DISCUSSION

1. A NEW MECHANISM OF DRUG RESISTANCE: EPIGENETIC REGULATION

1.1 EPIGENETIC MECHANISMS IN DRUG RESISTANCE DEVELOPMENT IN DIFFERENT MICROORGANISMS.

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 clonally variant genes happen, even if at low frequency, stochastically. Parasites presenting expression patterns that confer more fitness in a particular environment, such as a pattern resulting in lower permeability for toxic compounds, will be naturally selected. The selected parasites will then transmit their combination of repressed and active genes to the next generation of the asexual cycle by the epigenetic memory, contributing to the adaptation of the population to a specific environment.

Even if most of drug resistance mechanisms are related to mutations, non-genetic regulation has been previously suggested to play a role in drug resistance in other microorganisms, mainly in bacteria. Researchers have observed that resistance in bacterial populations is highly reversible, with populations that have developed resistance to an antibiotic, reverting after the drug is removed from the environment and becoming sensitive again after few generations. Most of the examples of non-genetic antibiotic resistance that have been documented describe a small sub-population of “persister-cells” that commonly remain largely unaffected by drug exposure (Bigger, 1944). Nowadays, the general thought is that bacterial populations produce a small fraction of phenotypically dormant offspring when environmental conditions become unfavourable. Persister-cells are not genetically distinct from normal cells and are usually viewed as a form of non-heritable drug tolerance, since they can readily revert to drug sensitivity. Indeed, Troy Day (Day, 2016) suggests that phenotypic antibiotic tolerance and the persister cell phenomenon might represent one of the best-studied empirical examples of evolution via non-genetic mechanisms.

Another example of a non-genetic drug-resistance mechanism in bacteria has been described in E. coli. Moreover, this mechanism has been suggested to be regulated at the epigenetic level (Adam et al, 2008). Exposure of an isogenic E. coli population to low concentrations of ampicillin,
tetracycline and nalidixic acid resulted in heritable variations in gene expression patterns that provided phenotypic diversity. Reversion rates to antibiotic sensitivity were extremely high, around 95% after removal of the drug, consistent with an epigenetic inheritance mode of resistance. Among other observations, the authors concluded that DNA methylation by the Deoxyadenosine methylase (an enzyme that adds a methyl group to the adenine of the sequence 5'-GATC-3' in newly synthesized DNA) correlated with the emergence of adaptive resistance, affecting the activity of efflux pumps.

Very little is known about drug resistance due to epigenetic mechanisms in eukaryotic parasites. To the best of my knowledge, there is only an indication of such a mechanism in Giardia (Ansell et al, 2015). G. duodenalis has developed resistance to the front line treatment that has been used for the last 40 years: nitroheterocyclics. These drugs, which are redox-active compounds, are believed to damage proteins and DNA after being activated by oxido-reductase enzymes. Due to the reversibility of nitroheterocyclic resistance found in the laboratory-derived drug-resistant G. duodenalis lines (Muller et al, 2008; Smith et al, 1988; Tejman-Yarden et al, 2011), Ansell and colleagues propose that resistance mechanisms in G. duodenalis are likely to include redox-sensitive epigenetic regulation, which would allow this parasite to tolerate oxidative stress leading to resistance (Ansell et al, 2015).

1.2 WHAT DOES A DRUG RESISTANCE MECHANISM REGULATED AT THE EPIGENETIC LEVEL IMPLY?

As explained in the introduction of this thesis, organisms can adapt through different mechanisms to changes in their environment: genetic mutations, epigenetic regulation and direct transcriptional response. In P. falciparum, adaptation to the presence of drugs is mainly due to genetic mutations. Indeed, all antimalarial resistance mechanisms in P. falciparum described to date are due to point mutations or rearrangements at the genetic level. In this context, the new mechanism described in this thesis is the first example of a drug resistance mechanism in P. falciparum that does not relate to genetic mutations.

clag3 genes play a key role in the drug resistance mechanism described here. They are regulated at the epigenetic level and present clonally variant expression. Of note, the transcriptional state of clonally variant genes changes stochastically, allowing the parasite population to increase its phenotypic diversity, strategy known as bet-hedging. Even if these switches from active to repressed
state and vice versa happen at low frequency, they happen at a higher rate than mutations. Besides, while for a parasite population to develop drug resistance by mutations a series of events must happen (accumulation of viable mutations, reproduction, selection, transmission), thus taking a long time, a drug-resistance mechanism that is regulated at the epigenetic level allows the parasite population to develop resistance in only a few intraerythrocytic cycles (as we have observed in our drug-selection experiments).

A common characteristic that epigenetic regulation shares with mutations in the DNA sequence is that both phenomena are heritable. Parasites presenting the phenotype that confers the best fitness, either by changes in the transcriptional state of the genes (regulated at the epigenetic level) or by changes in the DNA sequence, will be selected and transmitted to the next generation of the asexual cycle. This way, parasites are naturally selected and adapt to the environment. However, one of the main differences is that adaptations through epigenetic mechanisms are reversible, allowing the parasite population to adapt rapidly to different environmental conditions, while mutations are stable over time. Furthermore, genetic traits persist during transmission stages, which does not seem to be the case for epigenetic traits (see below).

1.3 Drug resistance mechanisms regulated at the epigenetic level in an elimination context.

Drug resistance in malaria parasites is currently threatening malaria elimination campaigns. As previously mentioned, to date, only drug resistance mechanisms due to mutations have been described in natural infections. Spontaneous mutations in the DNA sequence happen during parasite replication, some of them conferring a resistant phenotype to a specific drug. Continued exposure to drug pressure, for example in cases of defective drugs, poor adherence to treatment or mass drug administration (situation in which non-lethal concentrations of the drug are present in the bloodstream), selects for those parasites presenting a drug-resistance phenotype. These parasites will then transmit the resistance mechanism to the next generation. This selection process is creating a condition in which resistant parasites are spreading around the world.

Accumulation of independent mutations responsible for different drug resistance mechanisms in a single individual is generating parasite populations that present resistance to more than one drug at a time, such as to both drugs of an ACT. Nowadays, multidrug-resistant parasites present one of the greatest threats to the control and elimination of malaria. This phenomenon has been observed
in parasites presenting the mutation C580Y at K13 and amplification of the \textit{Pfplasmepsin} 2 and 3 genes, conferring resistance to the artemisinin derivative and to piperaquine, respectively (Imwong et al, 2017). These mutants were first observed in western Cambodia, where dihydroartemisinin-piperaquine was the first-line antimalarial treatment, and has spread to Northeastern Thailand, Southern Laos and Vietnam (Imwong et al, 2017; Thanh & Thuy-Nhien, 2017). Thus, by continued drug exposure, we are selecting for \textit{super-parasites} that will be more difficult to fight as time goes by, unless we find an alternative treatment. The best-known example of this kind of \textit{super-organism} is the multidrug-resistance tuberculosis (MDR-TB), which currently represents a major public health concern. In 2015, there were almost half a million incident cases of MDR-TB worldwide. Nowadays, MDR-TB is deriving to extensively drug-resistant tuberculosis (XDR-TB), which is still rare even if 117 countries worldwide had reported at least one case by the end of 2015 (WHO, 2017a).

To date, only selection of parasites presenting drug resistance due to mutations have been described in natural infections. However, knowing that for certain conditions adaptation through epigenetic mechanisms may potentially happen at an even higher frequency, we should wonder if we might be selecting phenotypes determined at the epigenetic level. In the setting of the drug resistance mechanism described in this thesis, we know that selection of \textit{clag3} expression patterns happens during the course of a single infection (\textit{i.e.} selection of \textit{clag3.2}-expressing parasites when they exit the liver). As we observed in our experiments, there is a \textbf{reset of the epigenetic memory} after going through the sexual cycle, meaning that there is no transmission of the \textit{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. In this regard, selection of \textit{clag3} phenotypes is comparable to selection of parasites for the expressed \textit{var} gene, the other well-known example of genes responsible for adaptation to fluctuant conditions by bet-hedging. While within an individual infection parasites expressing the \textit{var} gene that can escape the immune response are selected, the epigenetic memory that determines this pattern is reset during transmission stages genes (Bachmann et al, 2016; Dimonte et al, 2016). At the same time, this reset allows the parasite population to increase its repertoire of \textit{var} expression and thus to raise its probabilities of surviving in the new host, by presenting a more diverse population.

Although the \textit{clag3} expression pattern that confers drug resistance to the parasites is not transmitted from host to host, suggesting that this drug resistance mechanism cannot have an impact in an elimination context, we should not downplay the significance of such a mechanism. Recently, a study described a comparable phenomenon related to atovaquone resistance, which is associated to mutations at \textit{cytb} (Goodman et al, 2016). Parasites presenting this mutation are not
capable of continuing the life cycle to the sexual phase; hence, the mutation is not transmitted to the next human host. In spite of being heritable but not transmittable from one human host to the next, this mechanism is of significant concern since the cyt$^b$ mutation emerges rapidly, with mutant parasites observed even in areas of Africa without a history of atovaquone exposure (Ingasia et al, 2015). This suggests that cyt$^b$ mutations might occur spontaneously during asexual replication, and that their selection can occur during an individual atovaquone–proguanil treatment (Malarone®) (Cottrell et al, 2014; Goodman et al, 2016). So even if variant clag3 expression might not be responsible for the selection of a transmittable resistant population, since the expression pattern is not transmitted from one host to another, it should be taken into consideration as a drug resistance mechanism that can develop in a single infection. This would threaten treatment effectiveness in a single individual and, consequently, elimination of the human reservoir, obstructing elimination campaigns.

Traditional epidemiological studies on drug resistance look for the presence or absence of genetic mutations associated with treatment failure. Here, we have described a novel mechanism that can bring us to a fresh perspective: epigenetic epidemiology. This new discipline, deeply discussed and developed by Dr. Caroline L. Relton (University of Bristol), is being considered as a new public health research tool (Bakulski & Fallin, 2014). Even if it has been mainly approached from human epigenetics, discussing how it can be influenced by environmental factors and play a role in the development of diseases such as cancer or psychopathologies (Bustamante & Uddin, 2014; Verma et al, 2014), from now onwards this new tool should also be taken into consideration when studying drug resistance in microorganisms. In fact, during the consecution of this thesis we have already started with the first steps of this task; we have studied epigenetic patterns of clag3 genes in field isolates.
2. *clag3* GENES EXPRESSION DYNAMICS

2.1 *clag3* CLONALLY VARIANT EXPRESSION AS AN ADAPTIVE STRATEGY.

In this thesis, we have described a new drug resistance mechanism in malaria parasites in which *clag3* genes play a key role. *clag3* genes are regulated at the epigenetic level and they present clonally variant expression. Additionally, *clag3* genes are typically expressed in a mutually exclusive manner, *i.e.*, only one of the two paralogues is expressed at a time. The expression of one or the other parologue alters the efficiency of transport of some solutes through the membrane of the infected erythrocyte. However, the function that clonally variant expression in *clag3* genes presents is still under discussion.

While we have observed a functional role for clonally variant expression of *clag3* genes in lab experiments when selecting parasites with toxic compounds, we have not yet clearly found a role for this feature in natural infections. Parasites collected from infected patients all expressed *clag3.2*, which suggests that CLAG3.2 implies a better phenotype in human-blood circulation, probably conferring higher erythrocyte-membrane permeability to nutrients. The fact that we found predominant expression of one of the two paralogues suggests that in natural infections parasites expressing *clag3.2* are selected at the very beginning of a blood infection, since when they are released from the liver there is a mix of parasites expressing either one or the other paralogue (reset of the epigenetic memory). However, we hypothesize that there must be factors that select for parasites expressing *clag3.1*, and that clonal variant expression in *clag3* genes present a function in natural malaria infections.

In our study with field samples, we investigated adult and children patients with uncomplicated malaria. As mentioned above, parasites from all patient samples presented expression of the same paralogue: *clag3.2*. We did not find any significant difference in *clag3* expression in our study population: neither between adults and children, nor between patients presenting a first malaria infection vs those with repetitive exposure (presenting different levels of malaria immunity). We could not determine either whether other malaria outcomes such as presence of drugs in human blood, nutritional condition or disease severity might select for parasites expressing *clag3.1*. Indeed, parasites coming from patients that had been exposed to drugs, such as recrudescence cases, would be the most interesting ones for our research question. When we tried to analyze *clag3* expression in this kind of samples, maturation of these parasites was not possible and we could not proceed with RNA extraction and gene expression analysis (data not shown). Future studies on *clag3*
expression in natural infections should include *P. falciparum* infected patients with different clinical presentations in order to discover the physiological, toxic, clinic, etc. conditions that may select for parasites expressing clag3.1, as we observe in most lab-adapted parasites under culture conditions. Nevertheless, selection of parasites expressing one or the other paralogue may be determined by several factors at a time: features such as nutrients concentration, presence of toxic compounds and immune system, would define the most favourable expression pattern in each given situation, not being then determined by a single circumstance.

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 blood circulation environment, a property that could have been later leveraged for reducing permeability to drugs. Most of lab-adapted parasites express clag3.1 under culture conditions, where there is a surplus of nutrients in the media. The reason for parasites expressing this paralogue could be that the parasite has a propensity to express clag3.1, not necessarily implying a phenotypic advantage in culture conditions. For instance, expression of clag3.1 may be related to a more favourable configuration at the chromatin level. Then, in those situations in which expression of clag3.2 does not confer an advantage, *i.e.* when concentration of nutrients is high enough, parasites expressing clag3.1 would be predominant.

### 2.2 Drug resistance and fitness in parasites not expressing clag3 genes.

We currently know that mutually exclusive expression in clag3 genes is not strict (Mira-Martinez et al, 2013; Rovira-Graells et al, 2015; Sharma et al, 2013). Parasites can express both or none of the paralogues under strong selective pressure. Of note, parasites that have both clag3 genes silenced recover the mutual exclusion pattern after removal of drug pressure with BS, as it happened in our selection experiments when we removed the drug from the 10G-0.6-2 culture. This suggests that not expressing any of the clag3 genes has a fitness cost for the parasite. Indeed, the 10G-0.6-2 line presents a much lower growth rate than the parental 10G line.

Parasites developing drug resistance through genetic rearrangements have also been observed to present a fitness cost. It is known that some resistant parasites present extra genetic
rearrangements known as “compensating mutations”. This way, parasites balance out the fitness cost that the mutation or genetic rearrangement responsible for the drug-resistance phenotype implies itself. This is the case of parasites presenting resistance to anti-folates (by mutations at the \textit{dhfr} locus) and increased copy number of the \textit{gch1} gene, which encodes for GTP cyclohydrolase. This enzyme is located upstream of DHFR in the folate biosynthetic pathway, allowing to reinforce folate synthesis (Nair et al, 2008). If parasites can develop resistance to drugs in natural infections by not expressing \textit{clag3} genes, \textit{i.e.} reducing RBC membrane permeability, parasites may then need an alternative way for acquisition of nutrients.

There could be different possibilities for parasites not expressing \textit{clag3} genes to compensate for the fitness cost. One option would be the selection of compensatory mutations that could reinforce the uptake of nutrients, probably by strengthening other existing routes. We would be then selecting parasites that present a combination of genetic mutations and a specific epigenetic regulation pattern. Another mechanism to compensate for lack of nutrients uptake could be direct response. As explained in the introduction of this thesis, recent studies have described that the lack of essential compounds for parasite growth can trigger a directed transcriptional response that modifies the parasite phenotype, such as a reduction in the number of merozoites produced in every multiplication of the erythrocytic cycle or formation of gametocytes (Brancucci et al, 2017; Mancio-Silva et al, 2017). Something similar may happen for the uptake of nutrients; the parasite may increase the capacity of an alternative route or might adjust its metabolism to be able to grow with less nutrients. But the most plausible possibility for parasites to compensate for the fitness cost of not-expressing \textit{clag3} genes is that parasites stay in a dormant state. These parasites would decrease their metabolic rate, reducing their need of nutrients and avoiding the entrance of toxic compounds at the same time. Parasites that do not express \textit{clag3} genes could be non- or slow-replicating cells and dormancy would reduce their susceptibility to drugs. Something similar has been observed in parasites starving for isoleucine, an essential amino acid for \textit{P. falciparum}. The authors described that parasites responded to lack of this nutrient by slowing their growth, and this dormant state was reversed upon isoleucine re-supplementation (Babbitt et al, 2012). Indeed, delayed clearance of parasites in patients that have received artemisinin-based treatment has been related to quiescent forms of the parasite in lab experiments. Ring forms seem to survive to a pulse of artemisinin derivatives in a dormant phase and reactivate the cycle once the drug is removed (Witkowski et al, 2013). Interestingly, 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).
2.3 Future studies, challenges and perspectives in clag3 genes expression dynamics.

As explained above, there is yet much to investigate about clag3 expression dynamics and its effect in transport and drug resistance. A future perspective to reach a better understanding is to better mimic natural conditions in our experiments. Now we know that nutrients concentration might influence clag3 genes expression and parasite viability (Desai, 2013; Mancio-Silva et al., 2017). Even the general assumption of most lab-adapted strains preferentially expressing clag3.1 might not be factual if we reduce nutrient availability. Would then parasites preferentially express clag3.2 as we observed in parasites coming from human infections, where there are more restrictive nutrient conditions? If the concentration of nutrients is the determining factor, this would be the expected pattern. In order to prove this hypothesis, experiments in the lab could be conducted using PGIM medium, a modified RPMI that contains some of the essential nutrients for the parasite at physiological concentrations (Pillai et al., 2012). Culturing lab strains that express clag3.1 (3D7-ITM) and strains that express clag3.2 (10G) in PGMI media and comparison of the effects in growth rate and possible switches in clag3 expression pattern would provide us with an idea of whether restrictive nutrients conditions select for clag3.1.

Secondly, we still need to fully understand the role of mutually exclusive and clonally variant expression of clag3 genes as a surviving strategy and their effect on the transport of toxic compounds. The study of clag3 genes expression in field isolates has a singular complication. clag3 genes share 95% sequence identity and the different areas between them are also diverse among isolates. When working in the lab with culture-adapted parasites this matter is of less significance, since clag3 sequences are available on public database. That was the case for the work conducted in the first paper of this thesis. However, when we were challenged to study clag3 dynamics in natural infections we encountered with this difficulty. For this situation, we amplified and sequenced the HVR of each clag3 paralogue and isolate to design specific primers for expression analysis by qPCR. Thus, a future duty to simplify future studies on this topic, would be to design primers that are specific for clag3.1 and clag3.2 in all parasite isolates (ongoing). The paralogue-conserved regions found at the N-terminal end (NtCR) (see Fig. 3 Mira-Martínez et al., 2017. Article 2) are a good option to start the design of these standard primers. Since we already have a set of clag3.1-specific and clag3.2-specific primers designed for each of our field isolates, these primers could provide a reference to test performance of the new universal primers (ongoing). Now that we have already confirmed that mutually exclusive expression occurs in natural infections, with the
implementation of universal primers we could also analyse samples presenting MOI higher than 2, to identify situations that select for parasites expressing clag3.1.

Last, we have already observed that parasites in which clag3 expression does not comply with mutually exclusive expression exist and can be selected under particular situations. However, this pattern has only been observed in lab-adapted parasites. In order to detect parasites not expressing clag3 genes in field isolates, even at low rates, we could conduct IFA experiments in parasites coming from infected-patient blood samples upon maturation of parasites. By qPCR analysis we detect the expression of the gene that is mainly active in the population, but we cannot detect individual parasites that have their two clag3 genes in a repressed state. That way, even if this expression pattern is rare, we could identify parasites not expressing clag3, and confirm the existence of this pattern in natural infections.
3. **PSAC: A PARASITE-ENCODED CHANNEL PLACED AT THE MEMBRANE OF THE HOST ERYTHROCYTE**

Another characteristic of the drug resistance mechanism described in this thesis is that it implicates a parasite-encoded channel placed at the membrane of the host RBC (PSAC), which had not been involved in any other drug resistance mechanism to date. PSAC is responsible for the uptake of a broad range of solutes, from essential nutrients for the parasite to toxic compounds, such as drugs (Desai et al, 2000; Hill et al, 2007). Changes in the expression of clam3 genes modify the permeability of the infected RBC by alterations at the PSAC level, allowing the parasite to become resistant to some toxic compounds (Mira-Martinez et al, 2013; Sharma et al, 2013).

### 3.1 The PSAC Channel as an Antimalarial Target.

In addition to its role in drug resistance, PSAC is an attractive object of study, since it is responsible for the uptake of nutrients. Knowing that PSAC is necessary for nutrients transport, studying the possibility of blocking the channel as an antimalarial target is an attractive strategy. As it was observed at Sanjay Desai’s lab (NIH, Washington), lab-adapted parasites present reduced growth rates when blocking PSAC in a nutrient-restricted media, demonstrating that PSAC inhibitors might be promising antimalarial drug candidates (Pillai et al, 2012). To reach this goal, finding a compound that blocks PSAC and that is not toxic for human cells is necessary. Some work has already been done with this purpose. At Sanjay’s lab a high-throughput inhibitor screen to detect new PSAC inhibitors was conducted (Pillai et al, 2010). The authors presented novel scaffolds that interact directly with one or more sites on the PSAC channel. As they concluded, on the one hand these inhibitors can be used as starting points for the development of new antimalarial drugs; on the other hand, these compounds are a good tool to investigate the channel itself.

PSAC inhibitors have been indeed largely used to gain further understanding on the structure and function of the channel, mainly at Desai’s lab. For instance, researchers at this group described that some PSAC inhibitors present different efficacy against diverse solutes, suggesting the presence of two distinct mechanisms of solute transport through PSAC (Lisk et al, 2007). These mechanisms could correspond to either two physically separate routes within a single channel or to two distinct binding sites for subsets of solutes along a common transit pathway (Bokhari et al, 2008).
Further studies with PSAC inhibitors allowed determining that clag3 products contribute to PSAC activity (Nguitragool et al, 2011). The authors observed that some PSAC inhibitors present different affinities in different parasite lines. For instance, ISPA-28 inhibits PSAC activity in Dd2 parasites but has no effect in the HB3 line. Then, in order to explain this phenomenon, the authors studied the effect of ISPA-28 against PSAC in the offspring from the genetic cross of Dd2 and HB3. Most of the clones obtained in the offspring exhibited comparable channel inhibition to one or the other parental line, suggesting that the affinity of the PSAC inhibitor is genetically determined. Genetic mapping and the creation of transgenic lines, complementing Dd2 parasites with the HB3 allele of individual candidate genes, allowed the authors to identify clag3 genes as responsible for this different phenotype between the two parasites lines. To further confirm the role of these proposed genes, the authors created a transgenic parasite line in which the HVR of one strain (clag3.2-HB3) was replaced by the HVR of the other paralogue of the other line (clag3.1-Dd2). They observed that the effect of some PSAC inhibitors was comparable to the effect on Dd2 parasites, suggesting that these inhibitors probably bind to specific motifs in the HVR, which was later confirmed by other studies (Pillai et al, 2012).

Knowing that some PSAC inhibitors present different affinities according to the clag3 that is expressed and the genetic background, chemicals to be used as potential therapeutic strategies should ideally block the transport of as many solutes as possible, regardless of the clag3 gene that is expressed and the genetic background of the parasite, while sequence-specific inhibitors can be used as tools for research purposes.

3.2 clag3 EXPRESSION DETERMINES THE EFFICIENCY OF SOLUTE TRANSPORT IN PSAC.

Similar to the variable affinity showed by some PSAC inhibitors, we have observed that the efficiency of transport for some solutes through PSAC also depends on the clag3 being expressed and the parasite genetic background. During our BS selection experiments with 10G parasites we observed changes in the RBC membrane permeability to BS depending on the clag3 paralogue that was expressed, being clag3.1 the paralogue that determines less efficiency for BS transport (Mira-Martinez et al, 2013). However, drug pressure with the same compound resulted in selection of parasites with different clag3 expression patterns when working with parasites presenting a distinct genetic background, at least for BS (Fig. 15). For instance, in the P04 isolate we observed selection of parasites expressing clag3.1 after applying low-BS pressure, consistent with our previous results.
with 10G parasites; however, the P12 isolate expressed clag3.2 under low-BS selection (Mira-Martinez et al, 2017).

We conducted a phylogenetic analysis of the HVR of CLAG3.1 and CLAG3.2 including sequences from lab-adapted parasites and field isolates from our expression analysis study (Mira-Martinez et al, 2017. Article 2). We obtained a tree with two separate clades. In each of these clades, sequences from both CLAG3.1-HVR and CLAG3.2-HVR could be found, indicating that an HVR from CLAG3.1 can be as similar to a CLAG3.2-HVR as to another CLAG3.1-HVR. Interestingly, we observed that the paralogue expressed by parasites selected under BS-pressure on P04 and P12 is within the same clade as 3D7-CLAG3.1 (the one selected in our previous experiments with 10G parasites); i.e. 3D7-CLAG3.1, P04-CLAG3.1 and P12-CLAG3.2 were in the same clade in phylogenetic analysis of HVR sequences (Fig. 3 Mira-Martínez et al., 2017. Article 2). Altogether these results suggest that the HVR sequence might determine the transport efficiency for some solutes.

Besides, analysis of whole CLAG3.1 and CLAG3.2 sequences from lab-adapted parasite lines and two of the field isolates (P04 & P12) resulted in a tree with two distinct clades: one for CLAG3.1 and another for CLAG3.2. This result indicates that CLAG3.1 is always more similar to the CLAG3.1 from another strain than to a CLAG3.2 sequence. Further analysis of CLAG3 sequences revealed specific conserved features at the N-terminal of the protein that were always conserved among all CLAG3.1 or CLAG3.2 sequences but different between the two genes (Fig. 3, Mira-Martínez et al., 2017. Article 2). This region will be referred to as N-terminal paralogue-specific conserved region (NtCR). Since all parasites collected from patient blood samples expressed the same paralogue (clag3.2), we

![Figure 1. Schematic of clag3-dependent BS-permeability. Expression of clag3.2 in the 10G and P04 genetic background implies higher permeability to BS, while in P12 expression of clag3.1 confers higher permeability for this solute.](image-url)
concluded that the NtCR of CLAG3 may determine general permeability properties, which would confer increased fitness while parasites are in the human circulation. Figure 16 presents a schematic representation of CLAG3 proteins and proposes the regions that are likely to determine the efficiency of transport of specific compounds, such as BS, and the regions that might determine general permeability. However, this idea requires being tested with additional isolates in order to be confirmed.

Figure 2. Schematic representation of CLAG3.1 and CLAG3.2 genes, showing regions proposed to determine general permeability (NtCR) and efficiency of transport of specific solutes (HVR). Phylogenetic analysis of the whole sequence of both genes show a tree with two clear separate clades, split in CLAG3.1 and CLAG3.2 (left panel). Analysis of HVR sequences revealed two clades, with a mix of CLAG3.1 & CLAG3.2 HVR sequences in each clade, which may correspond to the clag3-selection behaviour when selecting parasites with BS (the paralogues expressed by parasites selected under BS-pressure are all placed in clade 1 (in circles), while the other clag3 gene of the same isolates are all in the clade 2). Figure adapted from Figure 3 - Mira-Martinez et al., 2017.
3.3 Future studies on the effect of CLAG3 sequences on the efficiency of solute transport.

To date we have studied clag3 expression patterns under different culture conditions in parasites with a 3D7 genetic background and two of the field isolates: P4 and P12. To be able to correlate HVR sequence and efficiency of solute transport, additional BS-selections in other isolates should be conducted. This way we would determine if the clag3 parologue expressed by the selected parasites is always within the clade 1 of the HVR sequences tree, like 10G-CLAG3.1, P04-CLAG3.1 and P12-CLAG3.2 (Fig. 16). These results would give us more information regarding whether the transport efficiency of some solutes is determined by the HVR sequence, at least for BS.

To further study the correlation between the HVR sequence and efficiency of solute transport, similar studies to those conducted by Desai and colleagues, creating chimeric clag3 genes (Nguitragool et al, 2011; Sharma et al, 2015), could be performed using the recently developed CRISPRcas9 technology. Exchange of HVR sequences between both genes in already characterized parasite lines and subsequent selection experiments would provide information about the determinants of the transport efficiency for specific solutes. If it is determined by the HVR sequence, parasites with the HVR sequences exchanged between paralogues would show different clag3 expression patterns than wild type parasites under BS pressure. For instance, if we used 10G parasites with exchanged HVR sequences between both paralogues we would expect that parasites expressing clag3.2 would be selected when applying BS pressure (instead of clag3.1 as previously observed in wild type parasites). In addition, the same kind of experiments will be soon conducted in our lab to study the CLAG3-NtCR. Exchange of that region between paralogues is expected to confirm if conserved motifs of CLAG3.2 determine higher general permeability, as we hypothesized from our results in experiments with field samples, in which parasites from all patients were expressing clag3.2.

In our study with field isolates (Mira-Martínez et al., 2017. Article 2), we excluded parasites presenting a single clag3 gene due to recombination. Of note, 20% of the samples collected presented a single clag3, a value that was unexpectedly high. Since parasites in natural infections preferentially express clag3.2, we would expect that parasites found in natural human infections would present a recombinant clag3 gene more similar to clag3.2. This is also expected from the process that generates the recombinant clag3 at the chromosome level, which creates a single clag3 gene that carries the 5’ UTR of clag3.2. Then, it is expected to find the NtCR of clag3.2 in this recombinant gene, for its close location to the 5’ end. Sequencing the clag3 gene from these recombinant parasites and including them in the phylogenetic analysis might provide us with information of the regions of the gene that determine a viable phenotype in human blood infections.
Besides, we could study the behaviour of these parasites under conditions that require a more restrictive permeability, such as drug pressure. How would these parasites adapt to increasing concentrations of BS if they cannot switch clag3 expression? Probably, their only possibility would be to silence the single clag3 gene in the presence of this antibiotic.

### 3.4 Transport of Drugs through PSAC

We and others have observed that PSAC is responsible for the acquisition of some compounds that are toxic for the parasite, such as sorbitol, LEUP and BS (Hill et al, 2007; Lisk et al, 2008; Wagner et al, 2003). Additionally, 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 because of its high toxicity to human cells. Thus, we investigated whether drugs that are in use or under development for the treatment of malaria require transport through PSAC. These compounds would be, in that case, subject to potential development of resistance through the same mechanism described for BS.

For our experiments, we generated a list of drugs that are expected to be transported through PSAC based on their logP (Partition coefficient), the preferred descriptor of molecular lipophilicity or hydrophilicity: lipophilic compounds, which present high logP values, are expected to cross the biological membranes by passive diffusion, whereas hydrophilic compounds (those presenting low logP values) require membrane-transport proteins for their uptake. Since BS and LEUP, which have a low logP value, require PSAC to enter the cell and their transport is affected by clag3 expression, drugs such as DOXY and FOSMI, which also have a low logP value, were selected for our study. FOSMI has additionally been experimentally demonstrated to accumulate in infected RBC as a consequence of parasite-induced NPPs (Baumeister et al, 2011). However, after conducting our experiments, we did not observe differences of IC50 for those compounds when comparing 10G and 10G-0.6-2 (expression of clag3.2 vs both clag3 silenced), meaning that these compounds do not require CLAG3 proteins for their transport into the cell. After this, we selected additional chemicals from a list of compounds that are suspected to require facilitated transport that was created based on computational analysis by Basore and colleagues: AZI, LEUP and LUM (Basore et al, 2015). We also selected other compounds that had been reported to be transported through NPPs (T3, T16 and PENTA) (Biagini et al, 2003; Stead et al, 2001; Wein et al, 2012). Among the drugs that we tested, only three of them (T3, T16 and LEUP) and our positive control (BS) showed differences of IC50 values between 10G and 10G-0.6-2 (Mira-Martínez et al., under preparation. Article 3). These results
suggest that the uptake of the rest of the drugs is mediated by an alternative route that does not involve CLAG3 proteins and that only experimental analysis will show which compounds require CLAG3-mediated uptake. (Fig. 17). We cannot dismiss though that these drugs use the parasite-induced NPPs, as predicted by the mathematical model of Basore and colleagues.

![Figure 3. Schematic of compounds that require CLAG3-containing PSAC and those that cross the membrane by passive diffusion or using other transport pathways.](image)

Interestingly, we found relatively modest differences in IC$_{50}$ values between 10G and 10G-0.6-2 for T3, T6 and LEUP (around 3 fold, whereas the difference was around 8 fold for BS), suggesting that these drugs might, in addition to CLAG3-mediated transport, use an alternative route not involving CLAG3 to enter the cell (Fig. 17). Our first hypothesis to explain the different increases observed in IC$_{50}$ values between BS and the rest of compounds was that 10G-0.6-2 had been created by selecting parasites under BS pressure; parasites could have developed additional resistance by another parallel mechanism. However, in experiments at Desai’s lab a LEUP-resistant mutant, which presents a mutation at $clag3.2$ that would modify the permeability characteristics of PSAC to LEUP, showed similar levels of resistance to 10G-0.6-2 (4 fold difference compared to the wild type), and this line was likewise created by LEUP-selection (Sharma et al, 2015). At the same time, this does not dismiss our first hypothesis of parasites using multiple pathways for acquiring resistance to the drug. Desai and colleagues observed that a transgenic line carrying the mutation responsible for LEUP-resistance was more sensitive to LEUP than the original line created by selection (Sharma et al, 2015). They concluded that additional genetic or epigenetic changes could be required for this greater resistance to LEUP, as we had suspected for our 10G-0.6-2 line for BS.

We have previously shown that in the 10G line resistance to BS at low concentration was associated with a switch from $clag3.2$ expression to $clag3.1$. Thus, we investigated whether 10G parasites can
also acquire resistance to sub lethal concentrations of other compounds by epigenetic changes in the expression of \textit{clag3} genes. We observed that parasites selected with T3 and T16 reduced drastically the total expression of \textit{clag3} genes after only two weeks of selection, demonstrating that parasites can become rapidly resistant to these compounds through changes in the \textit{clag3} epigenetic state. Additionally, experiments to determine if expression of one of the two \textit{clag3} paralogue determines more efficiency for the transport of these drugs are in progress. None of the compounds that we tested and seem to require CLAG3 for their transport 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 \textit{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.

**3.5 Other drugs might use alternative routes to enter the infected RBCs.**

The results of our IC₅₀ experiments suggest that the uptake of the rest of the drugs that we tested (AZI, DOXY, FOSMI, LUM and PENTA) is not affected by repression of both \textit{clag3} genes, even if their physical properties predicted that they require a channel to enter the cell. To further understand this observation, we first demonstrated with IFA experiments that the 10G-0.6-2 parasite line does not present CLAG3 proteins at the RBC surface, confirming that our line is an appropriate tool to test CLAG3 requirement for drug uptake (Mira-Martínez et al, in preparation. Article 3.). However, it is possible that other proteins can form the PSAC in the absence of CLAG3, such as other CLAG family member(s). Thus, even if we have proved that our parasite line (10G-0.6-2) is valid for testing parasites with non-functional CLAG3 proteins, we cannot determine if these drugs use PSAC.

Next, in order to discern whether 10G-0.6-02 is a valid tool to test PSAC functionality, we conducted an experiment using 5-aminolevulinic acid (5-ALA), a compound that is taken up by infected RBCs through PSAC and converted to fluorescent protoporphyrin IX (PPIX) (Beck et al, 2014; Sigala et al, 2015; Staines et al, 2004). Import of 5-ALA therefore acts as a convenient reporter for PSAC activity and can be easily determined through fluorescence measurement (Sherling et al, 2017). Unexpectedly, we observed that both 10G and 10G-0.6-2 imported 5-ALA at the same level, suggesting that other proteins participate in the formation of PSAC, possibly independently from the channel formed by CLAG3. Hence, our experiments using 10G and 10G-0.6-2 parasite lines are
valid to determine CLAG3 requirement of a drug to get into the cell, but not to test PSAC requirement.

According to the results of these experiments, we suggest that the drugs that could reach the cell in the absence of CLAG3 use a different transport route formed by CLAG8 or CLAG9 (of note, 10G-0.6-2 presents clag2 in a repressed state). It is known that the RhopH complex is formed by RHOPH2, RHOPH3 and one of the CLAG proteins (Kaneko et al., 2005). We suggest that both the complex and the channel can also be formed by another CLAG protein, defining a channel with different properties (Fig. 18). Thus, in case we want to study drugs that might require PSAC, the recently created *rhoph2* and *rhoph3* conditional knockdown parasite lines, which were not available when we started this project (Counihan et al., 2017; Ito et al., 2017; Sherling et al., 2017), would be a more adequate tool. Using these knockdown lines, the authors observed that RhopH2 and RhopH3 proteins are necessary for the formation of the whole RhopH complex and a functional PSAC. Therefore, in the absence of RhopH2 and RhopH3 no PSAC can be formed, being the knockdown lines excellent tools to determine which drugs use the PSAC to enter the cell. However, this is not the purpose of this thesis. We aimed to find drugs that can be susceptible of treatment failure through a mechanism regulated at the epigenetic level. Thus, our 10G-0.6-2 parasite line is still a valuable tool, since it can detect drugs that could be susceptible of treatment failure by epigenetic regulation of clag genes. Of note, this parasite line has clag3.1, clag3.2 and clag2 in a silenced state, genes that present clonally variant expression and that are regulated at the epigenetic level. clag8 and clag9 cannot be silenced at the chromatin level, thus being more unluckily for parasites to develop resistance against drugs that use these potential channels formed by CLAG8 or CLAG9.

Figure 4. Schematic representation of the model of different RhopH complexes and PSAC formed by RhopH1, Rhoph2 and one member of the RhopH1/CLAG family. Adapted from Ito et al., 2017.
3.6 Other clag genes and their role in drugs transport.

As explained above, recent studies confirmed that RHOPH2 and RHOPH3 are necessary for a functional PSAC, even if they might not be an integral part of the channel itself (Counihan et al., 2017; Ito et al., 2017; Sherling et al., 2017). However, whether other genes from the clag family (RhopH1) are involved in the PSAC formation is not known yet. The results obtained in our IC_{50} assays together with the results of S-ALA experiments suggest that other CLAGs can form PSAC in the absence of CLAG3. In *P. falciparum*, clag2, clag8 and clag9 are expressed at very low levels, compared to clag3 (Cortes et al., 2007; Mira-Martinez et al., 2017). Parasites could be presenting independent channels formed by these proteins, at lower levels, which may be responsible for the transport of other compounds, such as FOSMI, in the absence of CLAG3 (Fig. 19). Additional observations also suggest that other clag genes participate in PSAC/NPPs formation. For instance, clag genes are not present in other genus and neither is PSAC (Alkhalil et al., 2004), supporting the idea of clag family and the channel being associated. Then, sequence and structure similarity among these genes suggests that they are somehow related. As explained in the introduction of the thesis, the five members of the clag family are split in two groups. clag2, clag3.1, clag3.2 and clag8 are in the same group, but not all of them are present in all *Plasmodium* species. This suggests that they might be functionally related: the lack of one of the members could be compensated by other members of the same group. Furthermore, clag2, clag3, and clag8 have in common the presence of hypervariable domains at the same position (Fig. 10), which is not detectable in clag9 (Kaneko et al., 2005). This hypervariable region has been observed to be exposed at the membrane and to have a direct role in solute transport in clag3 (Nguitragool et al., 2011), making the hypothesis of clag2 and clag8 being involved in channel formation more plausible.
Figure 5. Schematic representing of the model of different PSAC channels formed by members of the CLAG family. Some compounds may be transported in the absence of CLAG3 by the action of PSAC formed with other CLAG proteins.

During the progress of this thesis, we studied the participation of other clag genes in solute transport and PSAC formation, we studied other clag genes transcript levels in 10G-0.6-2, the parasite line that presents both clag3 genes silenced and does not transport BS, among other compounds (Mira-Martinez et al, 2013). We did not observe differences in the expression of clag2, clag8 or clag9 between 10G-0.6-2 and its wild type 10G. If we had detected a reduction in transcript levels, it could mean that other clag genes participate in the transport of this drug. Besides, overexpression of other clag family members would have suggested that these genes compensate for the lack of transport of parasite-essential compounds, which was not observed. However, basal expression of other clag genes could be enough to compensate nutrients transport in an environment where nutrients concentration is excessive.

To date, clag2 is the only clag family member other than clag3 proposed to play a role in the formation of the channel. Contrary to our results, in Sharma et al., 2013 (Sharma et al, 2013) the authors observed a decrease of clag2 levels in a parasite line that, analogous to our 10G-0.6-2 line, was created by selection with BS and does not express clag3 genes. The authors concluded that clag2 might also be involved in channel formation or regulation, participating in BS uptake. However, clag2 presents clonally variant expression as clag3 genes do; i.e. there are lab-adapted parasite lines that express clag2 when maintained in standard culture conditions and other lines that present the gene in a silenced state (Cortes et al, 2007). The 10G parasite line, the line that we used in our selection experiments, does not express clag2. Thus, to study if clag2 is involved in PSAC formation, we selected 1.2B parasites, a line that is isogenic with 10G but has the clag2 gene in an
active state (Cortes et al, 2007; Rovira-Graells et al, 2012), with high concentrations of BS (Mira-Martinez et al, 2013). We did not observe any reduction in clag2 expression levels after selection with this line. Nevertheless, it is possible that clag2 presents different properties depending on the genetic background of the parasites, as clag3 genes do. In the study conducted by Sharma and colleagues they used parasites of the FCB line for their experiments, whereas we used parasites of the 3D7 genetic background (10G and 1.2B lines). Thus, we cannot dismiss the possibility that the different results obtained in the two studies are due to the genetic background of the parasite lines.

To better understand the role of other clag genes in PSAC activity, we also investigated their expression levels in human infections (both natural and experimental) (Mira-Martinez et al, 2017). We observed that clag2, clag8 and clag9 were in an active state in all samples analysed in our study. Additionally, field isolates expressed these genes at the same level when adapted to culture conditions. We neither found differences in transcript levels in samples from experimentally infected volunteers compared to the NF54 parental line maintained under culture conditions. Our results show that all the other clag genes are expressed in human and lab conditions in the studied population; however, other data suggest that these genes are not essential for parasite survival. First, clag2 presents clonally variant expression and some lab-adapted parasite lines do not express this gene. Then, spontaneous deletions of the clag9 locus have been observed during adaptation of parasite isolates to in vitro culture in other studies (Day et al, 1993) and clag9 knockout lines have also been generated by transfection (Nacer et al, 2011; Trenholme et al, 2000), demonstrating no essentiality of this gene. A potential explanation for clag8 and clag9 being always expressed is that, contrary to clag2 and clag3, they do not present clonally variant expression; it might be not possible to silence these genes by modifications at the chromatin level. However, the reason for clag2 presenting clonally variant expression and being silenced in some lab-adapted parasite lines is still unknown.

A possible explanation for clag2 presenting clonally variant expression is that there could be a condition that selects for parasites that present clag2 in a silenced state during human infections, as we concluded for clag3.1 expression. In fact, in case there is a reset in the epigenetic memory of clag2, as it happens with clag3 genes, there could be a mix of parasites having their clag2 in either an active or repressed state when parasites exit the liver. Subsequent selection of parasites expressing clag2 in peripheral blood after few cycles would explain the results we obtained in parasites collected from blood samples, analogous to what happens with selection of parasites expressing clag3.2 in human blood. If there is a subpopulation of parasites not expressing clag2 (comparable to the detected situation of parasites expressing clag3.1), it could not be determined by qPCR analysis (as we cannot detect parasites that do not express any of the two clag3
paralogues). Other experiments, at the single-cell level, such as IFA using antibodies that detect CLAG2, could be conducted to distinguish parasites that have clag2 in a silenced state.

Very little is known about other members of the clag family and their role in *P. falciparum* biology. Further investigation is needed to inquire into whether they are involved in formation of PSAC, transport of nutrients or uptake of toxic compounds, being candidates to participate in drug resistance mechanisms.
4. Drug Resistance is a Complex Problem

4.1 Filling the Gap Between the Lab and the Field.

It has been demonstrated that clag3 genes play key roles in parasite biology, such as nutrient and toxic compounds acquisition. However, very little is known about their role in natural infections. In this work we studied for the first time clag3 genes dynamics in natural and experimental human infections. clag3 genes are involved in a drug resistance mechanism that would be rapid and reversible. Thus, it is of key importance to anticipate if this mechanism could mean a threat to malaria control and elimination. Drug resistance can be defined as a complex problem since many factors and stakeholders are involved in its resolution (Annex 1). Indeed, one of the take-home lessons I get from this thesis is the importance of bridging basic science to real life.

As we observed in the execution of this work, sometimes what we observe in the lab does not correspond to what happens in natural conditions. In our lab in ISGlobal clag genes and epigenetic regulation had been studied in culture-adapted parasites for several years. But when we studied clag3 genes coming from human infections, apart from confirming some observations (such as the mutually exclusive expression property of clag3 genes), we observed different patterns of expression and reached new conclusions. For this, we support that the lab is a good model (with stable and controllable conditions) for exploratory studies; afterwards, results need to be confirmed in the natural environment, where conditions are not that stable or controlled.

Indeed, lab studies can provide a good prototype to study basic biological rules. Lab-adapted strains are an essential tool for the production of basic knowledge. However, it is important to adapt lab conditions, trying to create an environment as similar as possible to physiological conditions. It is already been proved how some variables, such as nutrients concentration, can modify the regulation of some genes (Desai, 2013; Mancio-Silva et al, 2017); thus, physiological conditions should be mimicked as much as possible. A strategy to take into account would be to adapt to the lab parasites coming from field isolates more often, instead of working with parasites that have been under culture conditions for longer than 30 years (as it is the case of the 3D7 strain). Continuously adapting field isolates would eliminate the stability of using the same line for all experiments; nevertheless, in a study conducted by Yeda and colleagues (Yeda et al, 2016) it was concluded that parasite genetic and phenotypic characteristics fluctuate in short- and long-term cultures. The most suitable situation is to work with field isolates, ex-vivo experiments, to get more reliable information. In fact, thanks to this kind of experiments we have observed that parasites in lab conditions prefer expressing clag3.1, while parasites collected from human infections are mostly expressing clag3.2, as we confirmed with NF54 parasites in the samples from the CHMI study.
Unfortunately, getting field isolates requires more complicated study design, approval of ethical committees and of course consent of volunteers. Conducting studies in the field is not an easy task. Thus, I would like to enhance the value of collaboration studies, as we did for this thesis.

4.2 Using different disciplines and collaboration studies for approaching a complex problem.

The work described here started in a molecular biology lab (Malaria Epigenetics – ISGlobal), where studies in epigenetic regulation in *P. falciparum* had been conducted for several years. Then we approached the project from an epidemiological perspective, conducting the studies in a molecular epidemiology group (Malariology Unit – ITM). This kind of research group is the perfect bridge between the lab and the field. Molecular epidemiology aims to define the molecular basis of diseases and to identify genetic determinants that predispose individuals to particular disease outcomes. Frequently this kind of research groups combine the strengths of collaborations between clinicians and basic scientists, as it happens at ITM, where molecular biology studies are conducted in close collaboration with the clinic of the institute and other research centers in malaria-endemic countries. Studies of this discipline are making significant advances in translational research areas, increasing our understanding of basic disease mechanisms and providing new knowledge that may be employed in clinical management strategies.

Working at the Malariology Unit at ITM allowed us to collect blood samples from travellers attending the clinic at ITM and UZA. The collaboration with the clinicians was very positive and reciprocal. On the one hand, we could reach patients that were being diagnosed, which facilitated the task of getting informed consent, have access to clinical data of the patients and obtaining fresh samples from volunteers, which would be cultured in the lab and used in our experiments. On the other hand, results from our analysis (such as MOI, drug resistance markers, demographic and phenotypic characterization) and the information we collected in the interviews with the patients (drug use, adherence, etc.) could be used for further description and epidemiological studies, interesting for the clinicians’ research.

Since the amount of samples obtained from the clinic and the hospital was not enough for our analysis, another lab was included in the study: MRC in The Gambia. This opportunity expanded our population of study; we collected samples from an ongoing drug-resistance study at MRC (PI: Joseph Okebe) in which the studied population was different from ours (children instead of adults). I would like to emphasize the possibility of conducting two different studies from the same study
group. As mentioned above, organizing studies in the field is logistically challenging. If we can make the most of these studies by sharing samples, lots of human and volunteer work could be economized. At the same time, by integrating studies we can also answer different research questions that could benefit both parts, by sharing results and information.

However, even if the samples obtained in The Gambia and at the clinics in Belgium were very valuable, getting samples from the field is not always the perfect situation. Because of the inclusion criteria that we had defined, for a simpler and exploratory study, we found some difficulties and limitations:

- A larger number of samples would have been desirable. We had to dismiss many samples for presence of recombination of clag3 genes, too low parasitaemia, MOI ≥ 2, etc. We could analyse only half of the samples that were collected.
- Because of polymorphisms present in different strains, studying clag3 genes in field isolates is quite laborious, as explained before. Besides the time invested in primer design, we also had to dismiss some samples from which no good gene sequences could be obtained.
- Patients that had been exposed to treatment are a hot subject for our studies. However, it was not possible to maturate parasites from these patient samples, so we could not get material for our analysis.
- Adaptation of parasites to lab conditions was an extra challenge. I tried to adapt 10 of the field isolates and 9 of them were successfully adapted for several cycles. Indeed, working with field isolates was challenging in many aspects. Maturation of parasites to schizont stage was more complicated than expected. Since parasites were collected at diverse times of the life cycle (ring stage, which is found in peripheral blood, lasts 20h) and parasites morphology is not identical in the different isolates, calculating the perfect time for harvesting the cultures (around 42h of life cycle) was arduous.
- We had to adapt some of our protocols (RNA extraction, qPCR analysis), which had been used in our lab for years. In our group in Barcelona we usually work with lab-adapted parasites, reaching very high parasitaemias in the cultures for genetic material extraction. In the study with field isolates we were working with very low parasitaemias and had little material for analysis, so protocols had to be optimized and validated, which took very long time.
- When getting blood sample from patients, most of the times the characteristics of the studied population must be restricted. It is much more troublesome to get samples from patients with severe malaria or from infants, for instance (because of ethical concerns). Primary infection in children would have been very interesting to study, or patients presenting specific characteristics, such as immunocompromised individuals, patients presenting complicated malaria, or people with nutritional deficiencies. But getting samples from these kind of patients
is usually not ethically acceptable, unless it is very well justified, which was not the case for our preliminary study.

To further study clag3 genes regulation in lab and human infection strains, we also took the advantage of another study conducted at ISGlobal: CHMI (PI: Pedro Alonso) (Gomez-Perez et al, 2015). The conduct of CHMI studies is a great progress. It facilitates the study of the parasite and it allows the comparison of the same strain in two different environments: human blood and lab culture. During the work of this thesis, other groups conducted similar experiments to ours, but in the study of var genes (Bachmann et al, 2016; Dimonte et al, 2016). These studies show the significance of this kind of tool. Even if the main aim of the development of CHMI is to conduct clinical studies in less complicated settings and under control conditions, researchers of basic science should take the advantage of this new tool for their work, such as for gene expression analysis or immunological studies. This methodology is very valuable to better understand the differences of what is happening in natural infections and what we observe in the lab. Thanks to the CHMI study conducted in Barcelona, we could go further in the gene dynamics study. For instance we could study the effect of the sexual phase of the parasite cycle in epigenetic memory, analysing clag3 expression in a lab strain after going through the sexual cycle and through the human host. We could also compare a lab strain in two different environments, lab and human, verifying that NF54 parasites prefer expressing clag3.1 in the lab while they prefer expressing clag3.2 in human blood.

Nevertheless, even if CHMI studies are very advantageous, the use of healthy volunteers for lab studies should also be reduced; even if minimum, there is always a risk when infecting someone with P. falciarpum. At the same time, there is no direct benefit for the population under study, and as stated at the Article 19 of the Declaration of Helsinki, Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research (WMA). Thus, once a study of this kind is conducted, we ought to take the most of it.

4.3 Transdisciplinariness: Crossing Boundaries of Disciplines and Research.

One of the definitions of “complex problem” is that many stakeholders should be taken into consideration for its resolution. Knowledge obtained from basic science should be transferred to the society. Indeed, after studying clag3 gene regulation in lab conditions and in human samples, we still wanted to go further into the impact of our work in clinical malaria.
We were interested in conducting a high-throughput screening using our parasite lines and testing drugs of the pipeline, such as those of MMV, in order to find compounds that could be susceptible of failure by the drug resistance mechanism described in this thesis. However, since our infrastructure was very limited we had to conduct all the tests manually, which was very time consuming. For this reason, we had to choose a set of compounds among those that were suspected to require PSAC for reaching their target (Basore et al, 2015). Collaboration with the industry, which counts with specialized robots for conducting this kind of test, would have been very valuable. Furthermore, from this collaboration, both parts could benefit from the results, since this screening would have helped in the investigation of new antimalarial candidates.

Our goal is to anticipate treatment failure by the drug-resistance mechanism described in this thesis on drugs that are still under research. Indeed, studies pursuing a similar aim have been already conducted in promising antimalarial candidates. Compounds such as KAE 609, KAF 156, OZ 439, and OZ 277, have already triggered fears that point mutations may lead to treatment failure (Magistrado et al, 2016; Phyo et al, 2016; Rottmann et al, 2010; Valecha et al, 2012). Testing drugs that are still under investigation could help us to prevent future treatment failure. Therefore, the importance of the collaboration among different stakeholders, such as industry and academia. Not only technology and infrastructure, but also knowledge should be transferred and shared from one field to the other. Indeed, as reported at Basore et al 2015., another problem for the prediction of drug features is that calculation or measurement of some parameters (such as polar surface area, polarizability, various measures of lipophilicity, molecular volume, which help to predict carrier-mediated transport) is not accessible to all sectors, especially to academia. Commercial software is available to calculate those features and to estimate pharmacodynamics properties; however, the authors claim that such software is expensive and typically available only to workers in the pharmaceutical sector (Basore et al, 2015). Thus, collaboration between industry and academia, together with organizations such as MMV is of key importance to get results of higher impact and to obtain information that we can transfer to society.

In addition, civil society is another important stakeholder to take into account in the resolution of a complex problem. If any epigenetic-regulated mechanism of drug resistance development was proved, we should then not underestimate the influence of the human factor, which is one of the causes for the development of drug resistance (Fig. 14). Lack of treatment adherence, fake or defective drugs and treating non-infected patients can trigger the selection of resistant parasites, meaning an impediment for malaria elimination (WHO, 2010). An epigenetic-related mechanisms would undergo a greater impact, for its rapidity to evolve, since the selection of resistant parasites happen even in a single individual (as in case of the selection of clag3.2-expressing parasites in human blood, for instance). Thus I would like to stress the importance of a correct treatment to
contain drug resistance. Educational campaigns for highlighting the importance of getting diagnosed and completing the treatment, both to citizens and to health-workers, should be strengthened as a tool for malaria control and elimination.

In conclusion, when trying to fight a complex problem, such as drug resistance, many stakeholders should be involved. It is important to fill the gap between the lab and the field, where we find not only patients and physicians, but also important stakeholders such us the industry, NGOs, academia, policy makers and the overall society (Fig. 20).

**Figure 6. Representation of the trandisciplinary approach to the complex problem of anti-malarial drug resistance.** Blue boxes represent scientific disciplines and red boxes represent different sectors of the society. Transparent-coloured boxes indicate the disciplines and social sectors that are still pendant to be included.