**Universidade Nova de Lisboa**

**Instituto de Higiene e Medicina Tropical**

# Interruption of gametocyte to ookinete development

**Jéssica de Jesus Fernandes**

**DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS**
**ESPECIALIZAÇÃO EM BIOLOGIA MOLECULAR EM MEDICINA TROPICAL E INTERNACIONAL**

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INSTITUTO DE HIGIENE E MEDICINA TROPICAL
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# Universidade Nova de Lisboa

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## Interruption of gametocyte to ookinete development

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# ABSTRACT

**Keywords:** Hybridomas, Malaria, Gametocyte, Ookinete, *in vitro* culture.

Malaria is one of the most life-threatening and deadliest diseases caused by a parasite from genus *Plasmodium*, reaping approximately 900,000 lives per year. Mostly pregnant woman and children under 5 years from sub-Saharan Africa are victims of this parasite. A subsequent issue related to control disease dissemination or its eradication, has been the parasite resistance to anti-malarial drugs and the resistance of, *Anopheles* mosquito´s (the vector responsible for malaria transmission) to the insecticides. The development of an efficient vaccine or the design of compounds capable of blocking the parasite development in the vector are promising strategies to reduce parasite transmission and infectivity. Transmission-blocking vaccines (TBV) have been developed as a tactic to block the development of sexual/sporogonic stages from the human host to the vector basing it on the stages of the sexual and sporogonic cycle of the parasite, such as, gametocytes and ookinete forms – the main subject of this work. Ookinetes are motile forms that penetrate the mosquito midgut epithelium and develop into oocysts carrying thousands of sporozoites which are responsible for infecting the human host during a subsequent mosquito blood meal. Creating vaccines aiming to target ookinetes and, by doing so, blocking its development and/or the egress in the mosquito midgut, is critical to hamper their progression to the next stages and their transmission. The cultivation of ookinete *in vitro* has been a great challenge. Generating *P. falciparum* and *P. berghei* ookinetes *in vitro,* allows us to obtain the parasite from the host and facilitates the study of their physiology, metabolism, and biological mechanisms to design several compounds against the parasite and fight the infection. Also, the knowledge of antigens involved is fundamental to develop transmission-blocking vaccines. In this work, we were able to produce *P. falciparum* and *P. berghei* ookinetes *in vitro,* by submitting the parasites to ookinete medium, at 21ºC and 26ºC. From the *P. falciparum* culture were obtained 500-800 ookinetes/mL at both temperatures and from *P. berghei* culture were obtained $1\times10^6$ ookinetes/mL. The SMFA (Standard Membrane Feeding Assay) using the *P. berghei* ookinete culture resulted in an infection rate of 11.1% and, for whole blood tested, 72%. Comparing the results from this work with other studies, the ookinete concentration obtained was the equivalent to $5\text{-}60\times10^5$ ookinetes/mL and to $1.5\times10^6$ ookinetes/mL. In both our experiments, the number of ookinetes obtained is much higher than in our experiment (500-800 ookinetes/mL). However, the ookinetes found were morphologically different from the expected, with some of them showing deformities probably due to inadequate conditions. Regarding to *P. berghei in vitro* culture, in previous studies, the ookinete concentration obtained was $3.75\pm0.48\times10^6$/mL, comparatively higher to the ookinete concentration present in this work, still we were able to obtain *P. berghei* ookinetes by *in vitro* culture. The attempt to induce *P. falciparum* and *P. berghei* ookinetes development, *in vitro,* was well accomplish, however the protocols used need to be improved.

# RESUMO

Palavra-chave: Hibridomas Malaria, Gametócito, Oocineto, cultura *in vitro*, atividade de bloqueio de transmissão.

A malária é uma doença provocada por um parasita do género *Plasmodium*, que ceifa aproximadamente 900.000 vidas por ano. Principalmente mulheres grávidas e crianças menores de 5 anos da África Subsaariana são vítimas deste parasita. Uma questão subsequente relacionada ao controlo da disseminação da doença ou mesmo erradicação da mesma, tem sido a resistência do parasita aos antimaláricos e a resistência do mosquito *Anopheles* (vetor responsável pela transmissão da malária), aos inseticidas. Desenvolver uma vacina eficiente ou compostos capazes de bloquear o desenvolvimento do parasita no vetor são estratégias promissoras para reduzir a transmissão e infectividade do parasita. As vacinas de bloqueio de transmissão têm sido desenvolvidas como uma tática para bloquear a transmissão do parasita, do hospedeiro humano para o vetor, baseando-se nos estadios do ciclo sexuado e esporogónico do parasita, tais como, os gametócitos e os oocineto – o principal foco desde trabalho. Os oocinetos são formas móveis que penetram no epitélio do intestino médio do mosquito e se desenvolveram em oocistos, que contém milhares de esporozoítos que serão responsáveis por infetar o hospedeiro humano durante uma subsequente refeição de sangue do mosquito. A criação de vacinas com o objetivo de atingir o oocineto bloqueando o seu desenvolvimento, ou a sua invasão do epitélio do intestino do mosquito, dificultam a sua progressão para os estágios seguintes e posteriormente a sua transmissão. Cultivar oocinetos *in vitro* tem sido um desafio. Produzir oocinetos de *P. falciparum* e de *P. berghei, in vitro,* permite-nos obter o parasita fora do hospedeiro e facilita o estudo da sua fisiologia, metabolismo e mecanismos biológicos, para que possam ser desenvolvidos diversos compostos que combatam o parasita. Além disso, o conhecimento dos antigénios envolvidos é fundamental para o desenvolvimento das vacinas de bloqueio de transmissão. Neste trabalho, foi possível produzir oocinetos de *P. falciparum* e *P. berghei in vitro* submetendo os parasitas ao meio de oocineto e a temperaturas de 21ºC e 26ºC. Da cultura de *P. falciparum* foram obtidos 500-800 oocinetos/mL em ambas as temperaturas e da cultura de P. berghei foram obtidos $1\times10^6$ oocinetos/mL. O SMFA (o ensaio padrão de alimentação por membrana) usando a cultura de oocinetos de *P. berghei* resultou numa taxa de infeção de 11.1%, e de 72% usando todo o sangue. Comparando os resultados deste trabalho com os de outros autores, a concentração de oocinetos que obtiveram foi de $5\text{-}60\times10^5$ oocinetos/mL., e de $1.5\times10^6$. Em ambas as nossas experiências os números de oocinetos obtidos *in vitro* foi muito superior ao número obtido nesta experiência (500-800 oocinetos/mL). No entanto, quando observados ao microscópio os oocinetos apresentavam alguma deformidade morfológicas provavelmente causadas por condições inadequadas. Em relação á cultura de *P. berghei* feito em estudos prévios, o número de oocinetos obtido foi de $3.75\pm0.48\times10^6$/mL, comparativamente maior do que o número de oocinetos presentes neste trabalho, ainda assim, foi possível obter oocinetos de *P. berghei in vitro*. A tentativa de produzir oocinetos de *P. falciparum* e de *P. berghei* por cultura *in vitro*, bem-sucedida, mas ainda assim, os protocolos precisam ser melhorados.

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## LIST OF ABREVIATIONS

**ACT -** Artemisinin combined therapy

**AHA –** Autoimmune haemolytic anaemia

**CTRP –** Circumsporozoite and TRAP – related protein

**DAPI –** 4,6 - diamino – 2 – fenil – indol

**DHFR –** Dihydrofolate reductase

**ELISA –** Enzyme – linked immunosorbent assay

**EM –** Endothelium midgut

**GAP –** Genetically attenuated sporozoites

**G6PD –** Glucose – 6 – phosphate dehydrogenase

**GPb –** *Plasmodium berghei* gametocyte

**Pf1 –** *Plasmodium falciparum* gametocyte

**IgG –** Immunoglobulin G

**IPT –** Intermittent preventive treatment

**IRS –** Indoor residual spraying

**ITM –** Insecticide treated nets

**MB –** Methylene blue

**MVIP –** Malaria vaccine implementation program

**OPb –** *Plasmodium berghei* ookinete

**OPf –** *Plasmodium falciparum* ookinete

**PfATP4 –** *Plasmodium falciparum* P- type ATP4

**PfCHT1 –** *Plasmodium falciparum* chitinase 1

**PfeEF2 –** *Plasmodium falciparum* elongation factor 2

**PM** – Peritrophic matrix

**Pfs25 –** *Plasmodium falciparum* specific protein 25

**RAS –** Radiation attenuated sporozoites

**RBC –** Red blood cells

**RBM –** Roll back malaria

**SDS – PAGE -** Sodium dodecyl-sulphate polyacrylamide *gel* electrophoresis

**SMFA –** Standard membrane feeding assay

**SOAP –** Secreted ookinetes adhesive protein

**SP –** Sulfadoxine – pyrimethamine

**TRA –** Transmission reducing activity

**TBS –** Transmission-blocking strategies

**TBV –** Transmission-blocking vaccine

**WHO –** World Health Organization

**WSV –** Whole sporozoite vaccine

**FBS –** Fetal bovin serum

**mAb –** Monoclonal antibody

# 1. INTRODUCTION

# 1. INTRODUCTION

Malaria is a disease caused by a protozoan parasite from the genus *Plasmodium.* There are 5 different *Plasmodium* species that infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* (Sato, 2021).

*Plasmodium falciparum* is the most pernicious species from the *Plasmodium genus* killing hundreds of people from tropical and sub-tropical countries. According to the recent data from December 2019 by WHO (WHO, 2020), 228 million people were infected and 405,000 died from malaria, mostly, in sub-Saharan Africa. These numbers place malaria as one of the major causes of morbidity and mortality around the globe. Not only African countries are affected by malaria but also regions of South-East Asia, Eastern Mediterranean, Western Pacific, and the Americas. Although *P. falciparum* is the most prevalent species of human malaria in Africa, *P. vivax* presents a higher geographic extension. In 2017, 82% of the *P. vivax* cases registered were from India, Pakistan, Ethiopia, and Sudan (Barber et al., 2017; Singh & Daneshvar, 2013).

*P. ovale* and *P. malariae* are less prevalent and the associated death rates are lower compared to *P. falciparum* and *P. vivax.* The distribution of *P. ovale* is restricted to tropical western Africa, only 1% of the cases of *P. ovale* infection were reported outside of Africa (Cho et al., 2012). *P. malariae* is commonly found in sub-Saharan Africa, South America, Indonesia, Southeast Asia, and western Pacific, coexisting with *P. falciparum* in these regions (Dondorp, 2008). The least common species of *Plasmodium* responsible for causing human malaria infection is *P. knowlesi*, known for infecting mainly monkeys - their natural reservoir. Several cases of human *P. knowlesi* infection were identified in Southeast Asia. Indeed, this area is the natural habitat of these hosts (*Macaca fascicularis, Macaca nemestrina*, *Macaca leonine*) and where the mosquito vector can be found (Barber et al., 2017; Singh & Daneshvar, 2013).

The five human malaria species mentioned above can cause severe and even fatal forms of malaria disease, but most of these malaria-associated deaths are due to *P. falciparum* infection.

# 1. INTRODUCTION

The symptoms of malaria appear 6-8 days, depending on the *Plasmodium* species, after an individual be bitten by an infected female mosquito vector *Anopheles.* The symptoms involve fever, headache, cough, and diarrhoea. In advanced cases, anaemia, respiratory issues, organ failure and convulsions (Miller et al., 2002).

*Plasmodium vivax* and *P. falciparum* can cause anaemia, the former causes a less severe form of infection than the latter. Severe anaemia is caused mainly by *P. falciparum* which reduces the levels of red blood cells (RBC) on circulation and its production by the bone marrow (Haldar & Mohandas, 2009). The deadliness of *P. falciparum* is also attributable to the ability of this parasite to sequestrate and accumulate infected red blood cells in blood vessels, including brain capillaries, consequently, obstructing the passage of blood to the organs (severe malaria), known as cytoadherence. Sequestration results from the adherence of infected RBC´s to the vascular endothelium, capillaries, and venules, through the interaction between specific antigens ligands present on infected RBC`s and receptor molecules located on uninfected RBC´s surface (a phenomenon known as Rosetting), endothelial cells and placental cells (Bruneel, 2019; Luzolo & Ngoyi, 2019).

Several complications are associated to this parasite congestion phenomenon, such as, organ failure or brain damages, leading to cerebral malaria (Cho et al., 2012), which accounts for most malaria-associated deaths in African children under 5-year-old (Molina-Franky et al., 2020). In pregnant women, *Plasmodium* accumulation and sequestration on the placenta blocks the blood flow between mother and foetus, causing severe anaemia in the newborn, stillbirth, low birth weight and eventually mothers' death (van Geertruyden et al., 2004).

In addition to children, pregnant women from endemic areas or living in low-transmission regions are also susceptible of contracting malaria. HIV/AIDS patients are also a group at risk, due to the vulnerability of their immune system. Migrants or travellers with low immunity against the parasite or individuals who doesn't take any chemoprophylactics are at higher risk (WHO, 2020).

## 1.1. *Plasmodium falciparum* life cycle

The *Plasmodium* life cycle requires two different hosts, involving a large range of surface proteins recognition in each host. This enables the parasite to invade hepatocytes and erythrocytes in the vertebrate host where the asexual development occurs (Schizogonic cycle) and the midgut and salivary glands of the mosquito host, where sexual reproduction occurs (Sporogonic cycle) (Figure 1).

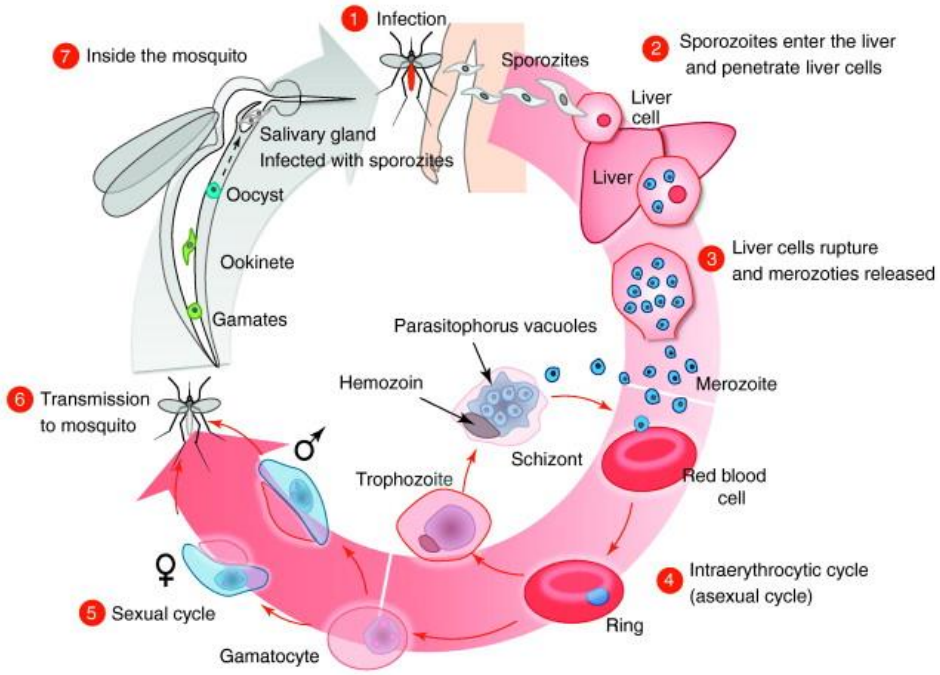


**Figure 1 – The life cycle of *Plasmodium falciparum*.** *Adapted from Cho et al, 2012.* The *plasmodium* life cycle begins when an infected female mosquito *anopheles* bites a human host during a blood meal. The sporozoites existent in the salivary glands of the mosquito are injected into the skin of the human host and reaches the bloodstream (1). In the circulation, the sporozoites will migrate until reach the liver cells and develop in thousands of merozoites (2). The liver cells rupture and release the merozoites to the blood stream and start to invade the RBC´s (3). Inside the RBC, the merozoites differentiate into ring form and develop into trophozoites (metabolically active stage) and finally, into schizonts - a multi-nucleate cell containing merozoites. After 48h, the schizont-infected RBC will burst and release the merozoites to invade new RBC´s initiating a new intraerythrocytic/schizogonic cycle (ring-trophozoite-Schizont) (4). Some of the merozoites change their course and develop into male and

female gametocyte (5). The gametocytes circulating in the human blood stream are collected by the mosquito while it feeds (6). Inside the mosquito midgut, the gametocytes differentiate into male and female gametes. Both gametes will fuse and originate the zygote. The zygote will develop into ookinete and later into oocyst. In the oocyst, several rounds of mitotic divisions result in the formation of new sporozoites in the salivary glands ready to be injected again by the new infected mosquito (7). The development inside the mosquito is nominated sporogonic cycle.

### 1.1.1. Schizogonic cycle

The Schizogonic cycle starts when an infected female *Anopheles* mosquito inoculates hundreds of sporozoites into the skin. In a few minutes, the sporozoites enters the bloodstream and reach the liver, where they need to cross a certain number of hepatocytes until they select one to invade and form the parasitophorous vacuole. Inside this, thousands of new invasive forms – merozoites, develop. Once mature, the merozoites are released within merosomes (vesicles containing the merozoites) to the blood vessels (Rankin et al., 2010a).

In *P. vivax and P. ovale* life cycle, some sporozoites reach the hepatocytes and develop into hypnozoites, a stage that remains dormant inside the liver cells and, weeks, months, or even years later cause reinfection (Robinson et al., 2015).

Once in the hepatic blood vessels, the merosomes reaches the right side of the heart and travel to the lungs where they lodge in the pulmonary capillaries and later rupture, releasing the merozoites into the blood stream (Rankin et al., 2010b). The merozoites will invade the erythrocytes, initiating the erythrocytic/schizogonic cycle. In the RBC´s, the parasite differentiates from ring stage into trophozoite and later into schizont. The trophozoite stage is characterized by a high metabolic activity and ends on a series of nuclear divisions culminating in schizont formation (Venugopal et al., 2020). One of the most relevant metabolic events implies the degradation of haemoglobin from the RBC´s, resulting in the accumulation of heme groups. The heme groups consist in two different components, an organic component known as protoporphyrin and an inorganic component consisting in a $Fe^{2+}$ atom responsible to deliver oxygen to the cells (Lin, 2015). The iron ($Fe^{2+}$) is a reactive atom that is

extremely toxic to the parasite and to overcome this, the parasite detoxifies the heme groups converting it into hemozoin, an inert crystal characterized as parasite pigment (Tuteja, 2007). *P. falciparum* takes up to 48h to complete the asexual cycle. A mature schizont filled with merozoites bursts, releasing 16-32 merozoites which will invade a new RBC´s initiating a new schizogonic cycle (Lamb et al., 2010).The schizonts bursting is characterized for causing periods of fever (the main symptom of malaria disease) and chills (Baron, 1996). The cycle leads to destruction and loss of red blood cells, causing anaemia. (Tuteja, 2007; Baron, 1996).

### 1.1.2. Gametocytogenesis

The successful transmission of the parasite from the human host to the mosquito vector comprehends the differentiation of invading merozoites into the sexual stages, process known as gametocytogenesis. Some environmental changes, such as, high parasitaemia and anaemia promote gametocyte formation. Besides, the influence of potentially antimalarial drugs, such as chloroquine, seems to promote gametocyte formation *in vitro* (Ngwa et al., 2016; Siciliano et al., 2020).

In the human host, the sexual commitment begins when the number of infected RBC´s increase in the blood stream. The immature gametocytes are sequestered to the bone marrow and to the spleen by the adherence of the parasite to the vasculature of these organs, through binding interactions between parasite surfaces and the cells on the organs surface (Joice et al., 2014). The Gametocyte maturation from stage I to stage IV occurs in the bone marrow and spleen, to protect the parasite from the host immune system, (de Niz et al., 2018). The infectivity of *P. falciparum* gametocytes depends on the stage V, the final stage of gametocyte maturation. This stage is the only present on the peripheral circulation (de Niz et al., 2018).

*P. falciparum* gametocytes develop from stage I to V in 8-12 days. In Stage I, gametocytes are not morphologically distinguishable from trophozoites. The stage II gametocytes have a slight elongation. In stage III, one side of the parasite extends with

the ends close to erythrocyte membrane resembling a D-shape. In stage IV, the extremities are longer and pointier exceeding the erythrocyte membrane and the male and female gametocytes can be already distinguished. A fully mature gametocyte stage V has rounded edges and crescent shape. A female mature gametocyte is more curved than the male gametocyte (Ngotho et al., 2019). The right moment when the parasite starts to differentiate into male or female gametocyte is not well understood, however, some authors describe a transcriptional signal mediated by AP2-G (transcription factor) that promotes the sexual differentiation on schizonts before gametocytogenesis commitment (Kafsack et al., 2014; Sinha et al., 2014). In addition to that, studies conclude that schizonts originated from the same clones, simultaneously become male or female gametocytes, which seems to be regulated by male/female, yet undiscovered, transcription factors (Silvestrini et al., 2000; Smith et al., 2000).

One interesting fact about producing gametocytes *in vitro* is the number of female gametocytes in relation to male gametocytes (generally the sex ratio is equivalent to 3.6 females to one male gametocyte) (Robert et al., 1996).

Female gametocytes have a single smaller nucleus with a nucleolus and the hemozoin is concentrated, whereas male gametocytes have a single larger nucleus and lack of nucleolus, and the hemozoin is dispersed (Dixon et al., 2012). On the optical microscope, and in Giemsa-stained blood smears, the female gametocytes appear bluish due to higher density of ribosomes and a red-stained distinct nucleus, whereas male gametocytes are pinkish due to less distinct and diffuse nucleus (Dixon et al., 2012; Jensen, 1979) (Figure 2**)**.

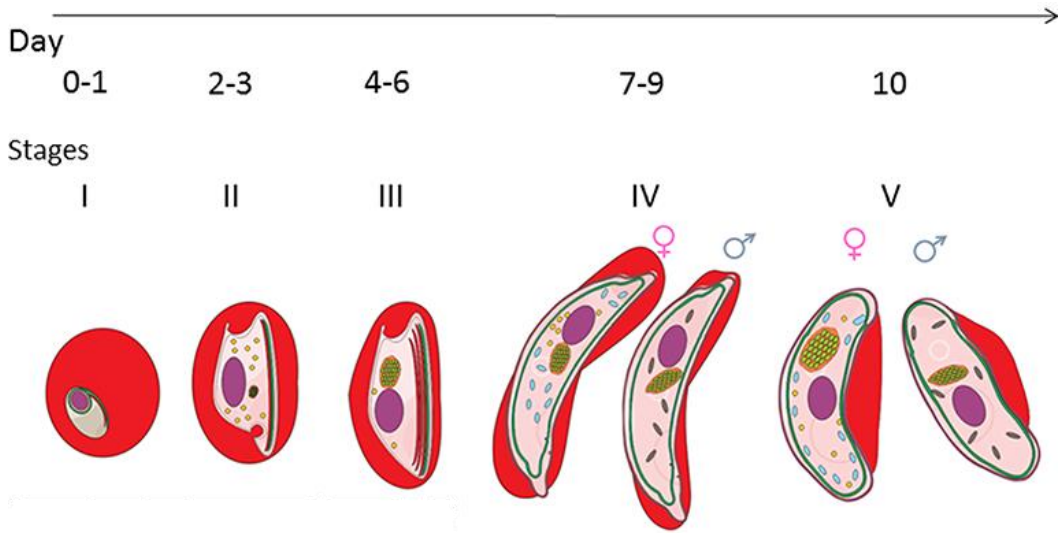


**Figure 2 – Morphology of *P. falciparum* gametocyte development.** *Adapted from Ngwa et al, 2016*. Stage I – rounded shape; Stage II – Extension of the edges and one of the sides resembling a D – character; Stage III – Oval shape from continue growing causing erythrocyte distortion (4 – 6 days); Stage IV – Long and thin format with pointed tips (7 – 9 days). The male and female can be distinguished by their pigment distribution. In males it´s scattered and in females is denser. Stage V – Both gametocytes resemble to a crescent shape. The female gametocyte is slightly curved while the male is thicker (10 – 12) days.

### 1.1.3. Sporogonic cycle

When a female mosquito takes up a blood meal, male and female gametocytes are ingested and, within the mosquito midgut, they transform into male and female gametes (gametogenesis). The gametogenesis is triggered by the variation of environmental factors, including a decrease of temperature, an increase of pH~8 and the presence of specific factors produced by the mosquito´s midgut, such as, xanthurenic acid (Bennink et al., 2016; Billker et al., 1998).

During male gametogenesis, the male gametocyte rounds up and egresses from the erythrocyte, simultaneously releasing the fertile gamete by the degradation of the parasitophorous membrane covering it. A round of three mitotic divisions occur on male

gametocyte, forming eight motile microgametes (exflagellation) (Janse et al., 1988; Sinden, 1983; Sologub et al., 2011). The flagellated microgamete fertilizes the female gamete, forming a diploid zygote (Sinden, 1999). The zygote develops into a motile ookinete which penetrates the peritrophic matrix (PM), crosses the midgut epithelium and lodges beneath the basal lamina, approximately 16-24h after blood ingestion.

The peritrophic matrix (PM) is a semi - permeable structure that separates the mosquito blood bolus from the epithelium midgut (EM), besides acting as a physical barrier. It is challenging for ookinetes to pass through the PM considering its composition in chitin, proteins, and proteoglycans (A. Ghosh et al., 2000). Previous studies demonstrate that *P. falciparum* and *P. gallinaceum* ookinetes secret chitinase, to facilitate the penetration through the PM (Huber et al., 1991; Langer & Vinetz, 2001). This enzyme disrupts the PM granting the parasite midgut invasion (Crompton et al., 2014). The ookinete establishment at the basal lamina may be associated with the ookinete surface ligands p25 and p28 that interact with laminin in the basal lamina enabling the ookinete passage through the EM. The exact function of p25 and p28 is not well clarified since some experiments indicate that these proteins are required for multiple functions including, the ookinete development into oocyst (Aly et al., 2009; Baton & Ranford-Cartwright, 2005; Shahabuddin, 1998; Tomas et al., 2001). Other surface proteins, such as, CTRP (circumsporozoite and TRAP-related protein) and SOAP (secreted ookinete adhesive protein) plays a role in midgut epithelial cell transversion to the basal lamina (Aly et al., 2009; Lal et al., 2009).

From ookinete to oocyst, the parasite is submitted to morphological modifications until complete maturation. Immature ookinete stages I to III, have a round shape with a small membrane projection that elongates until stage IV, forming a protuberance. At this moment, the protuberance extends at maximum range. At Stage V, the ookinete finalizes its maturation, displaying a banana-like or club-shape with a central accumulation of hemozoin (A. K. Ghosh et al., 2010; A. K. Ghosh & Jacobs-Lorena, 2013; Siciliano et al., 2020) (Figure 3).

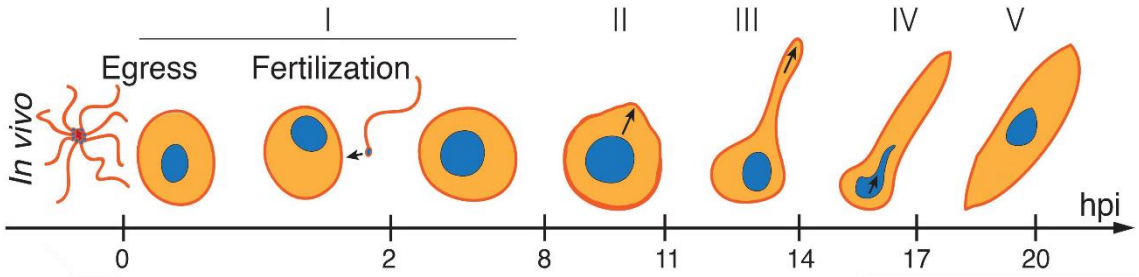


**Figure 3 - Morphology of *P. falciparum* ookinete development.** *Adapted from Siciliano et al, 2020.* ***In vivo*** development of ookinete formation. The male and female gametes egress from the erythrocyte. The male gamete exflagellates and fuses with the female gamete (fertilization) to form the zygote in the first 2 hours post infection (hpi) of the mosquito. Stage I - The zygote grows and transforms into a round shape ookinete (2 – 8 hpi); Stage II – knobs (rounded with a small protuberance) (8-11hpi); stage III – Stem (continuous extension of the protuberance) (11 – 14 hpi). Stage IV – enlarged stem form (enlargement of the stem) and nuclear migration to the stem (14 – 17 hpi); Stage V – Elongated form (mature ookinete) (17 – 20 hpi).

From zygote to ookinete, two meiotic divisions occur originating four haploid genomes from the diploid zygote (Sinden,1991).

The mature stage V ookinetes develop into oocysts within 10-12 days depending on the species and environmental conditions. Within the oocyst, multiple divisions (mitosis) occur to produce thousands of sporozoites. The motile sporozoites egress from the oocyst to the mosquito hemocoel and migrate through the haemolymph, where they invade the salivary glands, then, the sporozoites are ready to be injected by the female mosquito into the human host and a new cycle begins (Matuschewski, 2006; Mota et al., 2001)(Figure 4).

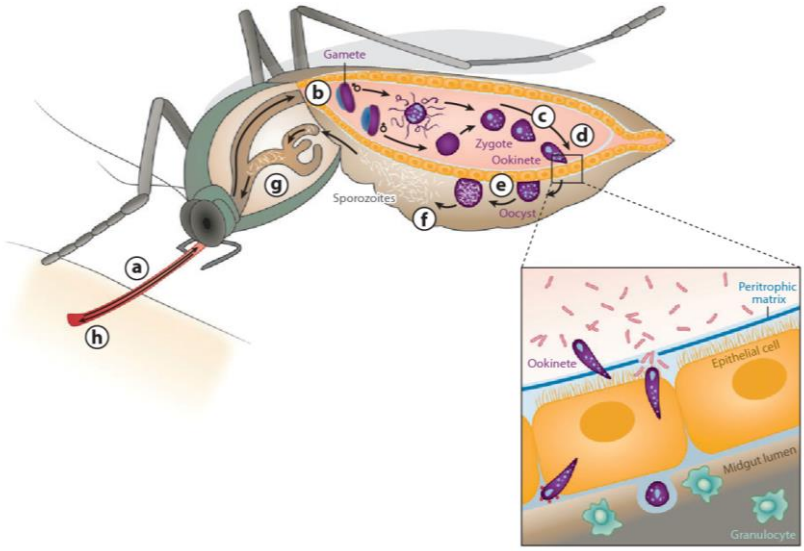


**Figure 4 – Development of *P. falciparum* sexual stages within the mosquito (Sporogonic cycle).** *Adapted from Crompton et al, 2014*. (a) An *Anopheles* mosquito feeds from blood infected with *P. falciparum* from an infected individual; (b) the gametocytes present in blood ingested by the mosquito undergoes morphological transformation to originate the male and female gametes in the midgut lumen; (c) After gamete exflagellation and gamete fertilization, the zygote forms (c) and develop into a motile mature ookinete (d). the ookinetes lodges in the basal lamina and develops into oocysts that undergoes a serious of mitotic divisions to develop thousands of sporozoites (e). The mature oocyst bursts and releases the sporozoites into the mosquito´s haemolymph (f). The sporozoites reaches the salivary glands where (g) they remain until be expelled by the mosquito into a new individual while it takes a blood meal (h).

*P. falciparum* cause numerous deaths on tropical and subtropical areas, however since 2000 the death rates decrease in 47% (WHO,2021), and it is expected that the number kept falling with the combination of new antimalarial drugs and vaccines, and vector control measures the transmission and infection caused by the parasite.

## 1.2. *Plasmodium berghei*

There are four different species that infect mice which are commonly use as murine models of malaria, including: *Plasmodium berghei, Plasmodium yoelii, Plasmodium chaboudi* and *Plasmodium vinckei* (Draper et al., 2018).

*P. berghei* is the model species of choice to study the whole parasite cycle, given that, it is easy to manipulate, and it shares most characteristics with the human parasites, namely genetics, biological and physiological processes (Janse, 2017).

### 1.2.1. *Plasmodium berghei* life cycle

The life cycle of *P. berghei* is similar to that of *P. falciparum*, however, *P. berghei* develops synchronously which means that it is possible to observe the same asexual stages in giemsa smears comparatively with of *P. falciparum* which develops asynchronous and for that reason, the different asexual stages can be found on Giemsa smears. Besides that, they are morphologically distinct and blood cells preferences are also distinct.

The hepatic schizogony takes 22h-23h and the released merozoites invade the RBCs, preferentially reticulocytes (immature erythrocytes), which contrasts to *P. falciparum*, that infects mature erythrocytes in the human host (Prudêncio et al., 2006).

As in *P. falciparum*, *P. berghei* digests the haemoglobin producing hemozoin crystals during its erythrocytic cycle. *P. berghei* mature gametocytes are round/oval, when compared to the banana-shaped mature gametocytes from *P. falciparum* (Leitner et al., 2010). The maturation time in *P. berghei* is shorter (26-30h) than *P. falciparum* (10-12days) (Janse & Waters, 1995). Surely the transition from asexual to sexual development is triggered by a decrease in temperature (20-22ºC), pH increase and host factors, such as xanthurenic acid along with other existent factors within the *Anopheles* vector (Carter et al., 2007; Yoeli & Upmanis, 1968)(Figure 5).

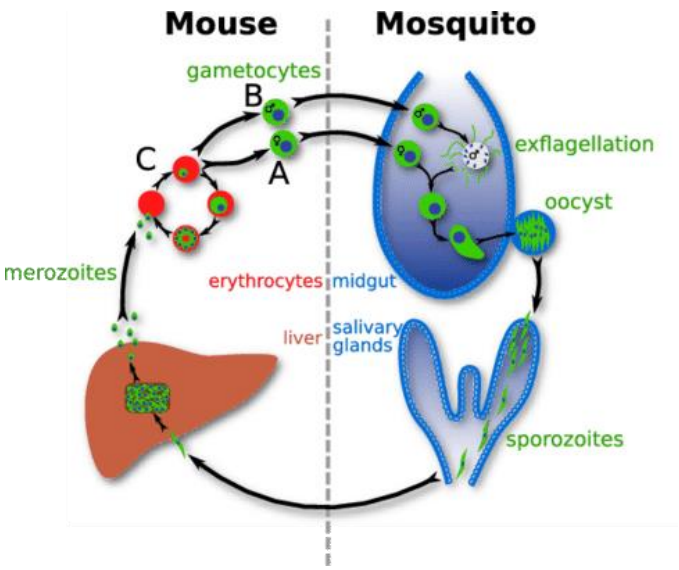


**Figure 5 – Schematic representation of *Plasmodium berghei* life cycle**. *Adapted from Yeoh et al., 2017*. (A) female gametocyte; (B) Male gametocyte; (C) asexual cycle.

In the mosquito, after the blood meal, the mature gametocytes evolve to fertile micro and macrogamete and both merge originating a zygote that undergoes differentiation into banana-shaped ookinete (18-24h). The motile ookinete penetrates the midgut, crosses the epithelium membrane (EM, and settles beneath the basal lamina where the oocyst formation and maturation occur (12-14 days) (Currà et al., 2016).

The ookinete entrance to the EM seems to be assisted by the p21 and p25 proteins expressed on the *P. berghei* ookinete surface (Siden-Kiamos et al., 2000). The mature oocyst filled with sporozoites (3,000-4,000) ruptures releasing the sporozoites that will enter the hemocoel and migrate to the salivary glands where they accumulate, 18-21 days after the blood meal, until being injected by the mosquito when it takes a new blood meal, restarting the cycle (Caldelari et al., 2019; Janse, 2017).

The study of malaria using rodent models is still a good alternative to understand the molecular and cellular mechanism involved in *P. falciparum* life cycle, and even, for examining different antimalarials drugs, since medicine testing on primates is forbidden by bioethics laws (Kalra et al., 2006).

## 1.3. Malaria control

The malaria control measures combine diverse approaches to defeat the parasite within the host, such as, avoiding the contact between human and vector preventing the transmission, performing early diagnosis, and developing treatments.

From 2000 to 2015, *P. falciparum* infections were averted by 68% and clinical cases by 40% due to malaria control measures implemented in sub-Saharan Africa (Bhatt et al., 2015). In 2015, some partnerships, such as, Millennium Development Goals and Roll Back Malaria (RBM), along with WHO, created initiatives to reduce the incidence and mortality rates by 90%, eradicating malaria in more than 30 countries and avoiding a new emergence of the disease in these areas until 2030 (*Global technical strategy for malaria 2016-2030*) (WHO, 2015a).

### 1.3.1. Vector control

Avert the mosquito contact among the communities is a great strategy to prevent malaria transmission. The insecticide treated nets (ITN) and indoor residual spraying (IRS) are good tools to provide protection to the community in malaria endemic countries.

The nets are impregnated with pyrethroids, a group of insecticides, recommended by WHO (WHO, 2002). They are placed around the bed, doors and windows creating a protective barrier, thus, reducing the physical contact with the mosquito (Greenwood, 2017; Hemingway et al., 2016). Indoor residual spraying is a vector control strategy consisting in spraying the inside of houses, with insecticide, at least two times per year, thereby, reducing mosquito population and *Plasmodium* transmission (Greenwood, 2017; Hemingway et al., 2016).

These two methods can provide protection to the African population against malaria, but in some areas, the pyrethroids have no effect because of insecticide resistant mosquito selection, allowing resistant mosquitoes to survive and reproduce.

This is a current problem and a life threat to communities, hampering the global goal of eliminating malaria (Moyes et al., 2020).

### 1.3.2. Diagnosis

Accurate diagnosis is crucial to determine infection and establish the proper treatment. A diagnosis based on signs or symptoms may result in misdiagnosis and inappropriate treatment for malaria or other diseases. A proper diagnosis for malaria comprehends the microscopic observation of thin and thick blood smears or the use of rapid diagnostic tests (RDT´s) designed to detect malaria antigens using specific antibodies (Ngasala et al., 2008; Zimmerman & Howes, 2015). The sensibility of RDT to detect *P. falciparum* has been improved but for *P. vivax* it has been challenging to progress (Nkrumah et al., 2011).

### 1.3.3. Antimalarial therapies

An effective treatment aims to eliminate the parasite from the patients and prevent the evolution of the disease to advanced stages (severe malaria). The treatment depends on several factors, such as, *Plasmodium* species, disease severity, health status of the patient and drug resistance.

The current treatment of *P. falciparum* infections consists in artemisinin-based combined therapy (ACT), a combination of artemisinin derivatives with assistant drugs. The ACT is the 1$^{st}$ line drug for *P. falciparum* treatment recommended by WHO and it includes five ACTs: artemether and lumefantrine; artesunate and amodiaquine; artesunate and mefloquine; artesunate and sulfadoxine-pyrimethamine (SP); dihydroartemisinin and piperaquine in a regiment of 3 days of administration for uncomplicated malaria (Draper et al., 2018). In cases of *P. vivax, P. ovale, P. malariae* or *P. knowlesi*, patients are treated with chloroquine, except for chloroquine-resistant

infections, in which case the ACT is exclusively recommended (WHO, 2019; World Health Organization, 2015).

Sulfadoxine and pyrimethamine (SP) are used as chemoprophylactic components in high-risk groups, as young children, or pregnant women. They are applied as an intermittent preventive treatment (IPT) (Chotsiri et al., 2019; Oranu et al., 2016; WHO, 2019).

### 1.3.4. Malaria drug resistance

Anti-malarial drug resistance has been reported since 1950-1960. The first drugs reported were chloroquine and sulfadoxine-pyrimethamine, which were the first implemented medicines to treat malaria patients from African countries (Menard & Dondorp, 2017). The concern about improving treatments to overcome anti-malarial drug resistance, led to the development of artemisinin-based combined therapies and a constant monitorization of drugs resistance cases. The first reports of ACTs resistance appeared in western Cambodia in 2008-2009 and later in all the Greater Mekong Subregion (Hanboonkunupakarn & White, 2015; Menard & Dondorp, 2017).

### 1.3.5. Malaria vaccination

The vaccination is a fundamental intervention tool to reduce malaria infections, aiming to target asexual, sexual, and sporogonic stages of the malaria life cycle.

The RTS, S/AS-01 is a single subunit vaccine specially designed to confer protection against pre-erythrocytic stages by inducing a humoral immune response to produce a range of antibodies to attack the parasite, blocking its development and preventing the progression of the infection within the human host (Gosling & von Seidlein, 2016; Laurens, 2019b). This vaccine is considered a pre-erythrocytic vaccine because it was design to target the circumsporozoite proteins on sporozoites surface targeting them in the circulatory system (Laurens, 2019a).

This vaccine is the most advanced and widely required to significantly reduce the infection and decrease the mortality rates among infants and children from endemic countries according to Malaria vaccine implementation program (MVIP) (WHO, 2020).

Recently, RTS, S/AS-01 has completed the phase III clinical trials (Guerra Mendoza et al., 2019). They demonstrate an efficacy of 39% after a fourth dose administration in young children aged 5-17 months in 4 years of following up (*Lancet.* 2015; Laurens, 2019a). Even with this percentage the vaccine needs to be improved both in terms of efficacy and safety since there is a risk of meningitis, cerebral malaria, and febrile convulsion.

The Whole sporozoites vaccines (WSV) use radiation attenuated sporozoites (RAS), genetically-attenuated sporozoites (GAS) or experimental infections combined with chloroquine chemoprophylaxis, inducing humoral and cellular responses without causing symptoms, but using these vaccines can result in unexpected infections in some individuals (Draper et al., 2018; Itsara et al., 2018).

The production of a highly effective vaccine able to induce an efficient immune response against pre-erythrocytic and erythrocytic stages is a big challenge for the scientific community but still achievable.

### 1.4. Transmission-blocking strategies (TBS)

Other perspectives based on blocking parasite transmission have been suggested and intend to target antigens from the sexual or sporogonic cycle.

Hampering or inhibiting the development of sexual/sporogonic stages within the vector, is considered an extra tool in the combat to malaria, helpful to achieve the goals of eliminating malaria, imposed by WHO. The strategies developed involve the generation of transmission-blocking drugs, transmission-blocking vaccines, and the

development of refractory mosquitoes. Transmission-blocking drugs and transmission-blocking vaccines will be referred in this work as a main subject.

### 1.4.1. Transmission-blocking drugs (TBDs)

Malaria transmission-blocking drugs design to target the sexual/sporogonic stages are difficult to evaluate since it is a very demanding task to test the efficacy of these compounds *in vitro*, however, there are some suggestive methods, available, for drugs screening. The standard membrane feedings assay (SMFA) is the gold standard assay to test compounds directly in the mosquito, allowing to evaluate the transmission-blocking activity of gametocytocidal and sporontocidal drugs or even the action of specific antibodies within the mosquito (Wadi et al., 2018, 2019). The assay consists in feeding mosquitoes with a *Plasmodium* gametocyte/ookinete culture and with the antibodies/compounds to test, through a thin parafilm membrane attached to a glass feeder containing the mix (culture-antibody/compound) and a week later examine the presence or not of oocysts in the midgut epithelium (Churcher et al., 2012).

Gametocytocidal drugs are designed to target gametocytes in the blood stream subsequently avoiding their transmission to the vector.

Sporontocidal drugs aim to target advanced sporogonic stages, such as, gametes or ookinetes, or even oocysts. Gametocytocidal/sporontocidal drugs applied synergistically can inhibit parasite development within the vector, therefore, preventing the transmission into a new human host. (Butcher, 1997; Wadi et al., 2019) . The disadvantage of the sporontocidal drugs is the reduction of drug concentration in the passage from human to vector. To bypass this problem, the drug must be administrated at a high enough dosage to reach the parasite in the mosquito and eliminate it without being harmful for the human host (Wadi et al., 2019).

#### 1.4.1.1. Artemisinin-Primaquine (AP)

Artemisinin affects younger gametocytes by inhibiting heme polymerization and haemoglobin catabolic pathways and impeding their maturation (Pukrittayakamee et al., 2004). Stages IV-V gametocytes are less metabolically active, so most of the gametocytocidal drugs as primaquine, one of 8-aminoquinolones that effects gametocytes, directly inhibits the metabolism by interfering with electron transport function in the respiratory chain of the mitochondria or by inducing oxidative stress by generating superoxide's (Hill et al., 2006; Schlesinger et al., 1988). WHO recommends the administration of 0.25mg base/kg of primaquine combined with ACT to patients with *P. falciparum* from low transmission areas except for pregnant women, infants <6 months old and patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. In terms of safety, patients with G6PD-deficiency have higher risk for acute autoimmune haemolytic anaemia (AHA) than G6PD-normal individual. The insufficient data about safety in pregnant women and infants does not allow to take any conclusion of these dosage in this groups. According to WHO recommendation, the dosage must be administered on the first day of ACT treatment to reduce the mature gametocytes in circulation and ensuring transmission-blocking (Global Malaria Program, 2015). Since primaquine only kills mature gametocytes, an additional dose in the end and 14 days after the ACT therapy could be administered to kill the remaining mature gametocytes developed later (Wilairatana et al., 2010).

#### 1.4.1.2. Methylone blue (MB)

Methylone blue (MB) is another drug that it has been shown to have gametocytocidal activity by interfering with the hemozoin polymerization and glutathione reductase (antioxidant enzyme) from *P. falciparum* (Schirmer et al., 2003; Coulibaly et al., 2009; Gallo et al., 2009). A previous study to evaluate the effectiveness of Methylone blue combined with artesunate (AS) and amodiaquine (AQ) in children (6-59month old) infected with *P. falciparum* from Burkina-Baso, demonstrated a significant reduction of gametocytes prevalence (1.2% by microscopy and 36.7% by

quantitative nucleic acid sequence-based amplification (QT-NASBA)), comparatively lower, if the children were treated, only with AS and AQ (8.9% by microscopy and 63.3% by QT-NASBA) (Coulibaly et al., 2015). The reason for the discrepancy between microscopic data and QT-NASBA is because the last one provides more accurate data than the microscope (Coulibaly et al., 2015). The dosage of 10 mg/kg/day for 3 days of MB combined with AS and AQ is tolerable by oral administration. The adverse events are related to gastrointestinal and respiratory symptoms when the drug is delivered by intravenous administration. It can also cause AHA in G6PD-dificient individuals (Calderón et al., 2017; Coulibaly et al., 2009, 2015). Another study by *Adjalley et al., 2011* demonstrated a reduction in oocysts of 72%, in infected mosquitoes. The experiment aimed to evaluate the transmission-blocking activity of MB using an *in vitro* gametocyte culture treated with MB. The results clearly show that MB is a potent gametocytocidal drug inhibiting all gametocyte stages and reducing mosquito infection. Further, the sporontocidal activity of MB was proved by indirect and direct SMFA in a study from *Vos et al 2015* indicating that a concentration of 5µM reduces oocyst formation in 99%.

#### 1.4.1.3. Atovaquone

Atovaquone is a hydroxy-1,4-naphthoquinone compound that interferes with mitochondrial electron transport of the parasite and it is used in combination with proguanil that inhibits the enzyme dihydrofolate reductase (DHFR), which is essential for pyrimidine syntheses, thus, compromising DNA synthesis and parasite reproduction (Srivastava & Vaidya, 1999). Both drugs act synergistically against the parasite.

The sporontocidal activity of atovaquone with proguanil in several studies including indirect and direct SMFA and *in vivo* experiments, where serum collected from volunteers treated with the combination of atovaquone and proguanil, suggests a sporontocidal activity for 7 days (Butcher & Sinden, 2003; Enosse et al., 2000). The same results were obtained by other antifolate chlorproguanil, 100% from indirect SMFA; 87% from direct SMFA. From the *in vivo* experiments, it showed reduction of

*P. falciparum* oocyst intensity by 57% and a prevalence of 32% at 0.5μg/kg (Vos et al., 2015). The safety of this compound still needs to be further evaluated.

#### 1.4.1.4. DDD107498 or M5717

DDD107498 is a quinoline-4-carboxamide that resulted from a series of optimizations from different compounds. It acts by blocking protein synthesis in *P. falciparum* via inhibition of the translation elongation factor 2 (*PfeEF2*). This drug was demonstrated to reduce oocyst prevalence by 50% at 3.7 nM (IC50= 3.7 nM) by indirect SMFA. The same results were obtained at 10 nM in direct SMFA. This drug seems to have sporontocidal effects on ookinetes at a concentration of 5nM (IC50= 3.7 nM) (Baragaña et al., 2015). It is now in phase I clinical trials to evaluate the safety and tolerability.

#### 1.4.1.5. Cipargamin

Cipargamin (KAE609) is an antimalarial drug that interacts with PfATP4 (P-type ATPase), an efflux pump responsible for keeping the $Na^+$ at low concentration in the parasite. The $Na^+$ efflux occurs simultaneously to an $H^+$ influx to maintain an electrochemical gradient. If the $Na^+$ ATPase are inhibited, there is an increase of $Na^+$ inside the parasite, consequently, compromising the electrochemical gradient essential to ions transportation (K. J. Saliba et al., 2006). It has been demonstrated the great potency of this drugs against all gametocyte stages as well as the sporogonic stages of *P. falciparum* (Spillman & Kirk, 2015). The effect of Cipargamin involves morphologic changes on gametocytes making them swollen and rounded at concentration of 5μM (van Pelt-Koops et al., 2012). The sporontocidal activity was tested by indirect SMFA and the observation demonstrated a significant reduction in oocyst and sporozoite prevalence (>85%) at a single dose of 8.1 mg/kg (Upton et al., 2015). Cipargamin is in phase II of clinical trials (Bouwman et al., 2020).

### 1.4.1.6. Ganaplacide

Ganaplacide (KAF156) is an anti-malarial drug from imidazolopiperazine class of antimalarials (White et al., 2016). This drug showed to have gametocytocidal effects in earlier stages of gametocytes development at 5nM, blocking the oocyst formation and sporontocidal activity, thus, reducing the oocysts formation in 90% in *P. falciparum* by direct SMFA assays. The *in vivo* experiment showed no mosquito infectivity at 100 mg/kg concentration in *P. berghei* infected mice (Kuhen et al., 2014). The Ganaplacide is in phase II trials for *P. falciparum* and *P. vivax* (White et al., 2016).

For the new generation of antimalarial drugs, the ivermectin not only showed to inhibit erythrocyte stages and late gametocytes at concentration of 558.7 nM (IC50 = 558.7 nM), but also, sporogonic stages development by killing the mosquito when it presents in the mosquito bolus (de Carvalho et al., 2019). Its mode of action involves interfere with glutamate-gated chloride channels that are involved in locomotion and feeding in invertebrates (Wolstenholme, 2012). The lethal concentration of 10.7 ng/mL kills 25% of the mosquitoes that feeds from gametocytaemic blood containing ivermectin ($LC_{25}$ = 10.7 ng/mL), thus, reducing oocyst and sporozoite prevalence. For the survival in the mosquito, ivermectin showed to inhibit oocyst latest stages at that concentration and reduce sporozoite prevalence 3 days prior infection (de Carvalho et al., 2019). Although the ivermectin has been demonstrated to be effective against multistage of asexual and sexual life cycle and toxic to the vector - *Anopheles gambiae,* it needs further investigation to evaluate the properly dosage adequate to kill the mosquito and in terms of safety which is a challenge since the drug remains circulating in the bloodstream, however, testing lethal concentration can lead to mosquito resistance and mutant selection due to frequent exposure to the drugs.

There are several potent compounds against liver and erythrocytic stages with gametocytocidal or sporontocidal effect which are promising drugs to use alongside ACTs as a complementary tool to fight the parasite. It also, contributes to prevent the emergence of new drug resistance since this has been a concern due to rapid spread of resistance parasites. The idea to create a single drug to target multistage from

schizogonic and sporogonic cycle enhance malaria treatment but there are issues to overcome, such as, establishing an adequate concentration and timing capable of blocking transmission since the delivery to the vector is indirect (the drugs are taken by the human and passes to the mosquito), thus, reducing vectorial capacity. The persistent search and design for novel antimalarial drugs with transmission-blocking activity are still ongoing. Some TBD´s already created, showed to be effective in laboratory but need to be studied for safety and tolerability or are in clinical trials, others have not yet been applied in the field.

### 1.4.2. Transmission-blocking vaccines

One way to prevent and reduce malaria transmission is producing an effective vaccine capable of inhibiting the parasite development inside its hosts. The transmission-blocking vaccines (TBV) aims to target proteins expressed by sexual/sporogonic stages of the parasite by inducing the immune system to produce antibodies able to recognize antigens expressed in those stages and by doing so, reducing viability, and interfering with its development within the mosquito midgut, thus, preventing the dissemination of the parasite through the community.

The antibodies produced by these vaccines aim to target *Plasmodium* antigens, such as, P230 and P48/45, proteins that are essential for male gamete fertilization.

During exflagellation process, the flagellar male gametes formed binds to the neighbouring uninfected RBC´s forming, the so-called, exflagellation centers. This binding interaction is promoted by the p230 protein (Marin-Mogollon et al., 2018; Rupp et al., 2008). The function of p48/45 remains unclear, but it is known that these proteins play an important role in epitope recognition and adherence to the female gamete (van Dijk et al., 2001).

P25/28 are expressed on the zygotes/ookinetes surface (Cowman et al., 2016). The expression of P25 occurs on female gametes persisting in the zygote and increasing in

ookinetes. This protein is essential for ookinete EM invasion and the establishment and maturation of the oocyst (McLeod et al., 2019).

Ookinetes also secrete chitinase, an enzyme responsible for disrupting the peritrophic matrix (PM), which the major component is chitin, allowing the ookinete to penetrate the PM. This enzyme is essential for ookinete establishment. Blocking the ookinete invasion by inhibiting chitinase function reduces the odds of oocyst formation (Cowman et al., 2016), hence, chitinase is a potential candidate for TBV. A monoclonal antibody designated 1C3 was shown to inhibit oocyst formation by neutralizing the enzymatic activity of *P. falciparum* chitinase (PfCHT1) (Langer et al., 2002; Tsai et al., 2001).

The female gametocyte/gamete antigen P47, a paralogue of P48/45 protein family, is a potential candidate for TBV development. However, it was recently showed that antibodies produced as immune response against this protein do not have transmission-blocking activity (Acquah et al., 2019; Canepa et al., 2018).

TBV targeting the P25 and P230 have achieved the human pre-clinical trials status (S.A. et al., 2015; Wu et al., 2008), but P25 is the only one that is in phase I (Chaturvedi et al., 2016).

The pfs25-CP VLPs with Alhydrogel® vaccine is the one in phase I clinical trials (https://clinicaltrials.gov/ct2/show/NCT02013687). This vaccine was formulated by creating a hybrid protein by fusing Pfs25 to the alpha mosaic virus coat protein (CP) (a virus that infects plants) and manufactured in Nicotiana benthamiana plants. Studies performed in murines demonstrate that, one or two doses of Pfs25-CP VLPs plus Alhydrogel® have a transmission-blocking activity >98% with a persistent immunization of the mice for at least 6 months. So, the antibodies produced by the mouse´s immune system induced by this vaccine, revealed a highly potential candidate for TBV (Jones et al., 2013).

Other studies based on the immunogenicity evaluation and safety of this vaccine in healthy humans showed no serious adverse events for the group administered with 30µg of the vaccine. In contrast, for 100µg dose group, 31% were adverted events reported but nothing related to dead or serious illness. The best results were for the 30µg group, showing a great immune response and acceptable safety after the 3$^{rd}$ dose, however, the transmission reducing activity (TRA) for this group was weak. Only the 100µg dosage afforded a significant value of 36.2% TRA after the 3$^{rd}$ vaccination. (Chichester et al., 2018).

The development of TBV´s is still in progress and so far, none of them have been used in the field. It is mandatory to find more molecules/epitopes for a large range of targets increasing the odds of producing more vaccines efficient to target as many antigens as possible, including those antigens describe above. A single vaccine able to induce the individual's immune system to produce antibodies able to recognize and bind to several epitopes from the antigen will present better results in preventing infection than just a vaccine that targets a single epitope, and this will mark the development of new TBVs, although, there is a long way to accomplish this idea.

In conclusion, it would be essential to discuss more TBS´s and concentrate the efforts to discover new TBD´s to be used, simultaneously with TBV´s for the advancement of malaria control.

### 1.4.3. How to produce monoclonal antibodies for further applications?

Specific antibodies designed to target several *Plasmodium* life cycle stages can be produced by generating hybridomas through the fusion of B cell lymphoblasts extracted from the mice (containing the antigen) with myeloma cells (Kruisbeek, 1997; Puligedda et al., 2019). The hybridomas when cultured produce monoclonal antibodies to target a specific antigen. The production of antibodies against antigens expressed by sexual/sporogonic stages can be used when conjugated with fluorophores, which confer fluorescence to the antibodies. These antibodies can be used in flow cytometry technology for the screening of different sexual/sporogonic stages, as well as to differentiate the male and female gametocytes/gametes and evaluate the viability of gametocytocidal/sporontocidal drugs (Chevalley et al., 2010). Those fluorescent antibodies also enable us to identify the sexual/sporogonic stages when observed on the immunofluorescence microscope as in this present work. Both techniques depend on the antibody used and the fluorescence distribution on the parasite to analyse the maturation progress.

The hybridomas have different expression levels of antibodies depending on the cell clone. The antibodies produced by a hybridoma cell culture can be detected and quantified for posterior purification. One way to quantify the antibodies is by ELISA assays.

The advantage of using hybridomas technology is to produce, constantly, larger amounts of antibodies *in vitro* as an alternative method to replace mice used for the purpose. Nowadays, it is applied in numerous applications, as well as the ELISA methodology to detect many molecules, including the antibodies.

# 2. OBJECTIVES

## 2. OBJECTIVES

1. To generate *in vitro P. berghei* ookinetes.

2. To generate *in vitro P. falciparum* ookinete.

3. To produce anti-pfs25 and anti-pbs21 monoclonal antibodies by persistent hybridoma culture.

# 3. MATERIALS AND METHODS

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## 3.1. Biological material

### 3.1.1 *Plasmodium species*

*Plasmodium falciparum* 3D7 strain (MRA-102, ATCC® Manassas Virginia). The strain came from Biodefense and Emerging Infections Research Repository (BEI Resources).

*Plasmodium berghei* (PbGFP) strain expressing green fluorescent protein (GFP) under the control of *Hsp70* promoter (Hliscs et al., 2013).

### 3.1.2. Mosquitoes

Female *Anopheles stephensi* mosquitoes grown in the insectary from IHMT (Instituto de Higiene e Medicina Tropical) with 12h light-dark cycle at 26ºC, 70-80% humidity and fed with 10% glucose (VWR chemicals®) solution.

### 3.1.3. Mice

*Mus musculus* female CD1 were bred at the Instituto de Higiene e Medicina Tropical animal house in agreement with Portuguese National Authority for Animal Health - DGAV (Direção Geral de Alimentação e Veterinária), according to license (009511 from 21 April 2019). The mice were used to obtain blood for *Plasmodium berghei* ookinete cultures according to Portuguese law (Decreto-lei nº 113/2013**)** and European legislation (DIRECTIVE 2010/63/EU).

### 3.1.4. Red blood cells

O-positive human blood was drawn intravenously from a donor after an informed consent agreement. The extraction was performed by a certified specialist, into blood

collection tubes containing EDTA to prevent blood clotting. 10 to 15 mL of blood were purified by washing 5 times with 1x PBS (same blood volume) and centrifuged for 3min at 2500rpm. Before starting, the plasma was collected and stored at -20ºC. The purified erythrocytes were diluted in cRPMI Medium making 50% hematocrit and stored at 4ºC.

### 3.1.5. Hybridoma cell lines

The 4B7 monoclonal cell line (MRA-28, ATCC® Manassas Virginia) designed to produce specific IgG antibodies anti-Pfs25 were kindly gifted by Dr. Xavier Fernandez-Busquets Nanomalaria group from Institute for Bioengineering of Catalonia (IBEC) was obtained originally from the BEI-Resources malaria depository for malaria research (BEI Reagent Search (beiresources.org).

The 13.1 monoclonal cell line was kindly gifted by Dr. Inga Siden-Kiamos Infections & Immunity group from FORTH Institute of Molecular Biology & Biotechnology (IMBB-FoRTH) and produces specific IgG antibodies anti-Pbs21.

The hybridomas were cultured in hybridomas medium as followed in the methods section.

## 3.2. Solutions

### 3.2.1. Hybridoma Culture

#### 3.2.1.1. Hybridoma Medium

Hybridoma medium was prepared by adding 5.2g of RPMI1640 (Biowest®) to 500mL of milli-Q water. After filtering, 10% sterile fetal bovine serum (FBS) (Biowest®) and 0.1% of penicillin/streptomycin were added to the final solution as a final concentration.

### 3.2.1.2. Freezing Medium

Freezing medium was made by mixing 10% (V/V) DMSO, 20% (V/V) of FBS (Biowest®) and 70% (V/V) of hybridomas medium.

### 3.2.3. Giemsa Solution

The Giemsa solution was used to stain the blood smears after being fixed with methanol for 20s. The 20% (V/V) solution was prepared by adding 20mL of Giemsa Azur eosin methylene blue (Merck®) and 80mL buffered water into a 100mL flask. The solution was filtered and stored at 4ºC.

### 3.2.4. D - Sorbitol Solution

50g of D-sorbitol (Sigma®) were dissolved in 1L of Milli-Q water making the final concentration of 5% (m/v) and autoclaved for sterilization.

### 3.2.5. Nycodenz Solution

55,2g of Nycodenz (Gibco®, Life Technologies™) were added to 200mL of sterile PBSx10 making a final solution of 55% (m/v) and filtered for sterilization.

### 3.2.6. Preparation of cRPMI Medium for parasite culture

The complete RPMI (cRPMI) medium was made by diluting the following components in Milli-Q water.

| Components | cRPMI medium |
|---|---|
| **RPMI 1640 (Biowest®)** | 10.44g/L |
| **Albumax (Gibco®, Life Technologies™)** | 5g/L |
| **Hepes (VWR®)** | 5.94g/L |
| **Hipoxantine (Sigma®)** | 0.1g/L |
| **$NaHCO_3$ (Merck®)** | 2g/L |
| **Ph** | ~7.4 |

**Table.1 – Preparation of cRPMI medium**

In the end, the final solution was filtered using a sterilized 0.22µm filter and stored at 4ºC.

### 3.2.7. Preparation of Ookinete Medium

The ookinete medium was prepared by mixing the following components:

| Components | Ookinete Medium |
|---|---|
| **RPMI 1640 (Biowest®)** | 10.44g/L |
| **Hepes (VWR®)** | 5.94g/L |
| **Hipoxantine (Sigma®)** | 0.1g/L |
| **$NaHCO_3$(Merck®)** | 2g/L |
| **Xanthurenic acid (Sigma®)** | 100µM |
| **FBS (Biowest®)** | 20% |
| **Penicillin (Gibco®)** | 50U/mL |
| **Streptomycin (Gibco®)** | 50µg/mL |

| | |
|---|---|
| **Ph** | ~7.4 |

**Table.2 – *Plasmodium* ookinete medium components**. This medium was used for *P. falciparum* and *P. berghei* ookinete culture.

### 3.3. Anti-body production

The antibodies can be produced by monoclonal hybridomas. The 4B7 cell line produce antibodies anti-pfs25 and 13.1 cell line produce antibodies anti-pbs21.

#### 3.3.1. Hybridoma thawing

An aliquot from frozen hybridomas was thawed by transferring 500µL from the aliquot to a 15mL Falcon tube and resuspended in 5mL of hybridoma medium. The hybridomas were centrifuged for 6min at 1200rpm. After the supernatant was removed, the pellet was resuspended in 6mL of new hybridoma medium and transferred into a 50cm$^2$ flask and incubated at 37ºC and 5% $CO_2$.

#### 3.3.2. Hybridoma culture

In order to maintain a healthy growth, the thawed hybridoma culture had to be treated by weekly changing the medium and checked under the inverted microscope (OLYMPUS CK40). At every medium change, the cells were transferred into a tube and centrifuged for 6min at 1200rpm. The supernatant containing the produced antibodies was collected into a new 50cm$^2$ flask and stored at 4ºC. The resuspension of the pellet was executed by adding 6 mL of hybridoma medium and the culture was placed back into the culture flask at 37ºC and 5% $CO_2$.

### 3.3.3. Hybridoma Freezing

When the concentration of the cultures was up to $1\times10^6$ cells/mL, the cultures were split or frozen.

For freezing, a solution with 10% DMSO, 20% FBS and 70% hybridoma medium was prepared and added to the cell pellet obtained after centrifuging the culture and removing the supernatant. The resuspended cells were divided into 1mL sterilized cryo-tubes and stored at -192ºC in liquid nitrogen.

Before freezing the hybridomas, the cells were counted in a Neubauer chamber and dye with cell titer blue to check cell viability. The cell concentration was adjusted to $\pm1x10^6$cells/mL.

### 3.4. *In vitro* development of *Plasmodium berghei* ookinetes

For the *P. berghei* culture, infected blood extracted from a mouse with a parasitaemia >20% was used. The mouse was anesthetized with 10µL/g of a solution previously prepared with 10% Rompun/20% Imalgene diluted in PBSx1, and its reflexes were periodically checked to see if the mouse was unconscious, then a cardiac puncture was performed to extract the blood and a sample of 300µL was directly transferred to a 25cm$^2$ flask containing 6mL of ookinete medium previously prepared (see section 3.2.7). The culture was left incubating for 24h at 21ºC. The syringe used for blood extraction had approximately 20UN of EDTA, to prevent blood clotting.

After 24h, the ookinete parasitaemia was observed under the microscope (1000x). For the observation, a slide was prepared with 10µL of the ookinete culture and by making swirling movements with the micropipette to disperse the cells. Then, a membrane-feeding assay was made to check ookinete viability.

### 3.5. Standard membrane feeding assay (SMFA) – *Plasmodium berghei*

For this experiment, 500µL of *P. berghei* culture was pipetted to the feeder. A mosquito container with, approximately, 30 female *An. stephensi* was placed under the glass membrane feeder. The mosquitoes were fed for 1h, covered with a blanket at room temperature, to create the basic environment for mosquito survival.

The fed mosquitoes were selected and fed onwards with 10% sugar feeding solution.

### 3.6. Mosquito dissection

Eight days after the SMFA, the mosquitoes were dissected after been aspirated and anesthetized by leaving them on the freezer for 1min. To proceed to the dissection, the mosquitoes were killed by placing them in 80% ethanol and washed in PBSx1. The midguts were extracted by making a slight incision between the thorax and the abdomen and gently pulling the thorax forward. Carefully, the midguts were placed in a PBS drop on the slide and covered with a cover slip to observe on fluorescence microscope.

### 3.7. Oocyst counting by fluorescence microscopy

The midguts were observed on the fluorescence microscope (Nikon Eclipse 80 I) and the oocysts present were counted to evaluate whether ookinetes from the *in vitro* culture were viable.

### 3.8. *In vitro* culture of *P. falciparum*

The *P. falciparum* culture protocol was adapted from *Trager and Jensen, 1976*. *Plasmodium falciparum* (3D7) were cultured in 25cm$^2$ flasks with 3mL of cRPMI medium and 150 µL of RBC to make a 5% hematocrit, and then incubated at 37ºC, 5% $CO_2$. The medium was changed daily for parasite survival. When the cultures reached around 3-5% parasitaemia, they were transferred to a Petri plate, to make a 10mL culture.

### 3.9. Parasitaemia calculation

To estimate parasitaemia, blood smears were prepared to be observed under optic microscope. The smears were fixed with methanol and then stained with 20% Giemsa solution for 15-20min. After being washed with water, the slides were observed in the microscope (1000x). The parasitaemia was estimated by calculating infected RBC's percentage, in 5 different regions of the smear, according to the following equation:

$$Parasitemia(\%) = \frac{\text{number of infected erythrocytes}}{total\ number\ of\ erythrocytes} \times 100$$

### 3.10. Synchronization of *P. falciparum* culture

The culture was transferred into a 15mL falcon tube and centrifuged for 2min at 2500rpm. After supernatant removal, re-suspension was done by adding 10mL of cRPMI medium to the pellet. The solution was then transferred to a 10mL culture plate along with 450µL of RBC, to attain 5% hematocrit. When a 10-12% parasitaemia was reached, the culture was synchronized to obtain the same parasite asexual stages (ring stages), allowing them to develop into schizonts simultaneously.

Synchronization started by the centrifugation of the culture for 2min at 2500rpm and once the supernatant was removed, 10mL of 5% D-sorbitol was added. Then, the

cells were lysed by agitation in the vortex and left to incubate for 10min at 37ºC. Finally, the culture was centrifuged at the same previous conditions to separate the supernatant, containing the sorbitol and lysed cells. The obtained pellet containing the ring stages was washed by adding 10mL of PBSx1 and then centrifuged for 3min, at 2500rpm. The synchronized culture was then placed in the plate with 10mL of cRPMI medium, and incubated at 37ºC, 5% $CO_2$.

### 3.11. Separation of *P. falciparum* schizonts

This step was performed to separate the schizont stages from the other asexual stages.

For this procedure the culture had a parasitaemia of schizonts between 8-12%. The medium was partially aspirated, and the cultures were transferred to a 15mL Falcon tube. The cultures were diluted in 5mL of PBS and 10mL of 55% Nycodenz were placed into another Falcon tube. The diluted cultures were placed drop by drop into the Nycodenz solution, creating a density gradient. The cultures were then centrifuged at 110rcf, at 27/28ºC for 20min, speed 5 and brake 0. This originated a 3 different layers solution, as in Figure 6.

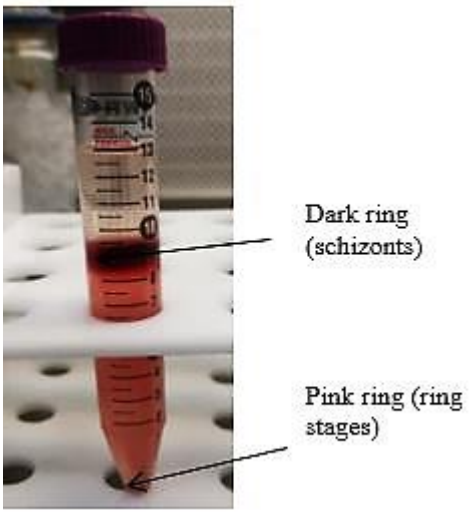


**Figure 6 – Isolation of *P. falciparum* schizonts generated by a density gradient.** The picture shows three different layers generated by the Nycodenz gradient. The bottom layer formed by a pink ring represents the ring stages of the parasite and on the top, the dark layer represents the Schizonts (latest stage).

The dark ring (containing the schizonts stages) was gently removed and placed in a new tube and then washed twice with 7mL of PBS 1x. The supernatant was discarded and 9mL of cRPMI medium was added to the cultures in each tube. The cultures were transferred to a 6 well plate, each one containing 3 mL of schizont culture to reduce the hematocrit to 0,5%, and then incubated for 24h at 37ºC, 5% $CO_2$.

### 3.12. *In vitro P. falciparum* gametocyte culture

To inhibit merozoite invasion and the start of a new asexual cycle, 5UN of heparin were added to the cultures during medium changing, 24h and 48h after the Nycodenz procedure. At this point the medium added was supplemented with 10% plasma. To prevent heat loss given that the gametocytes are sensitive to temperature variations, all the steps were performed above polystyrene. At this point, the parasites started to differentiate into gametocytes. The medium was changed daily, and a smear was prepared every 2 days to check gametocyte development.

Three days after applying the two heparin injections, the hematocrit had to be restored once it was reduced by the lysis of the dead schizonts.

### 3.13. *In vitro Plasmodium falciparum* ookinete culture

The protocol used for ookinete culture were adapted from *Bounkeua et al, 2010* and *A.K. Gosh & Jacobs-lorena, 2013b.*

Once gametocytes reached their mature form stage V, exflagellation was induced to promote male gametogenesis for further female fertilization. The cultures were centrifuged and the ookinete medium was added to a 6 well plate culture. 3mL of ookinete medium were plated in replicates.

The plate was incubated at 21ºC, for 30h under agitation (100rpm/min) inside an improvised humidified chamber. At the first 15min, the cultures were submitted to $CO_2$ presence to mimic mosquito's midgut environment (drop of temperature, $CO_2$ presence and pH rise) where the male gamete exflagellation occurs. Before this last step, a 100µL of the culture was observed under the light microscope to check for exflagellation by breathing on the sample every 2 min, for the previously mentioned reason. The following scheme represents the protocol steps established for the *in vitro* ookinete culture. The same protocol was performed at 26ºC.

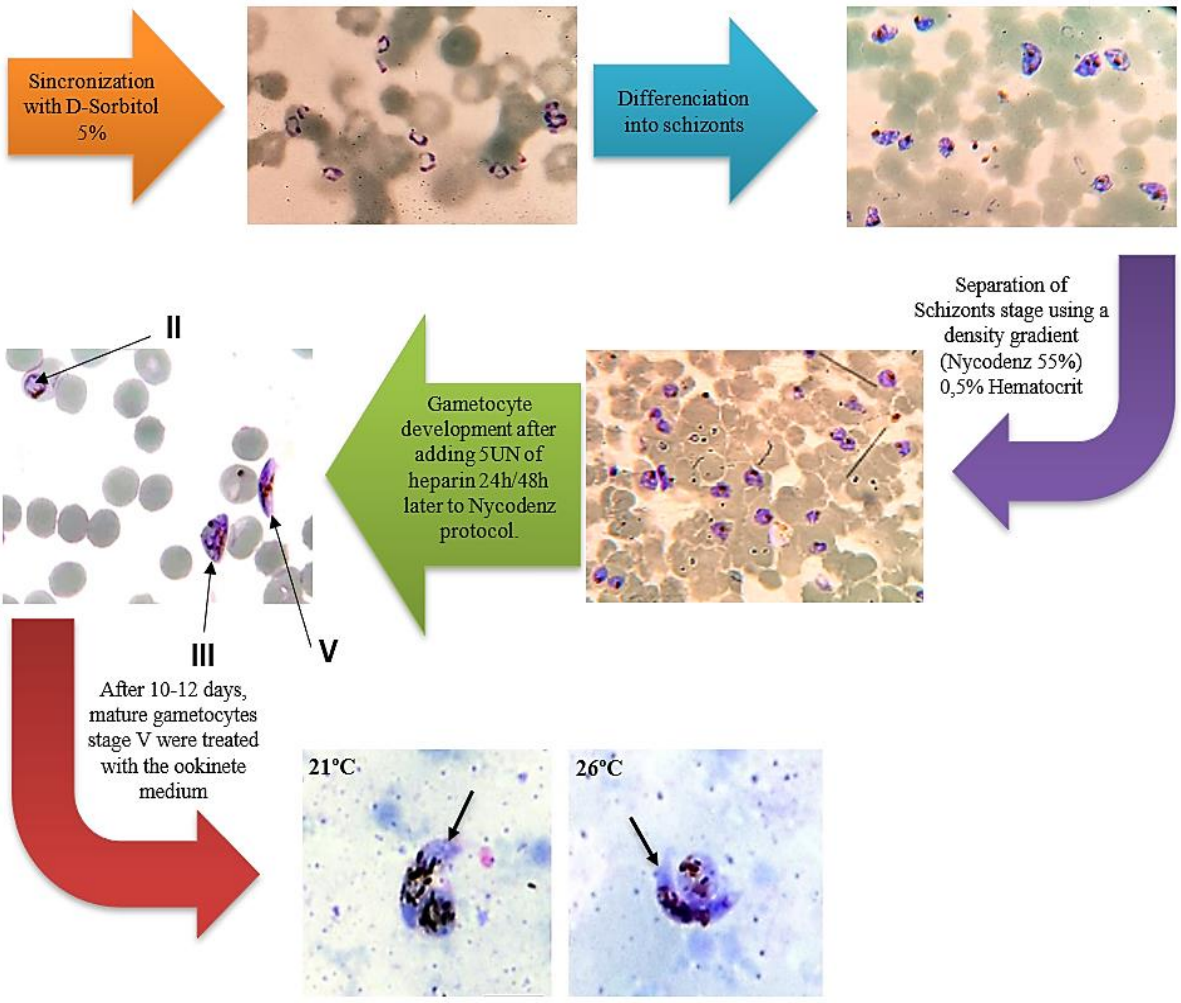


**Figure 7 – Schematic Representation of *in vitro P. falciparum* ookinete culture.** 3D7 *P. falciparum* strain was cultured and kept growing at 37ºC with 5.0% $CO_2$ and synchronized with 5% D-sorbitol after reaching a 5% parasitaemia. The cultures grew for a week and were then split into four cultures at 6-8% parasitaemia. After reaching a parasitaemia of 8-12% in schizonts stage, the cultures were submitted to a density gradient to separate the schizonts from the rest of the asexual stages. At this point, gametocytogenesis was induced and gametocytes started to develop. 24h and 48h after the Nycodenz, the cultures were treated with 5UN of heparin. The gametocytes took 10-12 days to mature into stage V gametocyte. The ookinete medium was added to the gametocyte cultures to induce exflagellation, fertilization and start ookinete development.

### 3.14. Identification of *Plasmodium falciparum* ookinetes

Ookinete cultures were centrifuged, and the medium was aspirated, leaving enough volume to dilute the concentrated cells.

To prepare blood smears, 10µL of each pellet were dropped into glass slides. Parasites were fixed with 4% paraformaldehyde and left for 30 min on the shaker at room temperature. Then, the smears were washed in 1x PBS, twice, for 2 min. After drying, slides were blocked with 2% BSA solution for 1h. After washing, an incubation step for 24h with anti-Pfs25 (0.64mg/mL) antibody was performed (except for the negative controls), following immunostaining steps with a secondary antibody anti-mouse IgG conjugated with FITC (Sigma) (1:500) for 1h and DAPI ((1:5000) diluted in PBSx1) for 10min at room temperature. The slides were washed with PBS and dried after applying each solution. The slides were placed in humid chamber with wet paper, protected from light. After two washes in PBS and one in Milli-Q water, the slides were mounted adding 15 µL of Prolong™ Gold Antifade Reagent (Invitrogen) on the bottom of the coverslip. The slides were visualized by immunofluorescence microscopy (Nikon Eclipse 80 I with DS-Ri1image system) and stored at 4ºC, protected from light.

# 4. RESULTS AND DISCUSSION

Ookinetes are motile cells that cross mosquito midgut epithelium and lodge beneath the basal lamina to develop into a mature oocyst where thousands of sporozoites are formed and will be released through the mosquito bite, during a blood meal, to infect the human host (Bounkeua et al., 2010).

Improving the methodology to obtain a large amount of ookinetes in culture is crucial, allowing to test different compounds and to design vaccines targeting ookinetes, thus, blocking their formation (TBV) in the mosquitoes.

### 4.1. *In vitro* differentiation of *Plasmodium berghei* ookinetes

Understanding the biological processes of *P. berghei* (a murine malaria model), permits studying the biology of *P. falciparum*, the deadliest human malaria specie.

*Plasmodium berghei* mature gametocytes are round while *P. falciparum* mature gametocytes are elongated, even so, sporogonic stages of *P. berghei* are morphologically similar to those of *P. falciparum*. The mature ookinete is slightly curved giving the appearance of a "banana" shape that is easily identified on Giemsa-stained smears. In the mature ookinete, the hemozoin can be found congregated into dense granules wrapped in vacuoles in one or both edges (Dessens et al., 2011; Recio-Tótoro et al., 2020), as illustrated in Figure 8 and Figure 9.

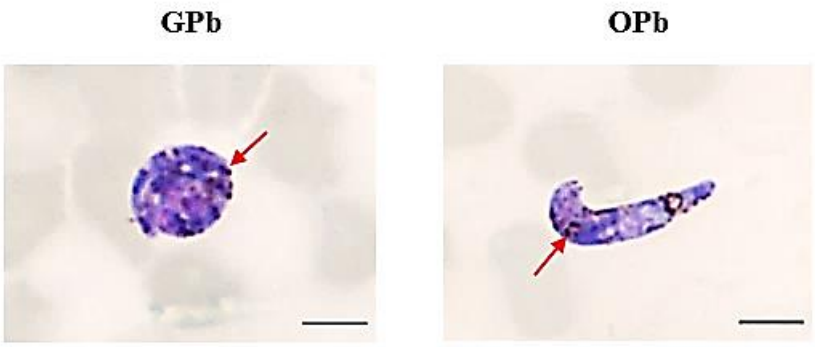


**Figure 8** – **Morphology of *Plasmodium berghei* gametocyte/ookinete observed on light microscopy** (1000x)**. GPb** - *P. berghei* gametocyte; **OPb** - *P. berghei* ookinete. The red arrows show the dense granules - black dots (hemozoin congregated) present on *P. berghei* ookinetes. Scale bar 5µm.

Approximately, $1\times10^6$ ookinetes/mL were obtained when *P. berghei* gametocytes were induced, *in vitro* for 24h at 21ºC to differentiate into ookinetes with the ookinete medium (described on materials and methods chapter 3.2.7.). The protocol used to obtain *P. berghei* ookinetes *in vitro* was from *Rodriguez et al, 2002*.

*Plasmodium berghei* ookinetes were originated from a GFP-expressing *P. berghei* strain (the same used in Giemsa-stained blood smears (Figure 8). Parasites were continuously grown in the mice and later collected by heart puncture for the purpose of *in vitro* culture of ookinetes. the fact that the *P. berghei* cycle is synchronous facilitated the experiment, not being necessary to perform a synchronization assay, as was done in the *in vitro P. falciparum* ookinete culture experiment.

The first column of figure 9 shows *P. berghei* ookinetes observed in bright field. The green staining corresponds to the GFP expression by *P. berghei* in the cytoplasm. The DNA is represented by the blue staining originated by DAPI staining.

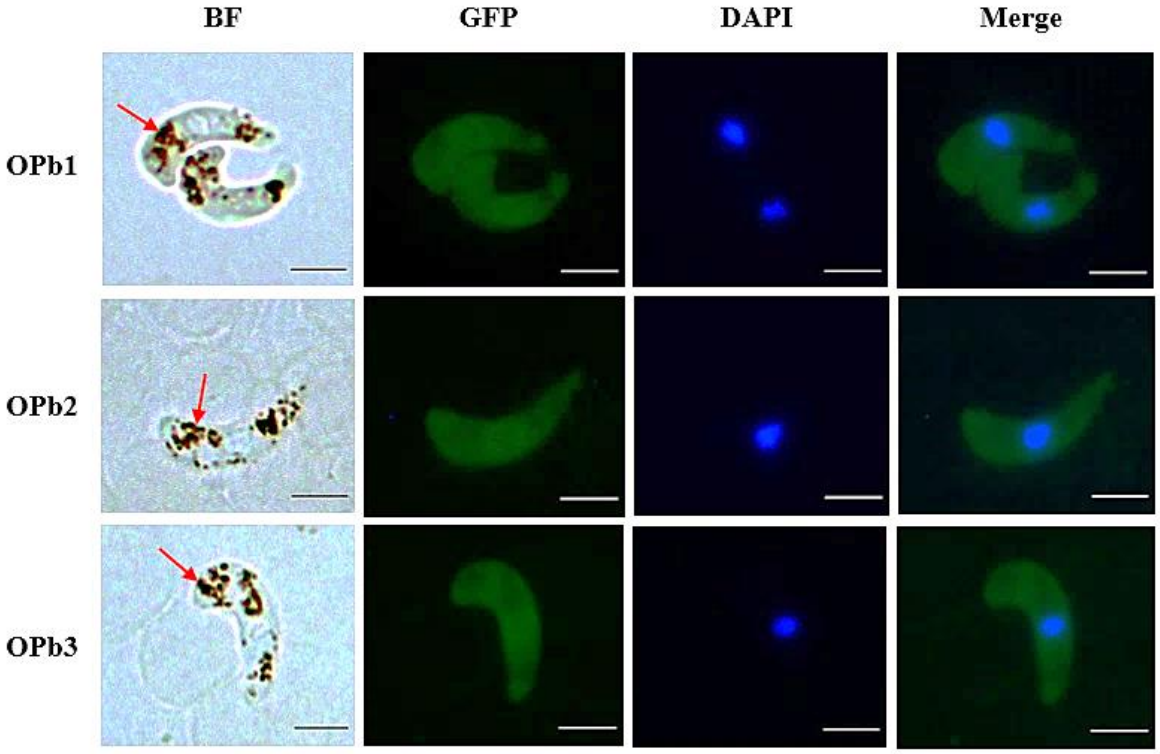

**Figure 9 – *Plasmodium berghei* mature ookinetes observed by fluorescence microscopy** (1000X)**.** In bright (BF) field it is visible the hemozoin congregation (dense granules) represented by the black dots, marked on the figure by the red arrows. The green staining corresponds to GFP expression (509 nm); DAPI - DNA staining (358 nm). OPb1-3 - mature ookinetes; Scale bar 5µm.

The ookinetes were identified based on the morphologic characteristics of these species, such as the "banana"-like shape and hemozoin distribution (Dessens et al., 2011; Recio-Tótoro et al., 2020), which can be identified on Figure 8OPb. Due to their characteristic shape, mature ookinetes are easily distinguishable from gametocytes or other asexual stages on Giemsa smears, as exhibited on Figure 8GPb, and by fluorescence microscopy (Figure 9).

The ookinete yield obtained from *in vitro* culture was $1\times10^6$ ookinetes/mL, which was relatively low compared to previous description in the literature. *Janse, Mon, et al., 1985* and *Carter et al., 2003* obtained an ookinete yield of $6.22\times10^6$ ookinetes/mL and $3.75\pm0.48\times10^6$ /mL, respectively.

This low number may be related to gametocyte death due to gametocyte sensitivity to temperature variation, hence, lowering the gametocytes yield. According to *Janse, Mon, et al, 1985*, the temperature which the gametocytes were submitted to was 21ºC (the same temperature at our ookinetes culture), on the other hand, *Carter et al, 2003*, submit the ookinete culture to 19ºC, both using the same ookinete medium components. The first obtained more ookinetes per mL than the last, suggesting that to obtain a higher ookinete yield cultures should be submitted to 21ºC; therefore, the protocol stablish in this work must be improved.

### 4.2. Testing the viability of *Plasmodium berghei* ookinetes from the *in vitro* culture

To test the viability of the *in vitro* produced ookinetes, the culture was fed to *An. stephensi* mosquitoes using a Standard Membrane Feeding Assay (SMFA). The mosquito infection rate was determined by counting the number of infected midguts, and the infection burden by counting the number of oocysts in each infected midgut, after mosquito dissection. In parallel, the SMFA were also performed with whole blood from an infected mouse (15-20% parasitemia) (Figure 10).

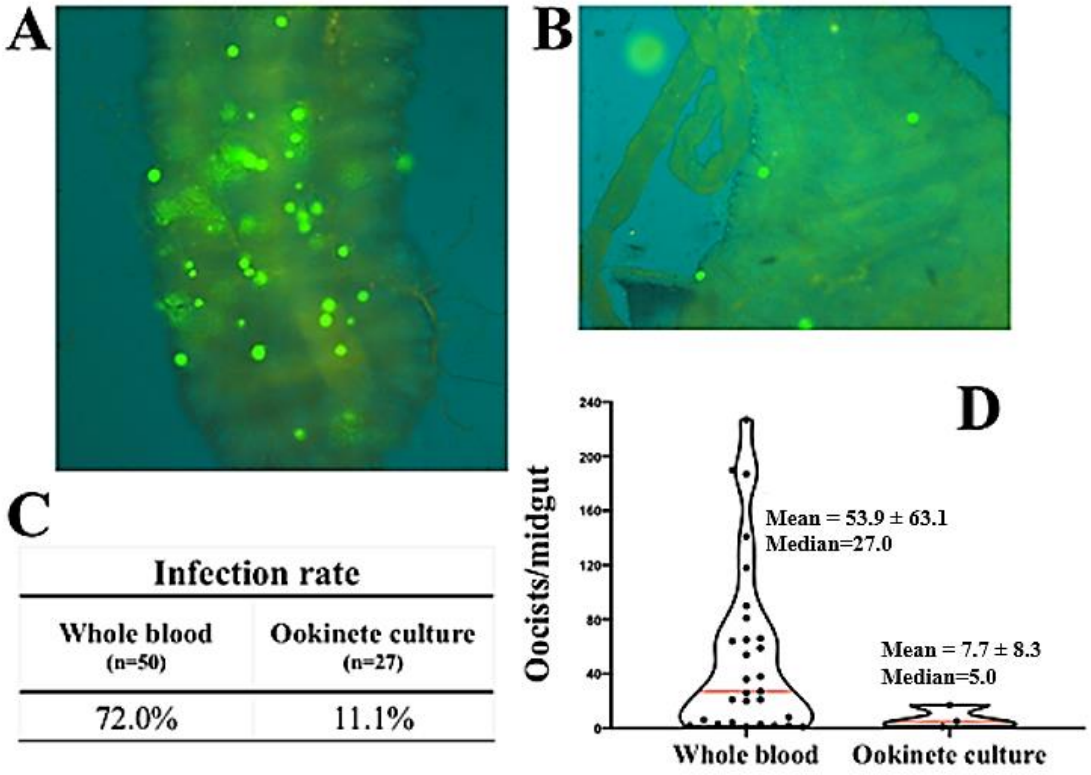


**Figure 10 – Mosquito infection by SMFA, whole blood vs ookinete culture.** The images illustrate two midguts from *An. stephensi* mosquitoes containing oocysts resulted from infected whole blood obtained by cardiac puncture (A) and *in vitro* ookinete culture (B); Infection rate, % of infected mosquitoes (C); Number of oocysts in each infected midgut, red line – mean (D).

The midguts in Figure 10 were dissected 8 days post-infection by SMFA. The infection rate (number of infected mosquitoes over the total number of the fed mosquitoes) was higher using whole blood 72% than the ookinete culture 11.1% (Figure 10C). The mean number of oocysts counted per midgut also followed the same tendency; For whole blood the mean was 53.9±63.1 and for the *in vitro* ookinete culture 7.7±8.3 (Figure 10D). The high SD can be justified by the broad range of oocysts counted per midgut. Similar results were observed by *Ifediba et al., 1982* who reported a mean of 69±30 oocysts per midgut for mosquitoes fed, through SMF, of hamster blood, and 19±12 for mosquitoes fed from *in vitro* formed ookinetes.

During the SMF using the ookinete culture, most mosquitoes didn't feed (just 3 females were feed from the 30 mosquitoes used) which may contribute for low infection rates and, consequently, to the poor number of oocysts developed. Those mosquitoes were probably already fed with sugar solution for which was placed in the mosquitoes cage, for mosquito survival, from where they were collected, making them feed less or not feeding at all from the culture. The mosquitoes fed with whole blood were in starvation since they were not fed from sugar solution. Another factor that may be contributed to reduce the oocysts formed is the mosquito pre-urination which is the process by which they expel blood from the bottom during the blood meal to avoid overheating (Lahondre & Lazzari, 2012). Releasing the ingested infected blood contribute for the loss of parasites ingested during the blood meal.

Feeding mosquitoes on mice blood allows the parasite to develop in their natural environment (host) and proceed to the next stages of their sexual and sporogonic cycle, thereafter. The number of oocysts developed in the mosquito's midgut fed with whole blood can be higher compared to the parasites developed *in vitro*, as ookinetes developed *in vivo* are under the influences of mosquitoes factors, such as, molecules derived from mosquitoes midgut. One of these factors is Xanthurenic acid which was added to the ookinete medium used in this experiment, even so, lower numbers were observed. In conclusion, the ookinetes generated *in vitro* were able to develop into oocysts.

### 4.3. *In vitro* differentiation of *Plasmodium falciparum* ookinetes

The ability to induce ookinete formation *in vitro* allows us to explore *P. falciparum* ookinetes biology without involving infected patients or infected mosquitoes (the later requires high security laboratory facilities BSL3). Initially, it was necessary to produce gametocytes from a synchronized *in vitro P. falciparum* culture. Schizonts were isolated using a density gradient and induced to differentiation into gametocytes. Figure 11 shows images of the morphology of the different stages of *P. falciparum* gametocytes.

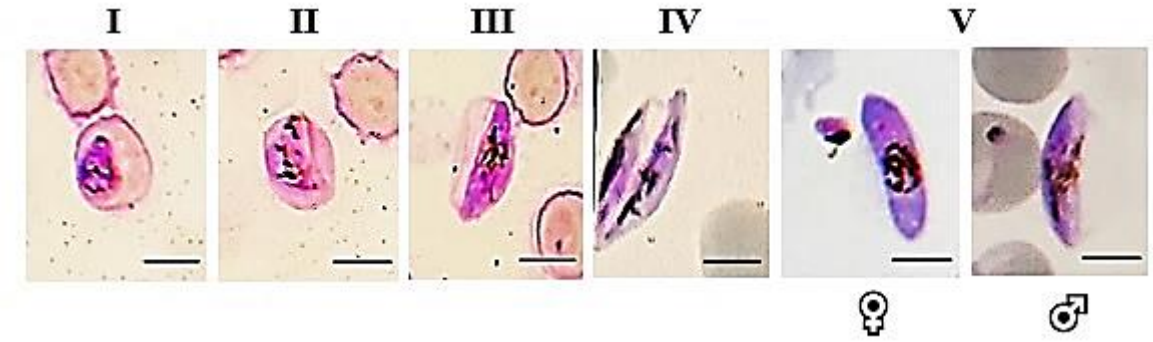


**Figure 11 – *In vitro* development of *P. falciparum* gametocytes.** Stages **I-V** stained with Giemsa on a smear observed by Light microscopy (1000x). **I –** Round shape; **II –** D - shape, extension of the gametocyte edges to the erythrocyte edges and expansion to one side closely to erythrocytes membrane; **III** – Elongated, exceeding the membrane of the erythrocyte; **IV** - Thin with pointed tips. At this stage, the male and female gametocyte are distinguished by their hemozoin distribution. In male gametocytes hemozoin crystals are scattered while in the female they are congregated and dense; **V** - Elongated and slightly curved giving a falciform shape. Scale bar 5µm.

The stage I and stage II gametocytes are round. At stage III, the gametocyte elongates to the erythrocyte edges, exceeding its membrane, and one side of the gametocyte expands reaching the erythrocyte membrane. The elongation continues until stage IV, forming thinner and pointed shape. At the final stage V, the gametocyte is elongated and present some curvature.

The differences of both sexes earlier can be observed in stage IV gametocyte which persists to the stage **V.** According to male and female gametocytes morphology,

on Giemsa-stained smears, the female is bluish due to high density of ribosomes and have a more defined and discrete nucleus while the male gametocytes have no ribosomes and has a less distinct and diffused nucleus giving the appearance of a pinkish cytoplasm. These characteristics can be observed on Figure 11(IV and V). The hemozoin distribution, along with those characteristics, allows us to determine the gametocyte gender. The same characteristics were described by *Delves et al., 2016; Jensen, 1979; K. S. Saliba & Jacobs-Lorena, 2012***.** These authors also induce gametocytogenesis *in vitro* and shown the same morphology, confirming our results. Indeed, we were able to produce gametocytes by *in vitro* culturing.

Figure 12 represents images of ookinetes produced *in vitro* using the ookinete medium described on materials and methods chapter section 3.2.7.

*P. falciparum* ookinetes are morphologically similar to stage V gametocytes. The resemblance between mature gametocytes and mature ookinetes obtained by *in vitro* culture, observed in Giemsa staining can be seen on Figure 12.

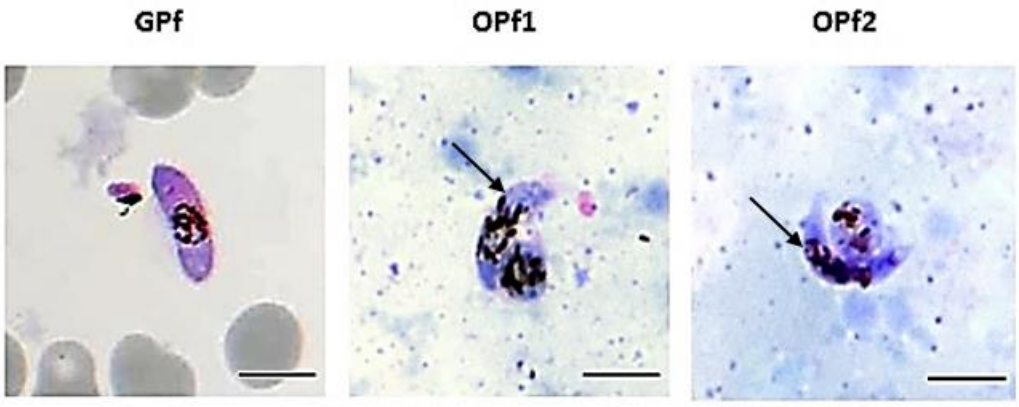


**Figure 12 - *In vitro* development of sexual/sporogonic stages of *P. falciparum* observed by light microscopy** (1000x). GPf- *P. falciparum* gametocyte; OPf1-2 - *P. falciparum* ookinetes obtained from *in vitro* ookinete culture, marked with the arrows. Scale bar 5µm.

The ookinetes OPf1 and OPf2 derived from the *in vitro P. falciparum* gametocyte culture.

Despite the morphologic similarities between both gametocyte and ookinete, when these are visualized on light microscopy, gametocytes are slightly different from the ookinetes. The edges of *P. falciparum* gametocytes are rounder than the ookinete edges (Delves et al., 2017). More so, the smearing process can induce parasite deformations leading to misidentification of ookinetes (Figure 12). Hence, in order to discriminate *P. falciparum* gametocytes from ookinetes, an immunofluorescence staining was performed using monoclonal antibodies (Ab) anti - pfs25 (a protein mainly expressed in ookinetes stages).

The number of *P. falciparum* ookinetes produced by *in vitro* culture, according to fluorescence microscopy observations, varied from 5-8 ookinetes/10 µL (equivalent to 500-800 ookinetes/mL of culture). Whereas, some authors, such as, *Bounkeua et al., 2010* and *Siciliano et al., 2020,* report ookinete concentrations of 5-60×$10^5$ ookinetes/mL and 1.5×$10^6$ ookinetes/mL, respectively, which take us to conclude that, the ookinete concentration obtained in our experiment was low, considering previously published data.

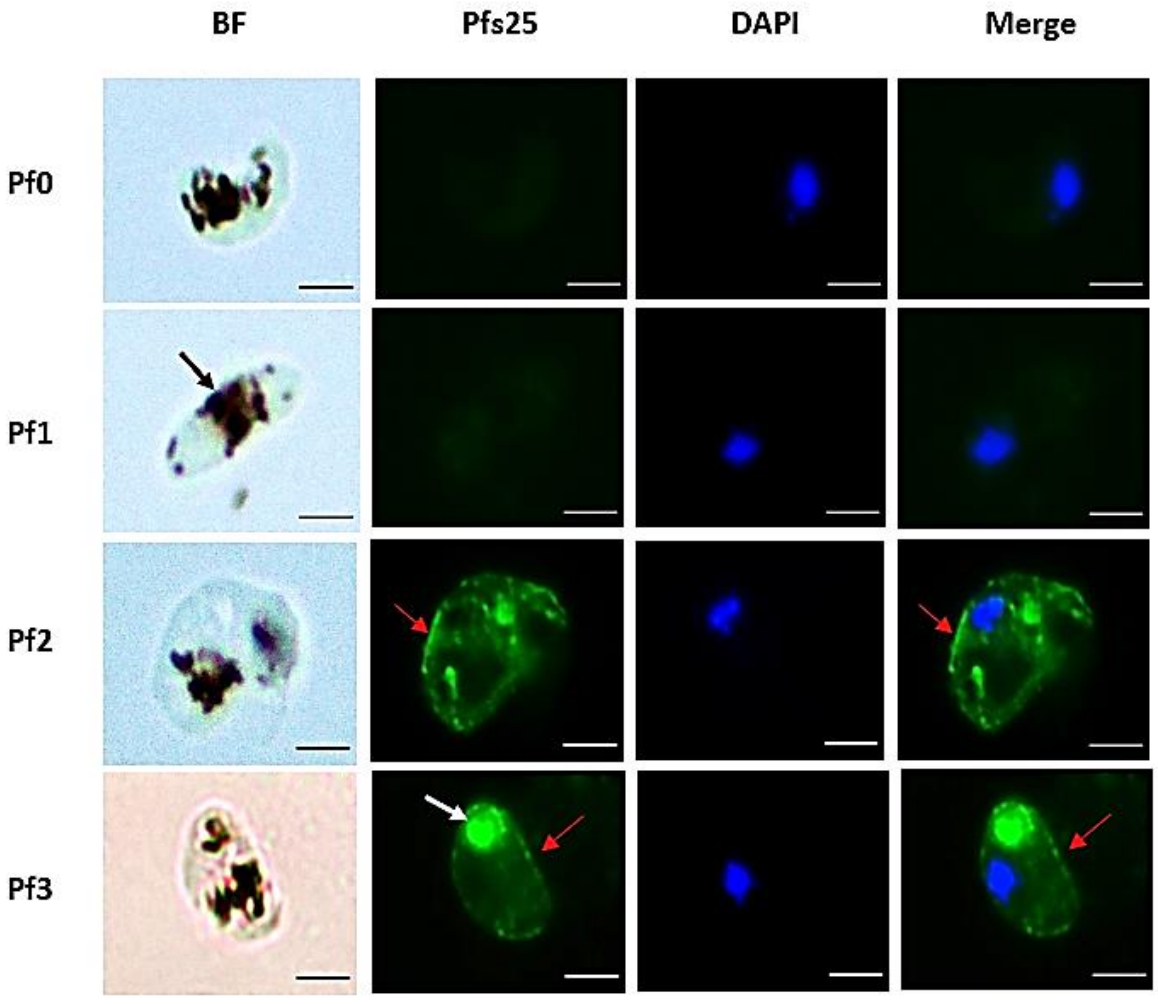

**Figure 13 - *Plasmodium falciparum* sexual/sporogonic stages resulted from *in vitro* ookinete culture observed by fluorescence microscopy** (1000X). BF - bright field; Pfs25 - anti-pfs25 IgG monoclonal antibody stained with a secondary monoclonal anti-mouse IgG conjugated with FITC (495 nm – wavelength); DAPI - DNA staining with DAPI (358 nm); Pf0-3 *P. falciparum* stages; Pf0 - *P. falciparum* sexual/sporogonic stage without the anti-pfs25 mAb (negative control); Pf1 - mature gametocyte (stage V gametocyte as a negative control accurate specificity to ookinete), the white arrows indicate the hemozoin concentration. Pf2 - *P. falciparum* mature ookinete originated at 21ºC; Pf3 - *P. falciparum* mature ookinete originated at 26ºC. The red arrows point to anti - pfs25 antibodies location on ookinete surfaces. Scale Bar 5µm.

Considering the image Pf0 (negative control), in Figure 13, no fluorescence is present due to lack of anti-pfs25 Ab in the staining buffer so, it is not clear if it is a gametocyte or an ookinete since both are indistinguishable chiefly when cultivated *in vitro*. Pf1 is a stage V gametocyte stained with anti-pfs25 Ab representing, also, a negative control to check if fluorescence is present, considering the Ab presence in the staining buffer, further suggesting that pf1 is a female gametocyte since no fluorescence is seen (Figure 13FITC) and the hemozoin is concentrated as seen in the bright field image displayed (Figure 13BF). Female gametocytes have the hemozoin agglomerated while male gametocytes have it scattered (Dixon et al., 2012).

The distribution of protein Pfs25 is at the surface of *P. falciparum* ookinetes, therefore, it can be used as a target to identify ookinetes by fluorescence microscopy (using anti - pfs25 antibodies) (Bounkeua et al., 2010). Parasite Pf2 and Pf3 on Figure 13(pfs25) are ookinete forms due to the presence of a green outline on the cell surface. The green outline on ookinetes surface is what it is expected to see by fluorescence microscopy as previously described by other authors as *Bounkeua et al.,2010* and *Siciliano et al., 2020*.

On the upper extremity of Pf3 [Figure 13(pfs25)] a round bright green fluorescence structure (arrow) is compatible with, what is known as, the residual spherical body that consists of a membrane that surrounds the microgamete and remains attached to it after the exflagellation (Delves et al., 2017; Kuehn & Pradel, 2010; Siciliano et al., 2020). However, Mature ookinetes are generally described as elongated forms with round edges and curve body resembling a “banana” shape (club or fluke)

(Bounkeua et al., 2010; A. K. Ghosh & Jacobs-Lorena, 2013b; Siciliano et al., 2020; Vinetz, 2005).

We could not observe such morphology clearly. Nevertheless, anti – Pfs25 mAb stained cells were seen (Figure 13pfs25), positively identifying ookinetes. The stages observed may are probably immature ookinetes form (Siciliano et al., 2020; Delves et al., 2017).

The gametocytes from the *in vitro* gametocyte culture were fully mature gametocytes. During the maturation process, dead parasites were found on the smears prepared with the gametocyte cultures. At least, one mature gametocyte was seen in every 5 fields. The lower the number of gametocytes in culture, the lower are the zygotes and, consequently, ookinetes formed. *In vivo,* the conversion rates decrease from gametocyte to gamete, and from gamete to ookinete, because, inside the midgut, the ookinetes must overcome the physical and chemical barriers to cross the peritrophic matrix (PM) (Delves et al., 2017; A. K. Ghosh & Jacobs-Lorena, 2013b). Inappropriate incubation temperatures at the gametocyte cultures to induce ookinete formation, might have contributed for the poor results (500-800 ookinetes/mL of culture). Perhaps, the incubation temperature of 21ºC was too low and 26ºC was too high. For the formation of ookinetes, it is mandatory a decrease in temperature of, approximately, 5ºC to activate *P. falciparum* gametocyte to differentiate into gametes within the mosquito midgut (Bennink et al., 2016b).

Improving the methodology to obtain a larger quantity of gametocytes *in vitro* will increase the probabilities to obtain more ookinetes.

A decrease in temperature, increase in pH and the presence of xanthurenic acid are crucial factors to induce gamete exflagellation, however, there is some non-identified important factors from the mosquito midgut that might be fundamental to the process.

# 4. RESULTS AND DISCUSSION

The protocols used to obtain *P. berghei* ookinetes worked, but still must be improved to increase the ookinete yield. The protocol used for *P. falciparum* culture also needs to be revised to enrich the number of ookinetes in the culture.

The exflagellation of *P. falciparum* male gametes was difficult to detect at light microscopy, so as counting the exflagellation centers for calculation of the conversion rates.

The slides prepared with the *P. falciparum* ookinete culture contained inactive plasma cells from the blood serum added to the gametocyte medium leaving the smears with some plasma cell, hampering the observation by light and fluorescence microscopy. This could be solved by improving serum pipetting after centrifugation, preventing cell suction.

The identification of *P. falciparum* ookinetes by immunofluorescence also needs to be optimized to improve the signal by reducing the anti-pfs25 antibody dilution from 1:1000 to 1:700 concentration.

The quantification of gametocytes and ookinetes is critical to calculate the conversion rates from immature to full mature stage V gametocytes; the exflagellation rates and zygote/stage I to full mature stage V ookinetes.

### 4.4. Antibody production

Both hybridomas cell lines (4B7 and 13.1) grew perfectly, resulting, mostly in round and transparent healthy cells. This conclusion was taken by observing the hybridoma cells on the inverted microscope after dyeing the cells with cell titer blue (a dye that stains blue dead cells while healthy cells remain colorless) (Kamiloglu et al., 2020; Zhang, 2012). The initial cell density of the hybridoma culture was $1.00\times10^6$ cells/mL and ended up reaching a cell density of $7{,}08\times10^6$ cells/mL for the hybridoma culture cell line 4B7 (design to produce anti – Pfs25 monoclonal antibodies) and $5.16\times10^6$ cells/mL for hybridoma culture cell line 13.1 (design to produce anti - Pbs21 monoclonal antibodies). The antibodies resulted from the hybridoma culture were not purified. Before purification a checkerboard ELISA assay for each cell line would be necessary to select the cells that efficiently produce antibodies, in such way, to obtain a higher concentration of anti-Pfs25 and anti-Pbs21 monoclonal antibodies and finally purify them for further experiments.

# 5. CONCLUSIONS AND FUTURE PERSPECTIVES

# 5. CONCLUSIONS AND FUTURE PERSPECTIVES

To improve the *P. berghei* ookinete yield, is suggestive to improve the timing between the blood extraction and the and it place in ookinete medium. It is very important take in consideration the body mice temperature since it drops when they are anesthetized, and gametocytes are sensitive to temperature variation. The mice must be still alive during blood extraction for the experiment otherwise it will not result to due gametocyte dead by the low temperature.

During the gametocyte culture, many gametocytes died probably due to temperature variations during medium changing. The gametocytes are sensitive to temperature decrease. The best way to prevent it is placing the culture in a warming plate at 37ºC.

The numbers of *P. falciparum* gametocytes and ookinetes obtained were low, so the protocols and techniques must be further improved to obtain a higher concentration of gametocytes/ookinetes for later evaluating compounds with potential transmission-blocking activity.

## 5.1. Suggestions to increase the number of *P. falciparum* mature ookinetes in culture:

- Concentrate stage IV-V gametocytes using density gradients or by magnetic isolation, will probably result in higher number of ookinetes formed. The same procedure can be used to concentrate *P. berghei* ookinetes).

The gametes fertilization is an important step since it promotes the formation of the zygote and the ookinete. Therefore:

- Incubating ookinete cultures with agitation to promote exflagellation of male gametes, may increase the likelihood of zygote formation.

The hybridoma cultures result in many colonies of cells, however, the antibodies produced by it were not quantified since the ELISA assay method used for the antibody

quantification needed to be optimized for better results. Also, the anti-pbs21 antibodies produced by hybridoma cell line 13.1, usually used to target *P. berghei* sexual/sporogonic stages (Sinden et al., 1987), were not applied in the present steps of this thesis.

### 5.2 The next steps would be:

Implementation of consistent generation of *P. berghei* ookinetes *in vitro.*

Improve the *in vitro* cultures of *P. falciparum* gametocytes.

Improve the *in vitro* cultures of *P. falciparum* ookinetes.

Implementation of in-house monoclonal antibodies anti-pfs25 protein production and purification protocols.

Perform a checkerboard ELISA assay for antibody screening.

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