for approximately one erythrocytic cycle) showed a mix of parasites expressing either *clag3.1* or *clag3.2*. Thus, we concluded that the epigenetic memory of *clag3* genes is reset during transmission stages and that parasites expressing *clag3.2* are selected in human blood. The observation of parasites preferentially expressing *clag3.2* in human blood, both in natural and experimental infections, was consistent with our previous hypothesis of *clag3.1* conferring lower erythrocyte membrane permeability. Nutrient concentration in the human blood stream is lower than that in the media used to grow parasites *in vitro*. Thus, parasites expressing the paralogue that confers higher permeability in a more nutrient-restrictive environment, such as the human blood, are expected to be selected.

We also adapted two parasite isolates from natural infections to standard culture conditions and selected them under BS pressure. We detected different patterns of selection depending on the isolate and: when adapted to culture conditions, one isolate kept expressing *clag3.2* when adapted to standard culture conditions, whereas in the other isolate *clag3.1*-expressing parasites were selected. The opposite pattern occurred in each isolate under BS selection. These results lead to the idea that expression of *clag3.1* does not imply less permeability to BS, but that transport efficiency for specific solutes, such as BS, depends on the genetic background of the parasite and is probably determined by the CLAG3 sequence, which.

Next, we performed a phylogenetic analysis of CLAG3 sequences from lab-adapted parasite lines and field isolates to help in the interpretation of our parasite-adaptation results. When the entire CLAG3.1 and CLAG3.2 gene sequences were analysed the resulting tree showed CLAG3.1 and CLAG3.2 sequences in two clearly separate clades. However, analysis of the HVR sequences showed a tree with two separate clades with a mix of branches belonging either to CLAG3.1-HVR and CLAG3.2-HVR. These findings together with the results of parasite adaptation experiments lead to the idea that paralogue-specific conserved regions may determine the selection of parasites expressing *clag3.2* in the human blood circulation, probably implying more general permeability characteristics, whereas HVR motifs could determine transport efficiency for specific solutes, such as for BS.

ARTICLE 3. IDENTIFICATION OF ANTIMALARIAL COMPOUNDS THAT REQUIRE CLAG3 FOR THEIR UPTAKE INTO *P. FALCIPARUM*-INFECTED ERYTHROCYTES.

Our experiments with BS demonstrated that *P. falciparum* parasites can develop resistance through changes in the transcriptional state of their genes at the epigenetic level. However, BS is a drug that is not clinically used to treat malaria infections because of its high toxicity to human cells. However, whether other drugs, clinically relevant for the treatment of malaria, use PSAC to enter the infected cell is not known yet. We tested drugs that could be susceptible of failure through the mechanism described in this thesis. First, we conducted IC_{50} assays, to compare the sensitivity to drugs in a strain that expresses *clag3.2* (10G; BS-sensitive) and a line that with supressed *clag3* expression (10G-06-2; BS-resistant). We selected a subset of drugs among a list of compounds that are suspected to use PSAC to enter the cell (Basore et al, 2015; Biagini et al, 2003; Stead et al, 2001; Wein et al, 2012). We observed differences in IC_{50} values between both strains in three of the drugs tested: LEUP, T3 and T16, indicating that *clag3* expression is required, at least partially, for the uptake of these drugs in order to reach their intracellular target. The rest of the drugs (azithromycin, doxycycline, fosmidomycin, lumefantrine and pentamidine) apparently do not require *clag3* expression to enter the cell.

Then, we selected parasites under drug pressure to study if parasites could develop resistance to these drugs through changes in *clag3* gene expression. For this, we used 10G parasites (*clag3.2*-expressing) and selected them under drug pressure for 3-15 weeks, according to the time needed for adaptation of the parasites. The drugs used in these experiments were selected based on our previous IC_{50} experiments; we selected drugs with significant IC_{50} differences between 10G and 10G-0.6-2 parasites and two drugs that show no significant IC50 differences, *i.e.* doxycycline and fosmidomycin, to additionally test whether selection experiments are a more sensitive tool to detect changes in clag3 expression. We observed that parasites selected with T3 and T16 reduced drastically the total expression of *clag3* genes after only two weeks of selection, demonstrating that parasites can become rapidly resistant to these compounds through changes in *clag3* epigenetic regulation.

In order to find an explanation for the other drugs reaching the cell in the absence of CLAG3, we first demonstrated the absence of these proteins in our 10G-0.6-2 parasite line by IFA experiments. Then, to study the functionality of PSAC in these parasites, we tested the uptake of 5-ALA, a compound that has been shown to require PSAC to enter the cell, by 10G-0.6-2 and its parental line 10G. Unexpectedly, we observed that this compound entered the infected RBCs with both lines at the same level, suggesting that PSAC can be formed independently of CLAG3. We suggest that other members of the CLAG family can form independent channels, which would be responsible of the transport of other compounds, such as FOSMI and PENTA, which have been demonstrated to require PSAC for their uptake.

DISCUSSION

The main particularity of the new drug resistance mechanism described in this thesis is that it is regulated at the epigenetic level. Epigenetic regulation of gene expression refers to heritable changes in transcription that occur in the absence of alterations in the primary sequence of DNA. Switches between the active and repressed state of the genes happen, even if at low frequency, stochastically. Parasites presenting the best expression pattern in a particular environment, such as a pattern that determines lower permeability for toxic compounds, can be naturally selected transmitting their expression pattern to the next generation (epigenetic memory). Thus, even if this type of resistance is heritable as drug resistance mechanisms due to mutations, a drug resistance mechanism regulated at the epigenetic level is **rapid and reversible**.

Drug resistance in malaria parasites is currently threatening malaria elimination efforts. Continued exposure to drug pressure selects for parasites that present mutations that confer resistance to the

drug, transmitting the resistance mechanism to the next generation. However, selection of *clag3* genes expression patterns happens during the course of a single infection (*i.e.* selection of *clag3.2*-expressing parasites in human blood when they exit the liver). As we observed in our experiments, there is a **reset of the epigenetic** memory after going through transmission stages, meaning that there is no transmission of the *clag3* transcription pattern from one host to another. Thus, in case the drug resistance mechanism described in this thesis happened in natural infections, it would only select for resistant parasites in a single infection, in the same individual. This fact emphasizes the significance of this mechanism as a rapid process, which could cause treatment failure in an individual infection.

clag3 genes present clonally variant and mutually exclusive expression. Thanks to these properties, parasites present a variety of phenotypes and, depending on the environment, parasites presenting the best fitness will be selected. Whereas we have observed a functional role for clonally variant expression in lab experiments when selecting parasites with toxic compounds, we have not clearly elucidated the advantage for this feature in natural infections yet. Parasites collected from infected patients were all expressing *clag3.2*, which suggests that CLAG3.2 implies a more favourable phenotype under the conditions of the human-blood circulation, probably conferring increased erythrocyte-membrane permeability to nutrients. We could not determine whether there is any malaria outcome that might select for parasites expressing *clag3.1*, such as presence of drugs, nutritional condition or disease severity. Future studies on *clag3* expression in natural infections should include *P. falciparum* infected patients with different clinical presentations in order to discover the physiological, drug treatment, clinical, etc., conditions that select for parasites expressing *clag3.1* in natural infections. The fact that *P. falciparum* evolved to present two *clag3* paralogues suggests that this represents an advantage for the parasite (of note, other *Plasmodium* species do not present two *clag3* genes). Thus, expression of *clag3.1* must represent an advantage in some situations that we have not identified yet. We hypothesize that the expression of *clag3.1* confers less general permeability to the RBC membrane, which would be advantageous in case of presence of toxic compounds in the human blood circulation.

We currently know that mutually exclusive expression is not strict (Mira-Martinez et al, 2013; Rovira-Graells et al, 2015; Sharma et al, 2013). Of note, parasites that have both *clag3* genes silenced recover the mutual expression pattern after removal of the selective pressure, which suggests that not expressing any of the *clag3* genes has a **fitness cost** for the parasite. It has been demonstrated that expression of *clag3* genes is necessary for the uptake of nutrients into infected RBCs (Nguitragool et al, 2011; Pillai et al, 2012; Sharma et al, 2013). If parasites became resistant to drugs in natural infections by silencing *clag3* genes, parasites would then need an alternative way for acquisition of nutrients. As it happens in parasites presenting drug resistance due to mutations,

there could be a selection of compensatory mutations that reinforce the uptake of nutrients. However, a more plausible possibility is that parasites not expressing *clag3* genes stay in a dormant state, decreasing their metabolic rate and reducing their need of nutrients. This way, parasites would avoid the entrance of toxic compounds. Indeed, delayed clearance of parasites after artemisinin-based treatment has been related to quiescent forms of the parasite in lab experiments. Ferreira and colleagues (Ferreira et al, 2013) suggested that the delay in clearance of artemisinin-resistant parasites could be due to epigenetic changes, since observations of increased gene expression of histones and transcription factors at late stages in artemisinin resistant parasites have been observed (Mok et al, 2011; Takala-Harrison et al, 2013).

The results of our studies with BS provide a proof of principle that *P. falciparum* can develop drug resistance by transcriptional alterations transmitted by epigenetic mechanisms. BS is a drug that is not clinically used to treat malaria infections. We investigated whether drugs that are in use or under development for the treatment of malaria require CLAG3 for their transport. Among the drugs that we tested, three of them (T3, T16 and LEUP) showed evidence of requiring CLAG3 proteins to reach their target, but none of them are currently used in clinical practice. Nonetheless, with these results we confirm that parasites can develop resistance to antimalarial compounds through changes in the epigenetic regulation of their genes. We have revealed the existence of selectable subpopulations of parasites with the two *clag3* genes silenced, at least under culture conditions, and we predict that they may exist, even if at low rate, and can be selected under human infection conditions. Administration of drugs that are susceptible of failure by this mechanisms could select for parasites presenting this phenotype.

Uptake of the rest of the drugs seems to not require CLAG3 proteins, suggesting that they use an alternative route to enter into the cell. However, recent results in our lab suggest the occurrence of PSAC channels not containing CLAG3, since the 10G-0.6-2 line, which does not express CLAG3, is able to import 5-ALA into the cell (a compound that has been previously proved to require PSAC to get into infected RBCs (Beck et al, 2014; Sigala et al, 2015; Staines et al, 2004)). Thus, we suggest that other members of the *clag* family may play a role in the formation of independent PSAC (Fig. 19).