INTRODUCTION

1. MALARIA GENERAL CONCEPTS

1.1 DEFINITION AND EPIDEMIOLOGY.

Malaria is a global disease that affects half of the world population. It is caused by protozoan parasites of the genus *Plasmodium* that are transmitted from human to human by the bite of female *Anopheles* mosquitoes. During the human infection parasites invade erythrocytes, causing symptoms that vary from mild to severe disease. In non-immune individuals, if not properly treated, malaria can cause death. There are five *Plasmodium* species that infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Among them, malaria due to *P. falciparum*, predominant in the African continent, is the most deadly form, while *P. vivax* is the most widespread species outside the African continent (Fig. 1). This thesis focuses on *P. falciparum* and thus, most of the information provided in the introduction refers to this species.

During the last 15 years the incidence and mortality of malaria has considerably decreased, thanks to control and elimination campaigns. However, there are still 91 countries with ongoing malaria transmission. Half of the world population is at risk of being infected by the parasite. The disease is spread around the tropical regions worldwide and in surrounding areas, such as the eastern Mediterranean region. *P. falciparum* is only found in tropical areas because its gametocytes require 10–18 days at a temperature of >21°C to mate and mature into infectious sporozoites inside the vector (Aly et al, 2009). This development timeline is only possible in hot, tropical conditions. *P. vivax* and *P. ovale* can develop in mosquitoes at ambient temperatures as low as 16 °C. The ability of these parasites to propagate at subtropical temperatures and to remain as quiescent forms at the hypnozoite state in the liver are likely to contribute to their ability to survive cold seasons, and the broader global distribution of these parasites (Gething et al, 2011). In 2015, there were an estimated 212 million cases of malaria and 429 000 deaths (WHO, 2016b). Approximately 80% of cases and 90% of deaths occurred in the African Region, being children under five years of age and pregnant women the population at higher risk. The intensity of transmission depends on diverse factors related to the parasite, the vector, the human host, and the environment. In areas with marked seasonality (rainy and dry seasons), malaria transmission presents a peak during and just after the rainy season. All these features make malaria complicated to control. Furthermore, malaria is strongly associated with poverty, with the highest mortality rates observed in countries with a
low Gross National Income (GNI) per capita. Therefore, malaria is a social and economic problem, which complicates even more control and elimination programmes.

**Figure 1. Map of malaria-endemic areas.** Blue areas represent countries where malaria was endemic in 2015, while yellow areas represent countries where malaria was endemic by 2000 but was eliminated by 2015. Continuous lines denote *P. falciparum* prevalence and discontinuous lines *P. vivax* prevalence. Countries coded ‘not applicable’ in the figure were not separately surveyed. The figure is based on data from the World malaria report 2015. Adapted from Philips et al., 2017.

**1.2 MALARIA LIFE CYCLE.**

Malaria parasites are transmitted from human to human by the bite of infected female mosquitoes of more than 30 different anopheline species. Alternative routes of transmission, although of less significance, are transfusion of infected blood and vertical transmission from infected mother to foetus, *i.e.* congenital malaria.

The *P. falciparum* life cycle is divided in two phases: asexual phase, which takes place in the human host, and the sexual phase, that starts in the human host and is completed in the mosquito (Fig. 2A). The asexual part of the cycle starts at the sporozoite stage. Sporozoites are injected through the mosquito bite into the human blood. These forms reach the liver, where they enter hepatocytes
starting a first exoerythrocytic multiplication phase. The hepatocytes burst after 6-7 days releasing thousands of merozoites into the blood circulation. In the case of *P. vivax* and *P. ovale*, some parasites stay in the liver in a dormant phase as hypnozoites, which will restart a new blood infection weeks to months after the primary infection. The newly formed merozoites will invade red blood cells (RBC) in the peripheral blood, starting then a second phase of asexual multiplication, *i.e.* the erythrocytic cycle. *P. vivax* invades young forms of RBCs, reticulocytes, while *P. falciparum* can invade RBCs of all ages. The first form of the parasite after invasion is known as ring, for its morphology, and lasts around 20h. Later, the parasite will evolve to the trophozoite stage, characterized by the accumulation of hemozoin pigment, which remains encapsulated within the food vacuole. In the last maturation step, parasites multiply their DNA content and evolve to multinucleated schizonts, forming up to 30 independent merozoites. Afterwards, the RBC bursts releasing new merozoites into the blood circulation, which will invade new RBC (Fig. 2B). The process of invasion is complex, with several interactions of host RBC receptors with parasite ligands (Cowman et al, 2016; Weiss et al, 2016). It starts with initial contact, followed by merozoite reorientation and apical attachment that leads to the formation of a moving junction that progresses until the merozoite is internalized (Weiss et al, 2015; Wright & Rayner, 2014). During invasion, components released from the rhoptries – organelles placed at the apical end of the merozoite – are secreted into the RBC and participate in the formation of the new parasite. This asexual cycle is continuously repeated in the human blood stream; in *P. falciparum*, *P. vivax* and *P. ovale* it lasts 48h, whereas in *P. malariae* parasites it lasts 72h and in *P. knowlesi* only 24h.

During the erythrocytic cycle some parasites differentiate into male and female gametocytes (transmission stages of the parasite), starting the sexual phase. Mature gametocytes from the blood circulation can be ingested by a mosquito during a new blood-feed. Within the mosquito, the sexual cycle will progress with the formation and fusion of a male and a female gamete and the subsequent zygote (2n). This is the only moment when sexual recombination takes place, allowing the exchange of genetic material and thus, the generation of genetic diversity in *Plasmodium* populations. Still in the midgut, the zygote differentiates into a motile ookinete that moves through the midgut epithelium of the mosquito host and forms an oocyst. The oocysts will generate thousands of sporozoites that will invade the salivary glands and be released into the human bloodstream during another bite. This way, the parasite is transmitted from one individual to another starting a new cycle (Fig. 2).
**Figure 2. Malaria life cycle.** (A) The *Plasmodium* spp. life cycle. The mosquito transmits the parasite in the sporozoite stage to the human host during a blood feed. Sporozoites invade liver cells, where they replicate and transform into merozoites, which will be released into the bloodstream, where they invade RBC and begin the asexual intraerythrocytic cycle. *P. vivax* and *P. ovale* can also enter a dormant state in the liver, the hypnozoite. Merozoites released from RBCs invade other RBCs and can continue to replicate. Some parasites differentiate into male or female gametocytes, which will be taken up by the mosquito vector in another blood meal. In the gut of the mosquito, the sexual part of the cycle continues. The new formed male and female gametocytes fuse, forming a diploid zygote, which elongates into an ookinete. This will transform into an oocyst, which undergoes cycles of replication and forms sporozoites. The new formed sporozoites travel to the salivary glands of the mosquito, ready to infect a new human host during the next blood-feed. Adapted from Phillips et al., 2017 (B) Thin blood films showing *Plasmodium falciparum* at different stages of blood-stage development. Ring stages are observed until 20 h post invasion. Trophozoites are present until 34h post-invasion and later schizonts develop up to 48h after invasion, when merozoites are released. Mature differentiated male and female gametocytes are represented in the second line. Adapted from Phillips et al., 2017.
1.3 CLINICAL MANIFESTATIONS OF MALARIA.

Symptomatology and severity of malaria vary according to the level of immunity of the infected individual. Immunity develops over repetitive exposure and is therefore dependent on age and exposure. Even if immunity rarely provides complete protection, it does reduce the risk of developing severe disease by regulating parasite density and modulating the virulence of the infection. Therefore, individuals with acquired immunity are more prone to carry low density and asymptomatic infections due to repetitive exposure to the parasite and to develop tolerance to the pathophysiological processes of the disease. In the African continent, due to the high transmission, many people are continuously exposed to the parasite. Protection against severe malaria is then developed by five years of age and most malaria deaths in this region occur in children below this age. In contrast, in areas with less transmission and low immunity, all age groups are at risk of developing severe malaria. This protection is generally thought to disappear within a few months or years of non-exposure, meaning a risk for migrants returning to endemic areas, although this has never been definitely established (Colbourne, 1955; Maegraith, 1989; Targett, 1984; Taylor & Strickland, 2000). While Jennings and colleagues (Jennings et al, 2006) found that severe disease was as common among patients who were assumed to present protective immunity (due to previous exposure) as among malaria-naïve patients, Bouchaud et al. (Bouchaud et al, 2005) defended that African migrants taking up long-term residence in Europe showed less susceptibility to falciparum malaria than European natives.

1.3.1 ASYMPTOMATIC MALARIA.

As mentioned above, repetitive exposure to the parasite leads to development of partial immunity. The immune system of these individuals maintains parasites at equilibrium levels, avoiding the development of severe malaria (Doolan et al, 2009). However, it has been observed that malaria caused by P. falciparum can evolve to a chronic infection, which can persist for months (Bousema et al, 2014). Recently, it has been argued that persistent infections with malaria parasites are frequently harmful to the individual, with serious health, developmental, and productivity consequences (Chen et al, 2016). Even if this kind of patients are more prone to carry low density parasitaemia, levels as high as 50,000 parasites per microliter have been reported in a study of asymptomatic pregnant women (range: 80–55,400 parasites per microlitre) (Phiri et al, 2016). This way, asymptomatic patients represent an important reservoir for infecting mosquitos, leading to continued transmission of the disease (even during the low transmission season). Thus, it is key to find and to treat asymptomatic carriers: on the one hand these patients require treatment for their
health status (“asymptomatic” malaria can result in chronic, low-grade haemolysis as well as intermittent, higher density symptomatic recurrences (Douglas et al, 2013)); on the other hand, it is a public health strategy, as it contributes to the elimination the human reservoir and reduces transmission.

1.3.2 Uncomplicated malaria.

The symptomatic phase of a malaria infection occurs only during the erythrocytic cycle. The incubation period starts from the moment the parasite is inoculated into the human host by the bite of a mosquito until parasite densities reach the fever threshold, which varies between 10-15 days depending on the level of immunity of the host. First symptoms, such as fever, headaches, chills and vomiting, might be mild and not disease-specific, making the clinical diagnosis less specific. Fever is often high, spiking up to 40º C in children and non-immune individuals (White et al, 2008), and it occurs at periodic intervals (48h), coinciding with the rupture of the RBCs. Fever can also be accompanied by other symptoms, such as abdominal pain, diarrhoea, back pain and myalgia. If not treated, the infection can evolve into severe malaria, especially in non-immune individuals. Pathophysiology processes comprise on one hand rupture of RBCs, which causes liberation of toxic compounds, such as nitric oxide (NO), responsible for fever and other symptoms. On the other hand, sequestration and cytoadherence of infected RBCs contribute to the most severe forms of malaria.

1.3.3 Severe malaria.

Severe malaria is defined as presence of asexual parasite forms in the bloodstream accompanied by one or more of the following features: impaired consciousness, prostration, generalized weakness, multiple convulsions, shock, pulmonary oedema, significant bleeding, hematemesis or melena, severe anaemia, jaundice, renal impairment, acidosis, hypoglycaemia and hyperparasitaemia (>10%) (WHO, 2015).

Untreated severe malaria is fatal in the majority of cases, but provided the appropriate treatment, mortality decreases to 12–20% (Sharma & Dutta, 2011). One of the most fatal forms of severe disease is cerebral malaria, i.e. coma with presence of P. falciparum parasites, and lack of other causes of coma. It is caused by the sequestration of the parasites in the cerebral vascular system, impairing blood circulation and generating a systemic inflammatory response, among other symptoms (Clark et al, 1995; Clark et al, 1993; White et al, 1985). The sequestration of parasites is caused by two pathophysiologic processes known as cytoadherence and rosetting, in which parasites adhere to either the endothelium or to non-infected erythrocytes, respectively.
(MacPherson et al, 1985; Maubert et al, 1997; Rowe et al, 1995). These processes will be further described in the next sections.

The factors that define the risk of severity of malaria have been reviewed by different authors (Crawley et al, 2010; Phillips et al, 2009; WHO, 2015), and can be summarize as follows:

1. *Plasmodium* species (*P. falciparum* presents the highest risk of severe malaria followed by *P. vivax* and *P. knowlesi*).

2. Genetic constitution and ethnicity. Some genetic factors are involved in susceptibility. For instance, sickle cell trait (haemoglobin S (HbS), haemoglobin C (HbC)) and ovalocytosis protect against severe malaria. This is due to selection of polymorphisms that malaria has exerted on the evolution of the human genome due to the high incidence (Lopez et al, 2010; Piel, 2016).

3. Malaria specific immunity. As explained above, in areas of stable and high malaria transmission, immunity is acquired by continued exposure to infections. In areas of fluctuant malaria transmission, both adults and children are at risk to develop severe malaria.

4. Immunocompromised patients. Malaria is a poverty-related disease. In areas where malaria is endemic, population, especially children, suffer of other pathologies, such as bacterial infections (pneumonia, meningitis, etc.) or intestinal parasites. These conditions compromise the immune system of the individual, making them more prone to develop severe disease. A circumstance of considerably higher risk is HIV infection. It worsens the prognostic of malaria, increasing the risk of severe malaria and death. At the same time, malaria may result in the worsening of clinical AIDS (Bentwich et al, 2000).

1.3.4 Malaria during pregnancy.

Malaria in pregnancy presents a specific pathophysiology, generating a complicated prognosis. Parasites get sequestrated in the placenta, blocking foetal blood circulation (Dorman et al, 2002; Suguitan et al, 2003). Women living in high transmission areas are partially protected against malaria disease, but when they are pregnant they are at a level of risk similar to non-immune individuals, since antigens presented during pregnancy are different than in the common forms of the disease (Goel et al, 2010). Partially-protective immunity against placental parasites is acquired during successive pregnancies. For this reason, pregnant women expecting their first child are the most vulnerable ones.
Malaria in pregnancy has adverse effects both in the mother and in the foetus (Desai et al, 2007). It often leads to abortions, still births, preterm births, intrauterine growth retardation and neonatal deaths. Besides, the infection can also be transmitted prenatal or perinatally to the foetus, resulting in congenital malaria. Surviving neonates that develop the disease become symptomatic after 10–30 days of life with fever, lethargy, poor feeding, pallor, jaundice and hepatosplenomegaly (John, 2016). However, congenital malaria is in most cases an asymptomatic infection and neonates clear the infection without developing symptoms.

1.4 MALARIA CONTROL AND ELIMINATION CONTEXT.

Malaria is a preventable and treatable disease, provided the currently recommended interventions are properly implemented. As the 20th century began, large areas of Europe and North America were still affected by malaria (Carter & Mendis, 2002). Regional malaria elimination campaigns were first conducted in the late 1940s. In 1955 the World Health Organization (WHO) launched the Global Malaria Eradication programme and by the early 1960s malaria was eliminated from Europe, North America, the Caribbean and parts of Asia and South-Central America (Carter & Mendis, 2002). However, elimination was not achieved in other regions, notably in Africa, and the malaria elimination goal was abandoned in 1972, resulting in a rebound of the intensity of malaria transmission in those areas. After few decades, from 1990s, malaria control was again implemented, with active campaigns such as The Roll Back Malaria initiative (1998) and the Millennium Development Goals (MDGs) (2000). It was in 2007 when the goal of malaria elimination was readopted with the Malaria Eradication Research Agenda (malERA)(Alonso & Tanner, 2013). Thanks to these and other initiatives, in the last 20 years, malaria incidence and mortality have decreased by 37% and 58%, respectively. In 2015, the United Nations established the Agenda for Sustainable Development, which includes the goal to eliminate malaria epidemics (UN, 2015).

Despite the effort and the promising results achieved in the last decades, malaria continues to be an important health problem for half of the world population (Fig. 1). In areas of high transmission, control interventions are focussed on reducing mortality and morbidity. Regions that have already reduced the burden of the disease are moving into (pre)elimination strategies, with the main aim of reducing and ultimately blocking transmission by the detection and treatment of the human reservoir (including asymptomatic individuals). Countries with three consecutive years of zero indigenous cases are considered to have eliminated malaria (WHO, 2017b).
Malaria control and elimination strategies can be divided into three categories:

a. **Vector control: environmental strategies.** These strategies include larval control, open field spraying and marsh draining. While these actions have been very effective in European settings, they are appropriate and advisable only in a minority of the current malaria scenarios, where mosquito breeding sites are few, fixed, and easy to identify, map and treat (Karunamoorthy, 2011).

b. **Human–vector contact control.** These strategies aim to reduce the physical contact between the mosquito and the human host by using mosquito nets, repellents and indoor residual spraying. These tools are simpler to use and more effective than the environmental strategies; however they can be difficult to implement among the population. The long-lasting insecticidal nets are among the most effective strategies to control malaria nowadays: they have a 3 year recommended use and reduce not only the contact but also the burden of female mosquitoes (Chanda et al, 2014). This was one of the main tools used in recent elimination campaigns (Bhatt et al, 2015).

c. **Diagnoses and treatment.** Prompt diagnosis and treatment is the main strategy for malaria control with the aim to reduce mortality and morbidity. In a malaria control context, intermittent preventive treatment (IPT) is also recommended in high risk populations, such as infants (IPTi) and pregnant women (IPTp) (WHO, 2012; WHO, 2015). Both malaria diagnosis and treatment will be discussed in more detail in the following sections. In elimination contexts, the main objective is to detect all parasite infections (human reservoir), regardless of symptoms, and to treat them in order to eliminate residual transmission. Thus strategies based in the active detection of infected individuals or distribution of drugs to a target population regardless of infection status (mass drug administration (MDA) in order to cure asymptomatic infections and to prevent re-infection during the post-treatment period are the focus in elimination (WHO, 2017b).

### 1.5 Malaria diagnosis.

Since the implementation of artemisinin combination therapies (ACTs) to combat drug resistance in 2003, WHO recommends diagnosis based on parasitological confirmation before treatment. Treating suspected cases on the basis of symptoms without parasitological confirmation contributes to the appearance of drug resistance, one of the main current threats for malaria elimination.
Furthermore, identifying the *Plasmodium* species contributes to a more specific treatment (e.g. primaquine against *P. vivax* or *P. ovale* hypnozoites), and allows to more accurately monitor the burden of each species. For these reasons, the interest on improving malaria diagnosis tools has raised in recent years.

Available diagnostic tools can be classified as:

1. **Light microscopy** (LM). It consists of the examination of a blood smear and the visualization of the parasite in the RBC. It is considered the standard diagnosis tool, although disadvantages include low sensitivity to detect parasite densities lower than 100 parasites/µL and the need of an expert microscopist for accurate diagnosis (Wongsrichanalai et al, 2007).

2. **Rapid diagnostic tests** (RDT). These tests are based in the detection of parasite antigens or antibodies against the parasite in peripheral blood. They are easy to use and relatively cheap to implement. The disadvantage of these tools is again their limit of detection (around 100 parasites/µL), not high enough to detect the majority of asymptomatic infections where low parasite densities are common (Bell et al, 2006). Furthermore, these tools are not able to distinguish past from current infections.

3. **Nucleic acid tests** (NAT). Also known as molecular tools, NATs detect parasite DNA and RNA in peripheral blood, being the most sensitive method of diagnostic able to detect parasitaemias of ≤1 parasites/µL. However, molecular techniques are still expensive and require technological facilities and highly trained staff, which is not common in malaria endemic areas (Schneider et al, 2005).

In elimination contexts, the detection of all infections is crucial as the proportion of asymptomatic infections (with low parasite densities) increases. Therefore, highly sensitive diagnostic tools, such as molecular methods, are needed to detect the human reservoir. It has already been described that an important proportion of all asymptomatic infections carry gametocytes and contribute to infectiousness for long periods of time (since these individuals do not seek treatment), thus sustaining malaria transmission (Alves et al, 2002; Lindblade et al, 2013) (Fig. 3). It has been shown that *P. falciparum* submicroscopic infections can account for up to 30% of mosquito transmissions in countries like Burkina Faso (Churcher et al, 2013; Ouedraogo et al, 2009; Ouedraogo et al, 2016), while a recent study showed median gametocyte densities similar to those in symptomatic individuals in Peru (Rovira-Vallbona et al, 2017). Furthermore, a third of all gametocytemic patients of the latter study were asymptomatic (human reservoir of infection).

Implementation of molecular tools is difficult or not be feasible in highly remote settings, and thus, new affordable and easy to perform tools are needed in these areas. Currently, loop-mediated
isothermal amplification (LAMP) technology provides the most efficient tool in terms of sensitivity-manageability balance. It provides sensitivity comparable to other NATs, such as quantitative-PCR (qPCR), being at the same time much easier to use (Serra-Casas et al, 2017). Furthermore, a high-sensitivity histidine-rich protein 2 (PfHRP2)-based RDT is currently in the field-testing phase (Das et al, 2017). This is a promising tool for malaria elimination strategies as it can detect submicroscopic parasitaemias. The test has shown so far a sensitivity of 84% in comparison to 62% for other RDTs, using qPCR as the reference standard method, and it was able to detect over 50% of specimens with parasite densities between 0.1 and 1 p/µL.

Figure 3. Limit of detection of the currently used diagnostic methods. LM and RDT can detect parasitaemias of ≥100 parasites/µL approximately. An expert microscopist could expand this limit to 5-10 parasites/µL. NATs can detect up to 0.005 parasites/µL in concentrated blood samples. Adapted from Zimmerman 2015.
2. MALARIA TREATMENT AND DRUG RESISTANCE

2.1 MALARIA TREATMENT.

Malaria treatment changes depending on severity, parasite species, region to be used and purpose of management. Treatment against non-complicated malaria typically consists of a three-day oral administration regime (which can vary according to the treatment administered), ideally starting within the first 24h after the appearance of the first symptoms (normally fever) for a good prognosis. The main aim of the therapy is to ensure the clearance of parasites from the blood of the patient in order to prevent complications that might lead to long term physical damage or even to death. At the same time, rapid treatment of malaria also functions as a public health strategy: it stops the generation of new gametocytes and thus reduces transmission of the parasite, being itself a control/elimination strategy.

During the last century, different drugs have been used against malaria (Table 1). These drugs present different mechanisms of action and some of them act at different points of the parasite life cycle; most of them act on the asexual form of schizonts, but other drugs have their target in different stages, such as primaquine, which kills hypnozoites (formed by *P. vivax* and *P. ovale*) and gametocytes. Since the implementation of artemisinin (ART) based therapies in 2001, WHO recommends the administration of drug combination treatments, using partner drugs with different mechanism of action and half-live in order to combat the development of drug resistance. Drug combinations consists of a fast-acting (ART derivative) and a long-acting drug (WHO, 2015) (Table 2).

Treatment strategies include:

- **Treatment of uncomplicated malaria.** Every country has its own treatment policies depending on the species prevalence and the context of drug resistance. The main recommended regimes by the WHO are artemisinin-based combination therapies (ACTs). There are currently 5 ACTs recommended against *P. falciparum* malaria. The choice should be based on the results of therapeutic efficacy studies against local strains of *P. falciparum* (Table 2). In the case of pregnant women, the antimalarial medicines considered safe in the first trimester of pregnancy are quinine, chloroquine (CQ), clindamycin and proguanil.

- **Treatment of severe malaria.** Injectable or rectal artesunate is administrated for at least 24h until the patient can tolerate oral administration, which consists of the normal ACT regime. In severe cases, hospitalization and palliative treatment is regularly required.
**Table 1. List of drugs used for malaria treatment.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Family</th>
<th>Mechanism of action* / Specific Target**</th>
<th>Introduced</th>
<th>First reported resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine</td>
<td>Aryl-amino alcohol</td>
<td>1** Pregnant women therapy.</td>
<td>1632 (cinchona) 1820 (quinine)</td>
<td>1908 (Neiva, 1910) 1910 (Nocht &amp; Werner, 1910)</td>
</tr>
<tr>
<td><strong>Sulfadoxine (SDX)</strong></td>
<td>Sulfonamide</td>
<td>2</td>
<td>1937</td>
<td>1970s (in association with PYR) (Peters, 1987)</td>
</tr>
<tr>
<td>Pyrimethamine (PYR)</td>
<td>Diaminopyrimidine derivative</td>
<td>2</td>
<td>1952</td>
<td>1959 (Burgess &amp; Young, 1959) 1970s (in association with SDX)</td>
</tr>
<tr>
<td>Proguanil</td>
<td>Biguanide</td>
<td>2</td>
<td>1940s</td>
<td>1949 (Peters, 1987)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4-Aminoquinoline</td>
<td>1 &amp; 2</td>
<td>1945</td>
<td>1957 (Sa et al, 2009)</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>4-Aminoquinoline</td>
<td>1</td>
<td>1945</td>
<td>1970s (Peters, 1987)</td>
</tr>
<tr>
<td>Primaquine</td>
<td>8-Aminoquinoline</td>
<td>3 &amp; 4 <strong>Gametocytes</strong></td>
<td>1950</td>
<td>NA</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>Bis-4-aminoquinolin</td>
<td>1 &amp; 2</td>
<td>1960s</td>
<td>1970s (Nosten &amp; White, 2007)</td>
</tr>
<tr>
<td>Artemisinin derivatives</td>
<td>Sesquiterpene lactone endoperoxid</td>
<td>5 <strong>Active against young gametocytes</strong></td>
<td>1980s</td>
<td>2008 (Dondorp et al, 2009)</td>
</tr>
<tr>
<td>Lumezantrine</td>
<td>Aryl-amino alcohol</td>
<td>1 &amp; 6</td>
<td>2000s (ACT)</td>
<td>NA (Blasco et al, 2017)</td>
</tr>
<tr>
<td><strong>Clindamycin</strong></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mechanism of action.*

1. Inhibition of intraparasitic heme detoxification in the parasite’s digestive vacuole.
2. Disruption of the replication of the parasite, by disrupting the synthesis of essential products.
3. Disruption of the metabolic processes of the mitochondria
4. Production of highly reactive metabolites generating toxic intracellular oxidative potentials
5. Active by involving cation-mediated generation of reactive intermediates and reduction of the peroxide bridge.
6. Inhibition of endocytosis of the cytosol by the parasite.
Table 1. Artemisinin-based combination therapies deployed for clinical use. (Adapted from Blasco et al., 2017)

<table>
<thead>
<tr>
<th>Artemisinin derivative</th>
<th>Non-artemisinin compound</th>
<th>Abbreviation</th>
<th>Brand name</th>
<th>Geographic use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemeter</td>
<td>Lumefantrine</td>
<td>AL</td>
<td>Riamet / Coartem</td>
<td>Most widely used ACT in Africa</td>
</tr>
<tr>
<td>Artesunate</td>
<td>Amodiaquine</td>
<td>ASAQ</td>
<td>Winthrop</td>
<td>Used mostly in western Africa</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>Piperaquine</td>
<td>DHA–PPQ</td>
<td>Artekin</td>
<td>First-line in several Southeast Asian countries</td>
</tr>
<tr>
<td>Artesunate</td>
<td>Mefloquine</td>
<td>ASMQ</td>
<td>Artequin</td>
<td>Preceded DHA-PPQ in Southeast Asia</td>
</tr>
<tr>
<td>Artesunate</td>
<td>Sulfadoxine Pyrimethamine</td>
<td>ASSP</td>
<td>NA</td>
<td>Used in India and some Eastern African countries</td>
</tr>
<tr>
<td>Artesunate</td>
<td>Pyronaridine</td>
<td>PA</td>
<td>Pyramax</td>
<td>Currently being registered in both Africa and Asia</td>
</tr>
</tbody>
</table>

- **Treatment against gametocyte stages to reduce transmission.** In low transmission areas, a single dose of 0.25 mg/kg of primaquine is administered (in addition to the normal ACT treatment) to patients with *P. falciparum* to reduce gametocytes carriage. Pregnant women, infants aged <6 months and breastfeeding women are excluded from this intervention. Although glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals are at risk of haemolysis when exposed to primaquine, studies have shown that the dose used for gametocyte elimination is safe and no G6PD-deficiency test is needed (Recht et al, 2014; WHO, 2015).

- **Intermittent Preventive Treatment.** In a malaria control context, intermittent treatment regimens are followed in different risk populations (WHO, 2017c).
  - **Pregnant women (IPTp):** intermittent preventive treatment with sulfadoxine-pyrimethamine (IPT-SP) is given to all pregnant women in regions with moderate to high malaria transmission in Africa and some other areas such as Papua New Guinea. The combination SP should be provided as early as possible in the second trimester, each dose given at least one month apart, with a minimum of 3 doses during each pregnancy. This strategy is followed even in areas where resistance to SP has been reported.
- **Children (IPTi):** in areas of moderate-to-high malaria transmission in sub-Saharan Africa infants below 12 months of age receive a complete treatment of SP (regardless of symptoms or infection status) usually at the time of vaccinations. WHO recommends to follow this regime in areas that have less than 50% prevalence of pfdhps 540 mutation in *P. falciparum*, responsible for resistance to sulfadoxine.

- **Seasonal malaria chemoprevention (SMC):** School children in areas with highly seasonal malaria transmission receive once a month, during the transmission season, a full course of an antimalarial treatment. The objective is to prevent malaria by maintaining therapeutic drug concentrations in the blood throughout the period of greatest malarial risk. WHO recommends IPT with SP + amodiaquine in the Sahel sub-region of sub-Saharan Africa, where *P. falciparum* is sensitive to both antimalarial medicines.

- **Asymptomatic patients.** In an elimination context, active campaigns to detect asymptomatic infections are conducted in order to detect and treat all infections. All individuals found positive for *Plasmodium* are treated usually with ACTs (according to the policy of each country) in order to prevent the formation of gametocytes and maintenance of malaria transmission.

- **Prophylaxis in travellers and non-immune returning migrants.** Depending on the malaria risk in the area to be visited, international travellers may also need to take preventive medication (chemoprophylaxis) prior to, during, and upon return from their travel. This is also recommended for migrants that have been away from malaria endemic areas for longer than a year, who might have lost their immunity. The recommended drugs for chemoprophylaxis are atovaquone/proguanil, doxycycline (DOXY) and mefloquine.

### 2.2 Drug Resistance.

Drug resistance is defined as the ability of a parasite strain to survive and/or to multiply despite the good absorption of a medicine given in doses equal to or higher than those usually recommended (WHO, 2016a). Despite the progress achieved in the last decades in reducing the burden of malaria, a looming threat to malaria control is the emergence of parasites that are resistant to currently used antimalarial medicines. To date drug resistance has been
described for almost all drugs that are in use or that were used in the past to fight malaria (Table 1 and Fig. 4).

The earliest anectodical reports of resistance to an antimalarial agent are those for quinine in 1844 (Elliotson, 1844). A century later, resistance to proguanil was observed just one year after its introduction in 1947 in peninsular Malaya (Peters, 1987), thus it stopped being administered. However, drug resistance started to be considered as a real threat with the appearance of resistance to CQ in the late 1950s (Fig. 4 and Table 1), which started independently in the Greater Mekong sub-region and in South America (Sa et al, 2009). CQ-resistant *P. falciparum* parasites entered Africa in the late 1970s and began to spread rapidly, causing a significant increase in morbidity and mortality (Trape et al, 1998). SP was then implemented as alternative treatment (1967), but the first parasites resistant to this regime were reported the same year (Peters, 1987; Wernsdorfer & Payne, 1991). Similarly, other antimalarials developed resistance rapidly:resistance to mefloquine was first reported in 1982, five years after it had been introduced (Nosten et al, 1991), whereas resistance to atovaquone developed in 1996, the same year the drug was introduced (Looareesuwan et al, 1996).

To combat resistant parasites, ART derivatives were developed and first used in Vietnam early 1990s (Fig. 4). This action implied a pronounced decrease in malaria mortality rates in the region (Schuften, 2000). Nowadays, as mentioned above, ACTs are the first line treatment against uncomplicated malaria, as recommended by WHO (WHO, 2016b). However, in 2008, the first reports of delayed parasite clearance for the ART derivatives came from Cambodia (Dondorp et al, 2009; WHO, 2010) (Fig. 4). Since then, delayed parasite clearance for ART derivatives has been reported in five countries of the Greater Mekong subregion: Cambodia, Lao People’s Democratic Republic, Myanmar, Thailand and Vietnam. Several studies have confirmed that this phenomenon has emerged independently in different locations (Anderson et al, 2017; Imwong et al, 2017; MalariaGEN, 2016; Menard et al, 2016; Miotto et al, 2015; Phyo et al, 2016; Takala-Harrison et al, 2015). Since resistance to CQ and SP all first emerged in the Greater Mekong sub-region and then spread to Africa and other regions, the biggest fear is that the same pattern could be repeated with ART delayed clearance. Consequently, the Malaria Policy Advisory Committee (WHO) launched specific ART resistance containment plans in September 2014. One of the main points of these plans is the goal to eliminate *P. falciparum* malaria in this area by 2030, as a strategy to decrease the risk that resistant phenotypes reach Africa. However, ART resistance could also appear independently in Africa, as it happened in Greater Mekon region.
For many years there was controversy in declaring ART resistance. The parasite clearance rate can be quantified as the time required to reduce the parasite biomass by two-fold, which for sensitive parasites is typically 1–3 h. Parasites responsible for treatment failure to ART were showing clearance rates of >5h (White et al, 2015). Mathematical modelling predicted that ART resistance was the result of ring stages becoming refractory to drug action (Saralamba et al, 2011). This prediction was consistent with results from in vitro ‘ring-stage survival assays’ (RSA 0–3h), in which the authors observed that parasites at ring stage survived after a 6-h pulse exposure to dihydroartemisinin by cell cycle arrest and resumed growth upon drug withdrawal. Exposed mature stages were though susceptible to the drug (Witkowski et al, 2013). From 2013, single nucleotide polymorphisms (SNPs) in the PF3D7_1343700 kelch propeller domain (K13) were identified as ART resistant markers, (Ariey et al, 2014; Witkowski et al, 2013). However, most of the studies investigating delayed parasite clearance after ACTs treatment in Africa did not find association with K13 mutations (Ashley et al, 2014; Menard & Dondorp, 2017; Menard et al, 2016; Taylor et al, 2015; WHO, 2001). In Africa non-synonymous K13 mutations are still rare, highly diverse and with no association with ART resistance. A recent report of a migrant worker with a P. falciparum K13-variant infection from western Africa with delayed parasite clearance following ACT treatment has again raised the alarm (Lu et al, 2017; MalariaGEN, 2016; Menard et al, 2016). Recently, two K13 mutations that have been related to ART resistance (R539T and P574L) have been described in China in patients coming from Angola and Equatorial Guinea, although these patients did not present treatment failure (Yang et al, 2017).

There are some hypotheses for the lack of impact of K13 mutations in Africa. First, the greater degree of acquired immunity might help control drug-resistant infections. Second, the incidence of polyclonal infections would select against drug-resistant parasites if they presented a fitness cost. Last, there is a great prevalence of asymptomatic infections that are not exposed to drug pressure. These reasons could also explain why almost every known drug-resistance event has started in the Asian region and not in Africa. Nevertheless, the potential spread of ART resistant parasites from Asia to Africa continues to be a concern, aggravated by the fact that mosquito-vector differences between Asia and Africa would not be an impediment for dissemination of mutated K13 alleles of Asian origin across Africa (St Laurent et al, 2015).

The rapid increase in the last years in the prevalence of mutant K13 strains in Asia has resulted in parasites being exposed to the ACT partner drugs almost as monotherapy agents. Subsequently to this increased selection pressure, there is a selection of parasites that present resistance to both drugs, threatening the aim of these combination therapies. It is
the case of resistance to the combination artesunate-mefloquine (WHO, 2014). Since 2008 this combination has been replaced by dihydroartemisinin-piperaquine (DHA-PPQ) in Cambodia. Nonetheless, resistance to the partner drug PPQ has now emerged and is spreading quickly in the country (Amaratunga et al, 2016; Duru et al, 2015). The partner drugs of other combinations, such as amodiaquine, have encountered only partial resistance in certain regions, and there is as yet little evidence of high-grade resistance to lumefantrine, probably because it has been used only in combination therapy. To date, no resistance has been reported to pyronaridine (Blasco et al, 2017).

Considering that drug resistance is the main threat to control and eliminate malaria, a better understanding of the molecular mechanisms underlying parasite resistance and discovery of new antimalarial drug candidates is of vital importance. The non-profit organization Medicines for Malaria Venture (MMV) and their collaborators play a key role in the achievement of this objective. Nowadays, they are working with a pipeline of 400 antimalarial drug candidates (Spangenberg et al, 2013).

Figure 4. History of the introduction of the principal antimalarials and of the first emergence of resistance in the field. Single bars refer to monotherapies; double- and triple-bars denote combination therapies. Colors refer to the chemical classes to which the antimalarials belong. Abbreviations indicate: ASSP: artesunate + sulfadoxine - pyrimethamine; AL: artemeter + lumefantrine; PA: artesunate + pyronaridine; DHA-PPQ: dihydroartemisinin + piperaquine; ASMQ: artesunate + mefloquine; ASAQ: artesunate + amodiaquine Adapted from Blasco et al., 2017.
2.3 MECHANISMS OF DRUG RESISTANCE.

To date, different molecular mechanisms have been described as responsible for treatment failure to several antimalarial compounds. SNPs or major genetic rearrangements allow the parasite to survive to the action of the drug. Drug resistance can develop through several mechanisms, including changes in drug permeability, transport or efflux, drug conversion to another form that becomes ineffective, increased expression of the drug target, or changes in the enzyme target that decrease the binding affinity of the inhibitor (Farooq & Mahajan, 2004). An important approach to fight drug resistance is to understand the molecular mechanisms responsible for it. Below I describe the main mechanisms described for antimalarial compounds that are or have been in use (Fig. 5).

Resistance to heme-binding agents.

Haemoglobin represents an important molecule for malaria parasites: on the one hand it is a source of amino acids for protein synthesis; on the other it constitutes a toxic burden because of the degradation products that are derived from its metabolism. Thus, multiple drugs used in first-line antimalarial treatments have haemoglobin catabolism as a primary target, such as CQ, amodiaquine, mefloquine and lumefantrine (LUM). Resistance to this kind of antimalarials, especially to CQ, has been reported in the past. Polymorphisms in the *P. falciparum* CQ resistance transporter (*pfcrt*) and the multidrug resistance transporter (*pfmdr1*) gene, which are membrane proteins from the digestive vacuole, are the main determinants of resistance to heme-binding drugs.
The massive use of CQ during the first decades of the past century selected for mutant \textit{pfcrt} alleles. Mutations in this gene (embracing from four to nine point mutations), mediate CQ resistance through active, H+-dependent drug efflux out of the digestive vacuole (Lehane et al, 2008; Petersen et al, 2015). Some \textit{pfcrt} variants also mediate resistance to the active metabolite of amodiaquine: monodesethyl-amodiaquine (Gabryszewski et al, 2016a; Sa et al, 2009). After CQ use was abandoned due to the resistance that parasites were presenting, the resistant genotype was reverted, indicating that \textit{pfcrt} mutations have a fitness cost for the parasite (Takala-Harrison & Laufer, 2015). This was confirmed in \textit{in vitro} experiments, where \textit{pfcrt}-mutant parasites showed reduced growth rates when compared to wild-type lines (Gabryszewski et al, 2016b; Lewis et al, 2014; Petersen et al, 2015).

The ABC transporter PfMDR1 is another key mediator of parasite susceptibility to multiple antimalarials (Veiga et al, 2016). \textit{pfmdr1} over-expression, increased copy number or sequence variants constitute a major determinant of parasite resistance to mefloquine and also reduce susceptibility to lumefantrine (Sidhu et al, 2006; Uhlemann et al, 2007). At the same time, distinct \textit{pfmdr1} haplotypes can modulate the efficacy of several antimalarials. For example, the N86Y mutation contributes to partial resistance to monodesethyl-amodiaquine and can augment the level of CQ resistance imparted by mutant PfCRT (Nawaz et al, 2009).

**Resistance to antifolates.**

Other drug resistance mechanisms imply changes in a metabolic route. Antifolates, such as sulfadoxine and pyrimethamine, interfere with folate synthesis. Pyrimethamine binds to dihydrofolate reductase (DHFR) enzyme and sulfadoxine to dihydropteroate synthase (DHPS) and, thereby, the two SP components inhibit the \textit{P. falciparum} life cycle by avoiding folate synthesis (Le Bras & Durand, 2003). However, acquisition of multiple SNPs at DHPS and DHFR prevent the binding and confer resistance to sulfadoxine and pyrimethamine, respectively (Anderson & Roper, 2005).

**Resistance to atovaquone–proguanil.**

Atovaquone–proguanil (Malarone) is a fixed-dose antimalarial combination that is prescribed as a prophylactic agent for travellers to malaria-endemic areas. Atovaquone–proguanil was also used recently as part of a plan to contain ART resistance in the Greater Mekong
subregion; however, its suitability as a second-line therapy has been debated because of the ease with which atovaquone resistance arises (Looareesuwan et al, 1996; Maude et al, 2014; Saunders et al, 2015).

Atovaquone inhibits the mitochondrial electron transport chain through inhibition of the malarial cytochrome bc1 complex (Birth et al, 2014). By this mechanism it prevents the synthesis of pyrimidines by the enzyme dihydroorotate dehydrogenase (DHODH). When proguanil is combined with atovaquone, it significantly increases the ability of the latter to collapse the mitochondrial membrane potential (Vaidya, 2012). However, none of these drugs are effective separately as antimalarials. A single point mutation in the cytochrome b subunit (CYTb) of the bc1 complex can confer resistance to atovaquone, which happens at high frequency. In addition, proguanil is metabolized into cycloguanil, which is a potent inhibitor of the DHFR enzyme in the folate pathway. Thus, parasites presenting either mutations at CYTb or at DHFR are not sensitive to the atovaquone–proguanil combination, since the action of both drugs is necessary for having a therapeutic effect (Krudsood et al, 2007; Kuhn et al, 2005; Vaidya, 2012)

**Resistance to ART.**

As mentioned above, different studies, from whole-genome sequence analysis of ART-resistant parasites to molecular epidemiological studies, have arrived to the conclusion that the K13 protein is a main mediator of ART resistance (Ariey et al, 2014; Straimer et al, 2015). ART resistance is associated with slow parasite clearance. The function of K13 and the mechanisms by which its mutations can protect ring stages still remain unclear.

Some hypothesis defend that ART-resistant rings have the capacity to regulate the cellular stress response to ART (Dogovski et al, 2015; Mok et al, 2015). Other hypotheses are based on the idea that the target of ART is *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K), which is the binding partner of wild type K13 (Mbengue et al, 2015). PI3K product is PI3-phosphate (PI3P), which is involved in membrane biogenesis and fusion events, both essential for parasite growth. Some authors have suggested that mutant K13 fails to interact with PfPI3K due to the action of ART, leading to increase its activity and producing high basal levels of PI3P. These high PI3P levels compensate for the action of ART, allowing the parasite to survive (Bhattacharjee et al, 2012).
Resistance to ACT partner drugs.

As previously mentioned, because of the increased prevalence of K13-mutant parasites with a delayed parasite clearance phenotype, parasites have been exposed to stronger selective pressure of partner drugs in ACT. As a result, parasites presenting multiple *pfmdr1* copies, a frequent mutation in Asian parasites that confers mefloquine resistance, showed resistance to the artemunate-mefloquine combination (Lim et al, 2009).

The artemunate-mefloquine combination was then replaced for DHA-PPQ in Cambodia. However, resistance to this combination has already emerged and is spreading quickly (Amaratunga et al, 2016; Duru et al, 2015; Saunders et al, 2014). Resistance to PPQ has been associated with amplification of the *PfPlasmepsin 2* and *3* genes in genome-wide association studies (Amato et al, 2017; Witkowski et al, 2017). Plasmepsin genes encode aspartic proteases that act as hemoglobinases. One hypothesis for the resistance mechanism is that plasmepsin amplification results in faster rates of Hb digestion (Witkowski et al, 2017), which counteract the ability of PPQ to inhibit Hb catabolism and heme detoxification (Duru et al, 2015). At the same time, *in vitro* evidence (Dhingra et al, 2017) and mutations observed in Cambodian PPQ-resistant isolates have suggested that PfCRT might act as a determinant of PPQ resistance as well.

Another important ACT is artemeter-lumefantrine. Even if *pfmdr1* increased copy number has been related to reduce susceptibility to lumefantrine in in-vitro and *ex vivo* experiments (Sidhu et al, 2006; Uhlemann et al, 2007), clinical resistance to lumefantrine has been reported only once and has not been confirmed (Plucinski et al, 2015), despite its massive clinical use.
3. Adaptation of parasites to the environment and epigenetic regulation

*P. falciparum* is an obligated intracellular parasite. The inside of the erythrocyte provides a relatively stable environment compared with the settings where free-living unicellular organisms reside. However, the parasite needs to adapt to fluctuant conditions among the different human hosts, such as nutrients concentration, presence of drugs, presence and magnitude of febrile episodes or the immune response (Mackinnon & Marsh, 2010). Malaria parasites, like the rest of the living organisms, have their own strategies to adapt to these environmental changes, among which epigenetic regulation has an important position.

### 3.1 Adaptation to changes in the environment.

In order to face changes in the environment, living organisms have different strategies to adapt. These strategies differ in the time required for adaptation, from direct response to years of accumulation of single mutations, and in their stability, varying from rapid reversibility to permanence through many generations. According to these features and their molecular basis they can be classified in three different categories, as described below:

a. **Genetic mutations.** Spontaneous mutations in the DNA typically occur during the replication of genetic material. Some of the new nucleotide combinations may confer a functional advantage, being selected and transmitted to the next generations. This way, organisms evolve and adapt to the environment. This mechanism is irreversible and slow, happening at a frequency of $10^{-8} - 10^{-9}$ per replication in unicellular organisms (Bopp et al, 2013; Lynch et al, 2008; Saxer et al, 2012; Sung et al, 2012). Generally, to confer a phenotypic advantage a series of independent mutations must happen and be selected. In *P. falciparum* a clear example of adaptation through mutations is the development of drug resistance. Spontaneous changes in the genome sequence lead to mechanisms capable of fighting the action of the drug, such as expulsing the drug from their place of action or changes in the enzyme target. For instance, mutations at *Pfcrt* confer to the parasite the ability to efflux CQ from the intracellular digestive vacuole, the site of drug action.

b. **Epigenetic variation.** Epigenetic changes are defined as inheritable differences between cells or organisms that have the same DNA sequence. Differing from genetic changes, this inheritable mechanism does not affect the primary DNA sequence, but the active or repressed
state of the genes. In *P. falciparum* epigenetic changes are determined at the chromatin level; histone modifications alter the accessibility of chromatin to be transcribed, defining the genes that will be expressed and the ones that will be silenced. This mechanism of adaptation is fast; transitions between the active and repressed states have been described to happen at a frequency of $10^{-2}$-$10^{-3}$ per replication (Grewal et al., 1998; Jablonka & Lamb, 1995; Thon & Friis, 1997; Zordan et al., 2006), and it is responsible for adaptations to fluctuating conditions.

In *P. falciparum*, the active or repressed state of the genes that participate in epigenetic variation switches stochastically at a low rate before any challenge occurs (Cortes et al., 2007; Roberts et al., 1993). Epigenetic variation in a population of genetically identical parasites imply translation of the same genome into alternative transcriptomes that result in diverse phenotypes. This diversity increases the plasticity of parasite populations and favours their survival and adaptation. These adaptive strategies, based on the stochastic generation of phenotypic diversity, are commonly referred to as **bet-hedging**. This way, among a population of genetically identical parasites, those presenting the best combination of active and repressed genes will survive in the conditions of a given environment. Selected parasites will transmit the same expression pattern to the next generation of the asexual cycle, functioning as a way of dynamic natural selection under fluctuating changes (Fig. 6). The fact that this survival approach is not related to DNA rearrangements makes it a rapid and reversible mechanism of adaptation. The most studied example of genes involved in bet-hedging are *var* and *clag3* genes, which will be further described in the next sections (Cortes & Deitsch, 2017).

**Figure 6. Schematic of adaptation via bet-hedging strategy.** Parasites of the same genetic background use their genes differently, displaying a variety of phenotypes. Big circles represent parasites presenting different phenotypic characteristics (distinguished by colours). This phenotypic condition is determined by the combination of active and repressed states of the genes (green arrows and red crosses at inner small circles, respectively). Parasites presenting the combination that confers the best fitness under a given environment will survive and therefore be selected (blue parasites in the example), transmitting their expression pattern to the next generation by epigenetic memory and adapting to the environment. Adapted from Rovira-Graells et al., 2012.
c. **Directed Transcriptional Response.** This mechanism consists of facing changes in the environment by an active transcriptional response. When organisms sense certain external conditions, a signalling cascade starts resulting in a change in the transcriptional state of specific genes that mediate adaptation to the new condition. Differing from the other two mechanisms, this one supposes a transient and thus non-heritable state. Even if this is a common mechanism in several organisms, including bacteria, yeast, and higher eukaryotes, evidence suggests that *P. falciparum* may have a limited capacity to generate directed transcriptional responses for rapid adaptation (Ganesan et al, 2008; Le Roch et al, 2008), although this remains controversial (Deitsch et al, 2007). Recently, a study identified a key parasite nutrient-sensing mechanism that is critical for modulating parasite replication (Mancio-Silva et al, 2017). The authors showed that *Plasmodium spp.* have an intrinsic capacity to respond to a nutrient-poor environment by reducing their multiplication rates, which appears to be regulated by KIN, a putative serine/threonine kinase. Additionally, another study linked the reduction of the host-derived lipid lysophosphatidylcholine (LysoPC), which drives the biosynthesis of an essential parasite-membrane component, with induction of the formation of gametocytes (Brancucci et al, 2017). These results provide the first clear examples of a direct transcriptional response in *P. falciparum*. Besides, unpublished results from our lab indicate that exposure of parasites to heat shock also induces a direct transcriptional response (Tintó-Font et al., unpublished).

### 3.2 **Epigenetic regulation in *P. falciparum.***

Apart from the already well-studied immune evasion process involving variably expressed genes regulated at the epigenetic level, such as var genes, recent findings have revealed an assorted range of roles for epigenetics in malaria parasite biology: lipid metabolism, protein folding, erythrocyte remodelling, or transcriptional regulation, among others. Epigenetic variation is involved not only in immune evasion but also in functional variation.

Epigenetic regulation in *P. falciparum* involves changes at the chromatin level, not affecting the primary DNA structure, and determining the active or repressed state of the genes. DNA is wrapped into nucleosomes in form of chromatin, which can be more or less condensed. When chromatin structure is in a condensed state (heterochromatin), it is less accessible to transcription complexes, silencing gene expression. On the contrary, in those regions where
genes are actively transcribed, chromatin is found in an “open” state (euchromatin). Nucleosomes are formed by protein subunits called histones, which undergo post-translational modifications that determine the chromatin state. The most common modifications are acetylation or methylation at the amino-terminal “tails” of histones H3 and H4 (Cortes & Deitsch, 2017).

3.2.1 Clonally variant expression.

There is a third type of chromatin, known as facultative heterochromatin or bistable chromatin, for which the expressed or silenced state varies among different cells within a population. Some regions of the genome can be found as either euchromatin or facultative heterochromatin, determining an active or repressed state of the genes, respectively. This state will be transmitted to the next generations, through a process known as epigenetic memory. Even if heritable, the state of the chromatin is not permanent. Silenced genes can revert to an active state and vice versa. This process determines clonally variant expression, a property that is present in some gene families. This property implies that individual parasites of the same genetic background and at the same stage of the life cycle use these genes differently. This way, parasites present different combinations of repressed or active genes among a population of identical parasites conferring them a variety of phenotypes.

The epigenetic regulation of different clonally variant genes shares certain characteristics. The histone mark H3K9me3 determines the silent state (Crowley et al, 2011; Howitt et al, 2009; Jiang et al, 2010; Kafsack et al, 2014; Lopez-Rubio et al, 2007; Lopez-Rubio et al, 2009). This mark is recognized by heterochromatin protein 1 (HP1) that begins the chromatin condensation process, preventing the transcription of the gene (Flueck et al, 2009; Perez-Toledo et al, 2009). Replacement of H3K9me3 by H3K9ac triggers a transcriptional switch, activating the expression of the gene (Crowley et al, 2011; Lopez-Rubio et al, 2007). The activating or silencing histone marks are maintained throughout the rest of the asexual cycle, even at points when the affected genes are normally inactive (Fig. 7).
Figure 7. Model for the regulation of variant expression and stage-specific expression of clonally variant genes. Green marks represent permissive histone modifications, such as H3K9ac, whereas red marks represent repressive histone modifications, such as H3K9me3. The permissive or repressive chromatin conformation is maintained along the full asexual cycle, even at points when the genes are normally inactive (in this illustrated example at ring/trophozoite stage). At the stage when the gene can be transcribed, a stage-specific transcription factor (TF) triggers the activation of the gene when permissive histone marks are present. Adapted from Crowley et al., 2011.

3.2.2 Mutually exclusive expression.

Some of the genes that present clonally variant expression belong to families that in addition present mutually exclusive expression. This property denotes that only one of the members of a multigenic family will be expressed at a time. The molecular mechanisms that allow the coordination among the members of the family presenting this property are not completely understood. Of note, for activation of one gene the simultaneous silencing of the previously active member of the family is required. To date only two families presenting mutually exclusive expression have been described in P. falciparum: var and clag3 genes. This property is related to bet-hedging in the case of immune evasion (var genes): by limiting the expression of genes of the same family at a time in a single parasite, parasites are more likely to escape the immune system, since they are not showing the whole possible repertoire.
3.3 Processes regulated at the epigenetic level in malaria parasites.

3.3.1 Antigenic variation and virulence.

The best known example of clonally variant expression is represented by the var family, formed by around 60 genes that encode the *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1). These proteins are displayed on the membrane of infected erythrocytes and are responsible for immune evasion (Fig. 8). var genes also present mutually exclusive expression: among the 60 genes that form this family only one of them is expressed at a time. Parasites presenting the best pattern of expression in a particular host to escape the attack of the immune system will transmit their expression pattern to the next generation.

PfEMP1 proteins also play a role in cytoadherence and rosetting mechanisms, which are involved in the development of the pathophysiology and virulence of malaria (Miller et al, 2002). All variants of PfEMP1 include a single transmembrane domain, and a hypervariable region that binds to endothelial surface receptors. By this mechanism, erythrocytes infected with mature parasites sequester in the host tissues avoiding the passage through the spleen, which would eliminate erythrocytes infected with mature parasites. The polymorphisms that PfEMP1 presents result in different binding specificities. Depending on the var paralogue that is expressed, parasites will use alternative host endothelial surface ligands for adhesion, resulting in erythrocytes sequestering at different host tissues (Montgomery et al, 2007). Depending on the tissue that is affected, the severity of the disease will vary. For instance, parasites presenting A group PfEMP1, which mediate the binding of infected erythrocytes to endothelial protein C receptor (EPCR) and intercellular adhesion molecule 1 (ICAM1) in the brain, will be responsible for causing cerebral malaria pathology (Bernabeu et al, 2016; Bernabeu & Smith, 2017; Gillrie et al, 2016; Wassmer & Grau, 2017). Those expressing VAR2CSA will adhere to the placenta in pregnant women, being responsible for pregnancy-associated malaria (Fried & Duffy, 1996; Salanti et al, 2003). Therefore, PfEMP1 are the best-characterized virulence factor of *P. falciparum* (Smith, 2014; Wassmer et al, 2015).

There are other examples in *P. falciparum* of large, multicopy gene families likely involved in antigenic variation: the rif/stevor/Pfmc-2tm (approximately 200 copies) and the surfins (approximately 10 copies) (del Portillo et al, 2001; Kyes et al, 1999; Sam-Yellowe et al, 2004; Weber, 1988; Winter et al, 2005). In other plasmodial species, multigene families have been described, such as vir genes in *P. vivax* (del Portillo et al, 2001) and kir genes in *P. knowlesi*.
These families are all thought to be regulated by epigenetic mechanisms and they all share the characteristic of expressing a limited subset of the members at a time.

3.3.2 Erythrocyte invasion.

Erythrocyte invasion by merozoites is a complex process that involves several steps and different host receptors and parasite ligands (Fig. 8). EBA and PFRH are important parasite-encoded proteins involved in this process. These small gene families, four and five members respectively, present clonally variant expression. They encode adhesins that are released from the rhoptries and micronemes during parasite invasion and interact with host-erythrocyte receptors to mediate merozoite reorientation and apical attachment. However, with the exception of Pfrh5, the members of these families appear to be functionally redundant and thus non-essential (Duraisingh et al, 2003; Maier et al, 2003; Reed et al, 2000; Stubbs et al, 2005; Wright & Rayner, 2014). Indeed, merozoites can use alternative pathways for invasion, involving different sets of ligands and erythrocyte receptors (Stubbs et al, 2005). The function of clonally variant expression in these genes is not completely understood. Nonetheless, it is generally accepted that changes in the regulation of these genes induce phenotypic variation, which could confer an advantage to the parasite in the process of invasion (Cortes, 2008; Duraisingh et al, 2003; Wright & Rayner, 2014). Additionally, it has been suggested that eba and pfrh variant expression, together with genetic polymorphisms, may have a role in evasion of the host immune system (Cortes, 2008; Wright & Rayner, 2014) (Fig. 8).

MSPDBL2 is also located at the merozoite surface and it may play a role in the initial steps of invasion. mspdbl2, member of small msp-3-like family, shows clonally variant expression and, interestingly, it appears to be silenced in most of the parasites, being active in only small subpopulations (around 1%) (Amambua-Ngwa et al, 2012). The phenotypic significance of the alternative transcriptional states of mspdbl2 remain unknown.

3.3.3 Sexual conversion.

Recently, sexual conversion has been described as an important biological process that is regulated at the epigenetic level. ap2-g has been identified as the gene that triggers gametocyte commitment. This gene is silenced at the epigenetic level by default; parasites
that stochastically switch the transcriptional state of the gene will generate the transmissible stage of the parasite (Kafsack et al, 2014) (Fig. 8).

Interestingly, it has also been observed that not only stochastic transitions but also environmental factors can trigger the formation of gametocytes (Bousema & Drakeley, 2011). This observation provides one of the first examples in *P. falciparum* of a direct transcriptional response as a survival strategy; upon changes in the host environment, such as reduction of nutrients, parasites would guarantee the survival of the population by formation of gametocytes. This has been observed in *P. falciparum* in vitro cultures where differentiation can be induced by depletion of nutrients in the culture media (Brancucci et al, 2015; Dyer & Day, 2000; Williams, 1999). Furthermore, a recent study has linked the depletion of LysoPC with ap2-g activation, inducing the formation of gametocytes (Brancucci et al, 2017). This is one of the first clear evidences of a direct response regulating gene transcription in *P. falciparum*.

### 3.3.4 Infected erythrocyte permeability.

Clonally variant expression of *clag3* genes (*clag3.1* and *clag3.2*) participates in the adaptation of the parasite to varying concentrations of nutrients and other solutes, such as toxic compounds, in the host plasma (Fig. 8). At the same time, it has also been suggested that clonally variant expression in *clag3* genes may play a role in antigenic variation and immune evasion, since *clag3* genes are exposed at the membrane of the infected erythrocyte (Nguitragool et al, 2011). *clag3* genes are the focus of this thesis, and they will be described in detail in the next chapter of this introduction.
Figure 8. Schematic of the different biological processes in *P. falciparum* regulated at the epigenetic level along the life cycle. Upon the trophozoite stage, several multigene families participate in antigenic variation and modification of the erythrocyte. From late rings (at around 20h after invasion), CLAG3 proteins participate in RBC membrane permeability, for the uptake of nutrients and other compounds. Last, the parasite can use alternative invasion pathways, depending on the expressed proteins, to invade a new erythrocyte. During this repetitive asexual cycle, some parasites will differentiate into gametocytes by the activation of AP2-G. Adapted from Cortés and Deitsch 2017.
4. clag3 GENES

4.1 RhopH1/CLAG.

The RhopH complex, which was identified in proteomic studies of invasive merozoites (Campbell et al, 1984; Holder et al, 1985), is initially localized at the rhoptries, i.e. specialized organelles at the apical end of the merozoites (Fig. 9). This high-molecular weight complex is formed by three proteins - RhopH1, RhopH2, and RhopH3 –, which are synthesized in mature schizonts and secreted, along with other rhopty contents, into the erythrocyte targeted for invasion. Each of the three proteins is conserved in all plasmodial species and none of them have significant homology to proteins from other genera; except for a domain shared with RON2, an invasion ligand also localized at rhoptries (Anantharaman et al, 2007; Kaneko, 2007; Richard et al, 2010).

While RhopH2 and RhopH3 are encoded by single genes, Rhoph1 is encoded by the clag gene family (Kaneko et al, 2001). The first clag gene to be described was clag9 (PF3D7_0935800). Barnes and colleagues described a deletion event at the chromosome 9 that was associated with loss of erythrocyte cytoadherence to melanoma cells. The authors mapped a candidate gene within the locus responsible for the loss of this capacity (Barnes et al, 1994) and they named it as clag9 (cytoadherence link asexual gene). Further genome sequencing and assembly identified 4 extra paralogues of this gene in *P. falciparum*: clag2, clag3.1, clag3.2 and clag8 (PF3D7_0220800, PF3D7_0302500, PF3D7_0302200 and PF3D7_0831600, respectively), named according to their location in the chromosomes. A few years later, mass spectrometry analysis of the RhopH complex determined that the clag genes encode the RhopH1 protein, being then designated as Rhoph1/CLAG (Kaneko et al, 2001).

In *P. falciparum*, all clag genes have 9 exons and are localized at the subtelomeric regions within 150 kb of the chromosome end (Fig. 10). Phylogenetic analysis of laboratory-adapted parasite lines from different continents and different plasmodia species revealed that the clag
gene family was split into two distinct groups early in the plasmodial lineage (Kaneko et al, 2005; Nguitragool et al, 2011; Sharma et al, 2013). The first group has a single member in each species, e.g. clag9 in *P. falciparum*. The expansion of the second group is quite variable, being *P. falciparum* the species with more paralogs: clag2, clag3.1, clag3.2, and clag8 (Kaneko et al, 2005). The genes from this group present hypervariable domains, located some 300 residues from the N terminus of the protein (Iriko et al, 2008) (Fig. 10).

**Figure 10. Schematic of the gene structures for the five members of the clag family, rhoph2 and rhoph3 in P. falciparum.** Exons are shown as grey boxes while introns are represented by black lines. Red sections represent coding sequences predicted to encode for a transmembrane domain (Gupta et al, 2015). Blue fragments in clag2, clag3.1, clag3.2 and clag8 represent the hypervariable region of each gene (Iriko et al, 2008). Scale bar represents 1000base pairs. Adapted from Gupta et al. 2015.

While clag9 is the most distantly related member of the clag family, clag3.1 and clag3.2 are the most similar genes, sharing 95% of sequence identity (Fig. 11A). clag3.1 and clag3.2 are neighbour genes localized at chromosome 3, in a region between 110 and 140 kb from the chromosome end in the left subtelomeric region, and are separated by only 10kb with a var-pseudogene located between them (Fig. 11B) (Bowman et al, 1999; Otto et al, 2010). Because of their subtelomeric localization and similarity, there are frequent recombination events, which contribute to sequence diversity among strains (Freitas-Junior et al, 2000; Iriko et al, 2008). 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. 11C). This recombinant gene carries the promoter of clag3.2 and the 3’ UTR of clag3.1.
Figure 11. clag family phylogenetic analysis and clag3 structure. (A) Bayesian phylogeny of CLAG protein sequences. Phylogenetic analysis was conducted using sequences from laboratory lines representing Africa, South America, and Southeast Asia. Branch lengths represent the expected number of substitutions per site (scale bars without shading); asterisks indicate a posterior probability (0.95). Coloured circles represent the mean pairwise diversity of protein sequence, with their diameter reflecting the number of amino acid substitutions per site (gray circle scale bar). CLAG3 subtree is expanded, showing that the two paralogs also segregate into discrete clades with high confidence (posterior probability 1.0). CLAG3.1 sequences are represented by red branches and CLAG3.2 by blue branches. Laboratory lines used for the analysis are specified in each branch. Adapted from Sharma et al., 2013. (B) Schematic of the clag3 loci, with the var-pseudogene PF3D7_0302300 (var ps) between the two clag3 genes. (C) Schematic of the proposed model of recombinant-clag3 generation. Recombination between clag3.1 and clag3.2 of the same chromosome generate a single clag3 gene, carrying the promoter of clag3.2 and the 3' UTR of clag3.1. Adapted from Iriko et al., 2008.

4.2 clag3 EXPRESSION DYNAMICS.

clag3 genes are expressed in mature schizonts, together with other members of the clag family and the other two components of the RhopH complex (rhoph1 and rhoph2). Transcription of all these genes begins at the mid-trophozoite stage (30-34h after invasion) and peaks during the mature schizont stage (42-46h after invasion) (Bozdech et al, 2003; Kaneko et al, 2005). As mentioned in the previous sections, clag3 genes present clonally
variant and mutually exclusive expression (Cortes et al, 2007). Several studies confirmed that the transcriptional state of clag3 genes involves histone modifications: silencing of one of the clag3 genes is mediated by H3K9me3-based heterochromatin, whereas activation is associated with an increase in the alternative modification at this position, H3K9ac (Comeaux et al, 2011; Crowley et al, 2011). These studies confirmed that clag3 variant expression dynamics is heterochromatin-mediated, and thus regulated at the epigenetic level. Additionally, Crowley and colleagues (Crowley et al, 2011) analysed the 5’ regions of clag3.1 and clag3.2 by micrococcal nuclease digestion, revealing that specific nucleosomes are positioned differently between the active and repressed states of clag3.1 and clag3.2., within regions that are important for promoter activity. Besides, the authors also observed that the histone modifications are preserved during non-schizont stages, suggesting that they are involved in maintaining the epigenetic memory along the successive generations of the asexual cycle.

Mutually exclusive expression was first described by Cortés and colleagues (Cortes et al, 2007). From a population of parasites expressing clag3.1 and clag3.2, 11 subclones were isolated. The authors observed that each clone preferentially expressed only one of the paralogues and that the expression pattern was maintained for at least 30 cycles of replication under culture conditions. Mutually exclusive expression in clag3 genes has been confirmed in culture-adapted parasite lines in other studies (Comeaux et al, 2011; Nguitragool et al, 2011; Pillai et al, 2012). Comeaux and colleagues (2011) also observed that clag3.1 and clag3.2 exhibit mutually exclusive expression in recently cloned parasites and that most of the subclones expressed clag3.1, suggesting a growth advantage in routine in vitro culture for parasites expressing clag3.1, at least in the 3D7 background. Interestingly, the same authors managed to create transgenic parasites presenting disruption of clag3.2 but were unsuccessful in generating clag3.1 disruptants.

To further study the mutual exclusion property, Comeaux and colleagues (2011) conducted experiments with this clag3.2-truncated line, which under standard conditions expressed clag3.1 (as expected from a disrupted clag3.2 coding-region). The promoter state at the clag3.2 promoter of this line affects the expression of the selectable marker-cassette human dihydrofolate reductase (hdhfr), which confers resistance to the drug WR99210. When parasites were selected under drug pressure, no clag3 transcripts were detectable, suggesting that activation of the disrupted-clag3.2 locus does affect expression of clag3.1 in a mutually exclusive manner and that mutually exclusive expression of clag3 genes is independent of protein production.
During the progress of this thesis, different expression patterns that do not comply with mutual exclusion, *i.e.* expression of more than one or none *clag3* promoter at the same time, have been observed under selective pressure (Rovira-Graells et al., 2015; Sharma et al., 2013). Interestingly, disruption of mutually exclusive expression only occurs transiently and under standard conditions the normal pattern is still for parasites to express only one of the *clag3* genes, since expression of a single *clag3* promoter is quickly recovered after removing the selective pressure. This feature indicates that mutually exclusive expression of *clag3* genes is not strict, but is strongly favoured.

The molecular mechanisms of simultaneous silencing of both *clag3* genes have been investigated in detail. On one hand Sharma and colleagues (2013) did not find enrichment of H3K9me3 in their *clag3*-silenced parasite line, the histone modification strongly associated with repression and heterochromatin formation in malaria (see section 2.2 EPIGENETIC REGULATION); thus, they propose differences between this mechanism and conventional silencing of a single *clag3* gene. However, Rovira-Graells and colleagues (2015) demonstrate that *clag3* epigenetic silencing relies on H3K9me3-based heterochromatin even when the two *clag3* genes are simultaneously silenced, suggesting that mutually exclusive expression depends on molecular interactions that favour heterochromatin formation in all but one of the *clag3* promoters.

Rovira-Graells and colleagues went further in the study of the molecular mechanisms that control the expression of *clag3* genes and that govern mutually exclusive expression. Among their observations, they show that the expression of a non-coding RNA (ncRNA) (PF3D7 0302400), located between the two clag3 genes, correlates with expression of clag3.1. However, the authors cannot conclude if this is a cause or a consequence of the expression of clag3.1; they suggest it could be part of the mechanisms that regulate mutually exclusive expression, even if further studies are necessary to confirm this hypothesis. Indeed, ncRNAs have been previously described in the epigenetic regulation of var genes (Amit-Avraham et al., 2015).

Despite the studies conducted in clag3 gene expression, the reason for these genes to present clonally variant and mutually exclusive expression remains unclear. Some authors suggested that clonally variant expression in clag3 genes may play a role in antigenic variation and immune evasion, since CLAG3 proteins are exposed at the membrane of the infected erythrocyte (Nguitragool et al., 2011). Nonetheless, clag3 genes have also been proposed to participate in a variety of roles, as will be explained in the next section. Clonally variant and
mutually exclusive expression might participate not only in immune evasion but also in functional variation.

**4.3 clag3 Possible Roles.**

A number of roles have been proposed for CLAGs and the interacting RhopH2 and RhopH3 proteins along the last decades. Upon their discovery, CLAG proteins were suspected to participate in cytoadherence, because a chromosome 9 deletion event associated with loss of cytoadherence, thereby their name (cytoadherence link asexual gene) (Barnes et al, 1994; Day et al, 1993). This function was later confirmed with the generation of a *clag9* knockout (Trenholme et al, 2000). However, the results of the experiments conducted by Trenholme and colleagues could not be reproduced in other laboratories, despite using the same laboratory parasite line and an essentially identical transfection strategy (Nacer et al, 2011). Another study suggested that CLAG9 may play a role in cytoadherence by interfering in the trafficking of PfEMP1 to the infected RBC surface, at least for VAR2CSA (Goel et al, 2010). Thus, whether CLAG proteins play a role in cytoadherence still remains uncertain.

Another proposal has been that CLAGs and the associated RhopH proteins play a role in erythrocyte invasion. This idea was partly motivated by the localization of these proteins to the rhoptries, in which we find other components that are secreted during parasite invasion (Cooper et al, 1988; Coppel et al, 1987; Etzion et al, 1991; Gardiner et al, 2004; Kaneko et al, 2001). In fact, recent studies with conditional knockdowns for *rhoph2* and/or *rhoph3* described a role of the RhopH complex in RBC invasion (Ito et al, 2017; Sherling et al, 2017). The results of these studies indicate that RhopH3 alone contributes to host-cell invasion, either by participating in interactions with RBC receptors or by facilitating parasite internalization. A direct link between erythrocyte invasion and CLAG proteins could not be demonstrated though, but the authors observed that the whole complex (RhopH1/CLAG, RhopH2 and RhopH3) is necessary for its trafficking and for viable parasites.

The last proposed role for CLAG3 is the formation of a transmembrane channel responsible for the marked increase in membrane permeability to diverse solutes in *Plasmodium* infected erythrocytes. This channel is known as Plasmodial Surface Anion Channel (PSAC) (Nguitragool et al, 2011) and is described in detail in the next section.


**4.4 ROLE OF CLAG3 IN SOLUTE TRANSPORT: PSAC.**

**4.4.1 INCREASED PERMEABILITY OF INFECTED ERYTHROCYTES: BACKGROUND AND MECHANISM.**

The intraerythrocytic niche of malaria asexual blood stages protects the parasite from the immune attack (Hafalla et al, 2011), but this advantage also implies that the parasite must develop a transport system to acquire nutrients that are not available inside the erythrocyte (Kutner et al, 1982; Neame & Homewood, 1975; Staines et al, 2007; Triglia et al, 1997). Indeed, it has been observed in several studies that RBCs infected with mature forms of *P. falciparum* (from 20h post-invasion) present increased membrane permeability to certain compounds compared to non-infected erythrocytes. The first indication of this phenomenon was obtained from *ex vivo* experiments using monkey blood, where the authors detected changes in the erythrocyte ionic content after infection with malaria parasites (Overman, 1948). Decades later, further studies confirmed increased membrane permeability to varying extents of infected erythrocytes to a range of solutes, such as sugars, amino acids, purines, organic cations, some vitamins, and inorganic monovalent ions (Elford et al, 1985; Ginsburg et al, 1985; Neame & Homewood, 1975). Interestingly, these new transport activities, which presented increased permeability to a broad range of solutes, showed at the same time high solute-specificity (Kirk et al, 1994; Saliba & Kirk, 1998; Staines et al, 2001). This increased membrane permeability in infected erythrocytes is mediated by the so called **new permeation pathways (NPPs)** (Desai, 2012; Elford et al, 1985; Ginsburg et al, 1985; Saliba & Kirk, 2001).

The identification of compounds that inhibit transport of each of the above solutes supported the idea of the presence of a specialized channel (Kirk et al, 1994; Kirk et al, 1993; Kutner et al, 1987). Nevertheless, how infected cells are able to internalize these solutes was unclear. Patch clamp studies, a collection of methods that capitalize on electrical currents associated with transmembrane ion movement, determined that the transport activity localizes at the host erythrocyte membrane and that it is mediated by one or more ion channels (Bouyer et al, 2011; Desai et al, 2005; Huber et al, 2004; Verloo et al, 2004). The nature of this channel(s) has been under discussion for several years: while some authors defended the idea of several distinct host-encoded channels activated by the parasite contributing to the increased permeability of infected erythrocytes (Bouyer et al, 2011; Staines et al, 2007; Winterberg et
al, 2012), others suggested a single parasite-encoded channel being responsible for NPPs (Alkhalil et al, 2004; Baumeister et al, 2006; Desai et al, 2000; Kirk et al, 1994). This new parasite-encoded proposed channel was PSAC (Fig. 12).

4.4.2 PSAC.

During the last 20 years, Sanjay Desai and colleagues (NIH, Washington) tried to identify the mechanistic basis of PSAC. Thanks to patch-clamp experiments, they observed that PSAC is an unusual broad selectivity channel that at the same time presents selectivity for some structurally similar solutes (Alkhalil et al, 2004; Cohn et al, 2003; Desai et al, 2000), consistent with previous descriptions of NPPs. Studies using erythrocytes from a single donor revealed changes in channel behaviour when infecting cells with different parasite lines (Alkhalil et al, 2004; Alkhalil et al, 2009). These studies supported the idea of parasite proteins being involved in the formation of NPPs rather than human-host-encoded proteins.

In 2011, the same group of researchers described for the first time a role for CLAG3 in the formation or regulation of the PSAC channel (Nguitragool et al, 2011). Studies using chemical inhibitors, linkage analysis of a genetic cross and transgenic parasite approaches, revealed a role of parasite encoded proteins CLAG3.1 and CLAG3.2 in the formation of the active PSAC. Thereafter, several studies at their lab have confirmed the involvement of clag3 genes in membrane permeability, nutrient acquisition and solute transport selectivity (Nguitragool et al, 2011; Pillai et al, 2012; Sharma et al, 2013). They also described CLAG3 as an integral membrane protein at the host erythrocyte with the HVR exposed at the surface (Nguitragool et al, 2014), supporting the idea that PSAC is a parasite encoded channel localized at the membrane of infected erythrocytes, responsible for the increased permeability in infected cells (Fig. 12).

**Figure 12. PSAC is a transmembrane channel exposed at the surface of the infected RBC.** This channel (yellow cylinders) is responsible for the uptake of several solutes that are necessary for parasite survival, such as sugars and amino acids among others. Parasite-encoded clag3 genes participate in the formation/regulation of this channel.

Despite all the studies that have been conducted, the structure and functionality of PSAC is not clearly defined yet. clag3 genes have been related to PSAC for several years, but the
relationship between the genes and the channel has not been completely clarified. It is not known for instance whether CLAG3 proteins conform the channel by their own or if they need the involvement of other proteins for its formation or regulation, such as other members of the CLAG family or of the RhopH complex.

The recent study on rhoph2 and rhoph3 knockdowns at Desai’s lab described a possible prototype of PSAC (Ito et al, 2017). In this study the authors suggest that CLAG3 proteins conform the PSAC channel and that RHOPH2 and RHOPH3 are also necessary for the formation of a viable channel. According to this model, the components of the RhopH complex would be released from the rhoptries of the invading merozoite, go to the parastiphorous vacuole and then transported via the Maurer’s clefts to the erythrocyte membrane, forming the PSAC. The new defined role for RhopH3 in erythrocyte invasion and the requirement of the whole complex for a functional channel explains the early synthesis and complicated trafficking of these proteins. Of note, clag genes, together with rhoph2 and rhoph3, are expressed at the mature schizont stage, with its peak at around 42-46h post-invasion, but increased permeability is observed at an earlier point (early trophozoites at around 20h post-invasion). This indicates that the proteins that form the channel are synthetized in the previous generation of parasites. Apparently, this complicated process of early protein synthesis and trafficking to the host-RBC membrane evolved to enable two distinct functions at separate points in the parasite cycle by the same complex: RBC invasion and solute transport (Fig. 13). This new proposed model for PSAC, in which RhopH2 and RhopH3 also participate, provides a more practical evidence in favour of the presence of a parasite-encoded channel.

The lack of strongly predicted transmembrane domains and of homology to other known channels raised scepticism about the idea that CLAG3 proteins form a PSAC. However, the new proposed model establishes a more plausible hypothesis for PSAC structure, for the association of CLAG3 with two other membrane proteins.
Figure 13. Model for the functions and trafficking of the RhopH complex proposed by Ito and colleagues (2017). The RhopH complex localizes to the rhoptries in the merozoite (1). Rhoph3 (yellow protein) participates in invasion (2) and the whole complex is deposited into the parastiphorous vacuole of the newly formed parasite (3). The Rhoph complex is then transported via the Maurer’s clefts to the RBC membrane (4), where CLAG3 (red component) participate in PSAC formation (5). Adapted from Ito et al., 2017.

4.4.3 TRANSPORT OF TOXIC COMPOUNDS.

Among the compounds that cross the RBC membrane there are several substances that present toxicity for the parasite. Most antimalarial drugs are active against the asexual blood stages of the parasite. To reach their specific targets, these chemicals must cross at least three membranes, beginning with the host RBC membrane, which is the limiting step. Crossing each membrane may involve partitioning and diffusion through the lipid bilayer or facilitated transport through channels or carriers. Computational analysis (Egan et al, 2000) suggests that most antimalarials have high intrinsic membrane permeability, obviating the need of facilitated transport (Basore et al, 2015). However, it has been demonstrated that some toxic compounds require NPPs for their transport, such as diamidine compounds (Stead et al, 2001), bis-quaternary ammonium compounds (Biagini et al, 2003), the antibiotics fosmidomycin (Baumeister et al, 2011) and blasticidine S (BS) (Hill et al., 2007), and the protease leupeptin (LEUP) (Lisk et al, 2008). Besides, computational analysis also suggests that a subset of compounds among currently used and under investigation drugs also require facilitated uptake (Basore et al, 2015).
Recently, it has been demonstrated that *P. falciparum* parasites can acquire resistance to the antimalarial compounds BS and LEUP by alterations in PSAC activity (Hill & Desai, 2010; Hill et al., 2007; Lisk et al., 2008; Lisk et al., 2007). Resistant parasites showed, in addition, altered permeability to other compounds that are known to require PSAC for their entry, such as sorbitol and alanine. These results establish that PSAC is involved in the uptake of at least some toxic compounds, and that it may play a role in drug resistance by changes in the channel activity.

The fact that PSAC seems to be encoded by two genes, which additionally present mutually exclusive expression, leads to the idea that each *clag3* gene confers different permeability characteristics to the erythrocytic membrane. This function may play a role in the adaptation of the parasite to varying concentrations of nutrients and other solutes, such as toxic compounds, in the host plasma. Indeed, *clag3* genes regulation is a well-studied example of bet-hedging and, as it will be further described in this thesis, changes in *clag3* expression have been described as a mechanism to avoid the uptake of toxic compounds into the cell.
5. Drug Resistance is a complex problem

Drug resistance in malaria parasites, as mentioned in previous chapters, is one of the main threats to fight malaria. An issue of this magnitude can be dealt with from different perspectives. On the one hand, it can be seen as a structured problem, defined as a problem that “is to be solved by standardized (quantitative) techniques and procedures” (Hisschemöller & Hoppe, 1995). Different disciplines can be applied to deal with drug resistance:

- Molecular biology. The study of the biological characteristics of the parasite and the molecular processes that it applies to escape the mechanism of action of the drugs is of vital importance to find a way to combat drug resistance.
- Chemistry. The design of new drugs that can escape from the current mechanisms of drug resistance is essential to succeed in the elimination of malaria through chemotherapy.
- Epidemiology. Another perspective is to study the patterns, causes and consequences of drug resistance in defined populations, to determine the relationships between these variables and the incidence of the problem.

On the other hand, drug resistance can also be conceived as an unstructured problem: biological and chemical factors are not the only cause of development of drug resistance; it also concerns many other elements, such as human activity and environmental conditions. Moreover, many sectors of the society are involved in this problem and they should be taken into account for its resolution. For this reason, “technical methods for problem solving appear inadequate” (Hisschemöller & Hoppe, 1995), or at least insufficient, which turns drug resistance into a complex problem.

After literature review (Hastings & Watkins, 2005; Malisa et al, 2011; Nsagha et al, 2011), different factors influencing the development of drug resistance by malaria parasites have been collected in a mind map (Fig. 14). As reflected in this map, all these factors are related in a complex system, where the variation of a single element can affect the rest. These factors can be divided into five categories: (i) parasite biology, (ii) host immune characteristics, (iii) human action, (iv) environmental factors, and (v) institutional programmes, policies and their implementation. In the mind map, it is shown that also different social sectors are involved in the drug resistance problematic, e.g. citizens, health workers, politics, media, etc. Other actors, such as researchers, industry, NGOs, donors, etc. play an important role as well, since their collaboration is crucial in the advancement of knowledge on the topic, contributing to the resolution of the problem.
Drug resistance in malaria parasites, beyond its complexity for all the actors involved, presents additional characteristics that make its resolution an arduous challenge:

- Development of drug resistance in one particular moment and place can spread all over the world.
- Malaria is a poverty-related disease, affecting the most disadvantaged countries of the world.
- Transmission of drug resistance is faster than research progress. The parasite genome and epigenome change constantly, becoming a very complex issue.
- Policy and institutional level are important stakeholders in containing drug resistance.
- Individual use of drugs affects everyone: personal responsibility becomes a Public Health issue.

For all of these reasons, drug resistance is considered a complex problem. Therefore, it should be addressed from an inter- and transdisciplinary perspective. Quantitative (lab work) and qualitative (social studies) research are both necessary and significant in order to address the problem effectively.
Figure 14. Mind map of factors involved in Drug Resistance in Malaria Parasites.