Development of molecular assays on *Plasmodium falciparum* gametocytes for functional analysis and novel diagnostics on malaria parasite transmission

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Sommario

1. INTRODUCTION

Malaria is a mosquito-borne parasitic disease that is endemic in 84 countries and causes an estimated 247 million clinical infections and 619 000 deaths annually (World Malaria Report, 2023).

The infectious agent is a protozoan belonging to the genus Plasmodium. Six species are known to infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium brasilianum*. *P. falciparum* causes 95% of the cases and the great majority of the severe disease prevalently in sub-Saharan Africa where the age group most affected are children under 5 years of age (World Malaria Report, 2023).

Early diagnosis and prompt effective treatment of malaria prevents severe sequelae and death. Prevention strategies for malaria include various forms of chemoprophylaxis, mosquito vector control, and, in some regions of sub-Saharan Africa, vaccination.

In the early 2000s, the introduction of artemisinin-based combination therapies (ACTs) for uncomplicated malaria and intravenous artesunate for severe malaria and improved parasitological diagnosis contributed to a substantial decline in global malaria mortality (*Dondrop et al., 2005*; Dondrop et al., 2010). Unfortunately, global malaria incidence has increased in the past 5 years due to interruption of malaria control strategies in some areas due to COVID-19 pandemic (Gonzàles- Sanz et al., 2023). Additionally, rising temperatures have expanded malaria to highland areas, exemplified by the alarming fivefold increase in cases in Pakistan following extreme monsoon rains attributed to climate change (Shaikh et al., 2023; World Malaria Report, 2023). Currently,

Malaria is endemic in sub-Saharan Africa, south-east Asia, in the easternmost regions of the Mediterranean, in west of the Pacific Ocean and in South America (Figure 1).

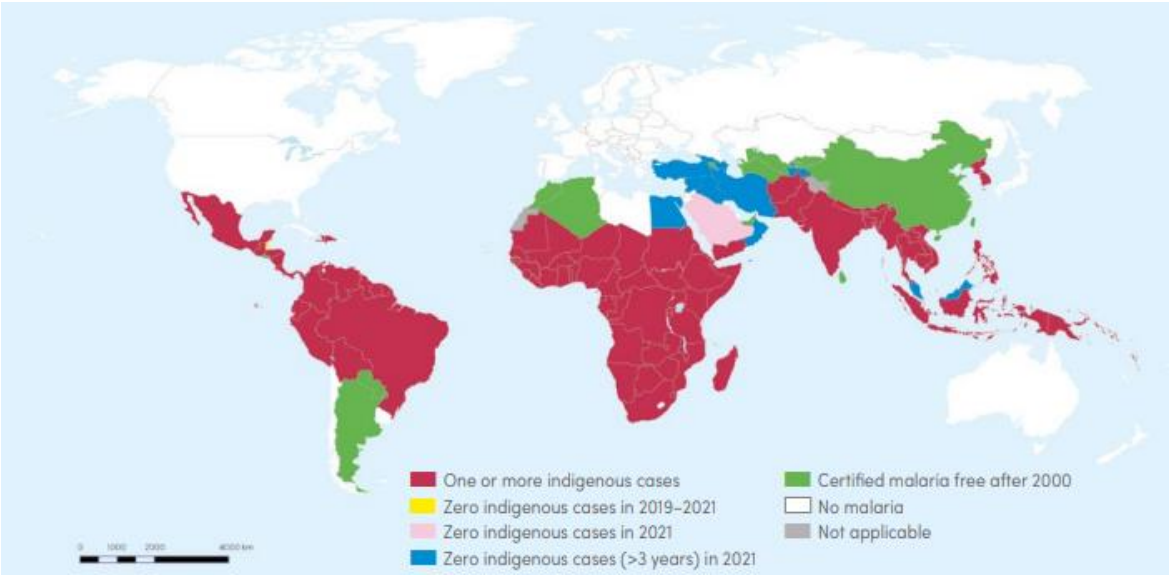


Figure 1. Countries with indigenous cases in 2000 and their status by 2021 (World Malaria Report, 2023)

1.1.1. *Plasmodium falciparum* life cycle and pathogenesis

P. falciparum presents a complex life cycle which takes place into two different hosts: the human and the *Anopheles* mosquito (Figure 2). In the human host the infection starts when a female *Anopheles* mosquito transmits microscopic motile sporozoites during its blood meal. Sporozoites are end stages of the Plasmodium sexual development. Sporozoites after being injected into the skin, enter the bloodstream, travel to the liver and invade hepatocytes. Inside the hepatocyte they multiply asexually in an asymptomatic hepatic cycle, differentiating into the liver stage schizonts, with thousands of nuclei. In the hepatic cycle the single asexual replication stage lasts approximately 8 days. With the rupture of the hepatic schizont all the merozoites are released in the human bloodstream where

they invade red blood cells (RBCs), starting the erythrocytic asexual cycle of *P. falciparum* infection, which is the responsible for the symptoms of malaria. Each intraerythrocytic asexual cycle takes around 48 hours. As the merozoite invades the erythrocyte, it spreads itself into a thin biconcave disc appearing like a ring in

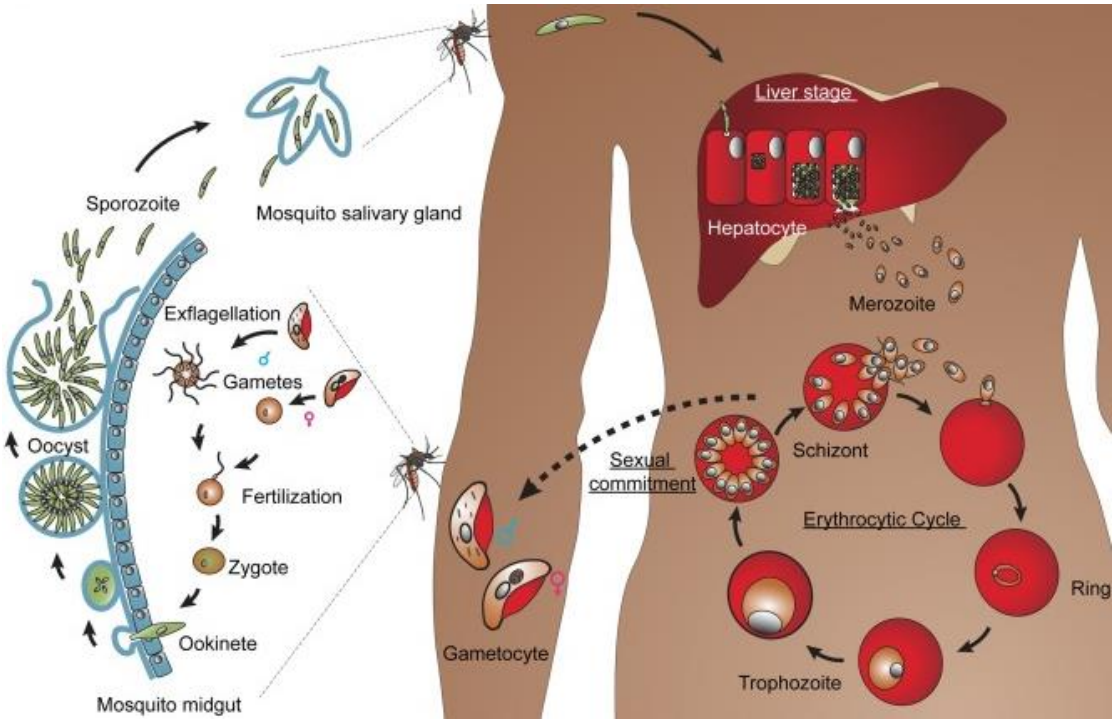


***Figure 2**. Plasmodium falciparum life cycle (Dixon and Tilley, 2021)*

Giemsa-stained blood smears. At the end of the annular stage, a parasitophorous vacuole, a membranous structure formed by the invagination of the RBC membrane at the time of merozoite invasion, which contains the parasite, is fully developed. Very fine hemozoin granules, derived from the haemoglobin catabolism, appear within the parasite digestive vacuole. The ring stage grows into a mature trophozoite in a period of active haemoglobin digestion and RBC changes. Then, the parasite undergoes a series of nuclear divisions and intense

DNA synthesis, becoming a schizont and assembling molecules needed for the next erythrocyte invasion. The rupture of the host cell releases from 10 to 20 merozoites with a concomitant fever attack, caused by the release of pyrogen substances from infected RBCs (Lawrence et al., 2003). Initially, clinical symptoms of malaria occur with cycles of chills and fever every three days. *P. falciparum* parasites cause severe anemia due to red blood cell lysis, a reduced medullary erythropoiesis and accelerated senescence of uninfected RBCs which are eliminated inside the spleen. The spleen often appears swollen, hyperaemic and so frail to undergo ruptures.

Typical of *P. falciparum* infections is the phenomenon of cytoadherence: only rings can be found within the circulating peripheral blood. A single parasite protein, the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed on both the trophozoite- and the schizont-infected erythrocyte surface, mediates binding of the infected RBC to the vascular endothelium, interacting with various receptors on the membrane of the host endothelial cells, such as CD36, thrombospondin receptor, intracellular adhesion molecule 1 (ICAM-1) and chondroitin sulfate A (CSA) (Tilley et al., 2011) (Tiburcio et al., 2015). This adherence leads to parasite sequestration in several organs, which protects the parasite from destruction, as the non-adherent mature parasitized RBCs are rapidly cleared within the spleen.

PfEMP1 is encoded by the large and diverse *var* gene family which undergoes a clonal antigenic variation, in which only one variant is expressed by the parasite. This plays a central role in *P. falciparum* pathogenesis, allowing the parasite to evade the host's immune system (Merrick et al., 2012) (Looker et al., 2019). The adhesion of infected erythrocytes to the endothelium causes slowing of blood

flow and may lead to vascular occlusion, with tissue hypoxia and ischemia. The binding of infected erythrocytes to brain venule endothelial cells can cause cerebral malaria with loss of consciousness, cerebral edema, coma and often death (Newbold C. et al., 1999).

1.1.2. *Plasmodium falciparum* gametocytogenesis

Merozoites can invade new erythrocytes to start a new asexual cycle, spreading the infection into the human host. However, a small subset of merozoites develops after invasion into male and female sexual forms called gametocytes, which are responsible for parasite transmission from its human host to the *Anopheles* mosquito. Gametocytes mature in 10-12 days during which they pass through five stages of morphological maturation (stage I-V) (Figure 3). Only mature gametocytes (stage V) are present in the human blood peripheral circulation, whereas immature gametocyte development takes place sequestered in tissues. Once gametocytes have gained maturity, they can be picked up by an *Anopheles* mosquito during its blood meal (Smalley M.E. et al., 1981) (Thompson J.G. et al., 1935). In the mosquito midgut, mature gametocytes receive the necessary signals to start gametogenesis: they lose their elongated shape (round up), egress from the erythrocyte and differentiate into male and female gametes. The male gametocyte undergoes three cellular divisions and produces up to eight flagellated and motile microgametes (exflagellation). The female gametocyte turns into a single macrogamete which is fertilized by one microgamete forming a zygote. The zygote further matures into a motile ookinete, which penetrates the gut epithelium and subsequently develops into an oocyst, whose maturation

results in the formation and release of thousands of sporozoites, which migrate to the salivary glands of the mosquito, ready to be transmitted to another human host during its next blood meal (Kuehn et al., 2010). Development within the mosquito lasts from one week to about one month, depending both on the species and climatic factors (WHO World Malaria Report 2018).

All the erythrocytic stage parasites (both asexual stages and gametocytes) can be cultured *in vitro* and liver stage parasites can grow *in vitro* inside human hepatocytes. Also, the first part of mosquito development (formation of male and female gametes) can be reproduced *in vitro*. Furthermore, *in vitro* formation and growth of the subsequent mosquito stages such as zygotes and ookinetes have been attempted, but these are inefficient in *P. falciparum* (Sinden R. E. et al.,2009) (Aly A.S.I. et al., 2009).

1.1.3. Commitment to gametocytogenesis and to sexual differentiation in *P. falciparum*

The process of switching from the asexual blood stage replication to the gametocyte differentiation is called gametocyte commitment. Commitment to gametocyte development is believed to start before schizogony, during which each schizont produces a progeny of merozoites that develop into either sexual forms or asexual blood stage parasites (Josling G. A. et al., 2015).

The 10-day-long process of gametocyte maturation is conventionally divided in five morphological stages (I-V). In the stages of maturation after stage I, gametocytes take the falciform shape from which the name *P. falciparum* derived. A stage I gametocyte is morphologically indistinguishable from the asexual

trophozoite and until a few years ago no specific marker could identify this early sexual stage (Tiburcio et al., 2015). However, recent studies using fluorescent antibodies have revealed the expression of the *P. falciparum* EXported Protein-5 (PfGEXP5) in the cytoplasm of the infected erythrocyte 14 hours after the invasion of a stage I gametocyte. PfGEXP5 export in the infected RBC is the first detectable molecular event of the parasite sexual development. PfGEXP5 expression is present during all the process of maturation and development of the gametocyte up to stage IV and then decreases in stage V (Tiburcio et al., 2015). Between 24 to 30 hours post invasion (stage I), the young gametocyte starts to produce early stage- specific proteins such as Pfs16 and Pfg27. Pfs16 is a protein expressed on the membrane of the parasitophorous vacuole (Bruce et al., 1994), whereas Pfg27 is a cytoplasmatic phosphoprotein with binding sites for RNA (Alano et al., 1991) (Sharma et al., 2003). The expression of both Pfs16 and Pfg27 proteins is controlled by the activity of the transcription factor AP2G, the most important regulatory factor for the sexual development of *P. falciparum* parasites (Kafsack B.F.C. et al., 2014) (Henry N.B. et al., 2019) (Ngwa C. J. et al., 2016).

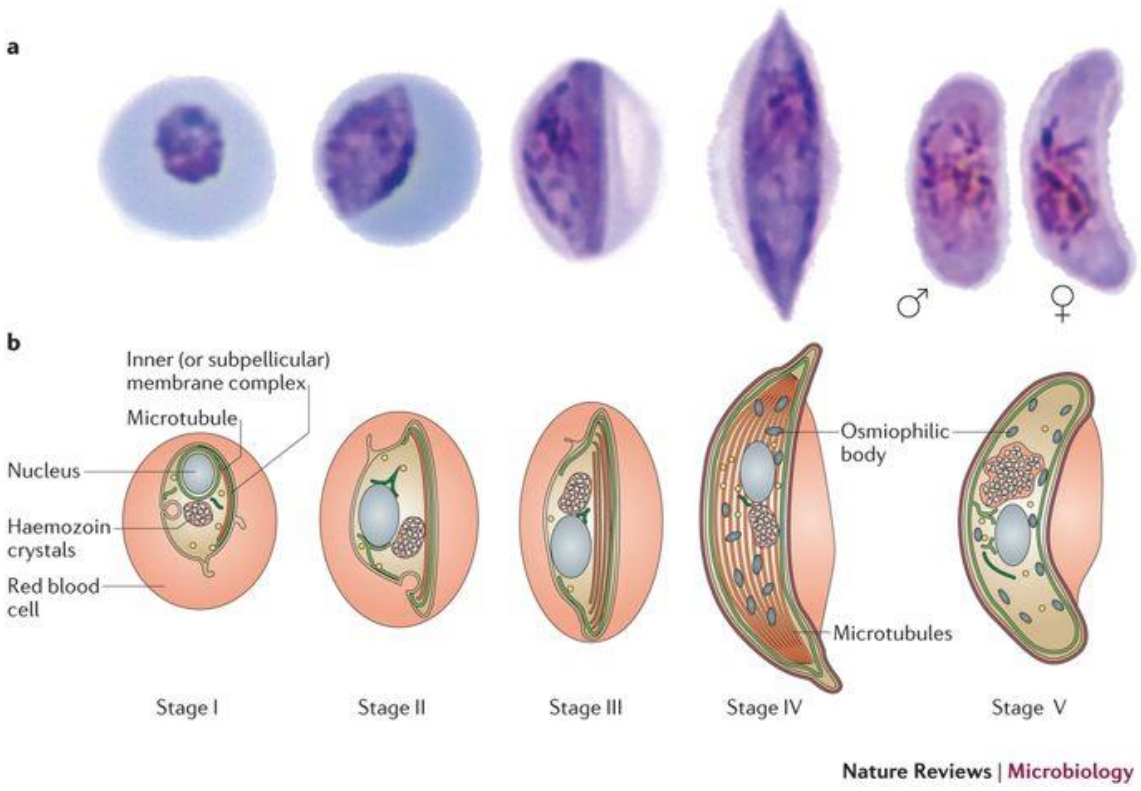


Figure 3. *Stages of maturation of P. falciparum gametocytes (shown as photographs (part a) and schematics (part b)) (Josling G. A. et al., 2015).*

In *P. falciparum*, only stage V gametocytes, are able to circulate in the peripheral blood vessels, where they are picked up from the mosquito during its blood-meal, fulfilling their essential role in malaria transmission. All immature stages of gametocytogenesis are found in the bone marrow and in the internal organs. The first evidences date back alredy in 1899 by Bastianelli and Bignami. Furthermore, even in the recent years, analysis of autoptic specimens (Joice et al., 2014), bone marrow aspirates (Aguilar et al., 2014) and one clinical case (Farfour et al., 2012) confirmed that the *P. falciparum* gametocytes preferentially accumulate in this organs and are not only confined inside the microvasculature but are also found within the bone marrow extravascular compartment, the stroma (Farfour et al.,

2012; Joice et al., 2014). This mechanism of sequestration of immature gametocyte-infected RBCs away from the general circulation is considered a survival strategy to escape mechanical filtration by the spleen. Unlike immature gametocytes from stage I to stage IV, whose development increases the stiffness of infected erythrocytes, mature gametocytes are deformable and can therefore circulate without being retained in the spleen (Farfour et al., 2012).

Stiffness may help retention in the bone marrow environment, whereas relaxing of the parasite-induced stiffness of the infected host erythrocyte may explain the presence of mature stage V into the peripheral blood, as this process may lead mature gametocytes to cross the vascular endothelium.

The mechanisms involved in the immature gametocyte's stiffness consist in a network of microtubules placed below their membrane and an actin cytoskeleton at the poles that make them the elongate shape. In the last part of their maturation, the microtubule network depolymerizes, and the actin cytoskeleton dissociates giving stage V gametocytes a greater flexibility. However, the rigidity of immature gametocytes does not depend on the cytoskeletal organization, as a treatment with an agent that destabilises microtubules, trifluralin, does not restore cell deformability. The change in mechanical properties of gametocyte-infected erythrocytes is linked to a reorganization of skeleton nanostructure and to reversible changes in the interaction level between the skeleton and the plasma membrane.

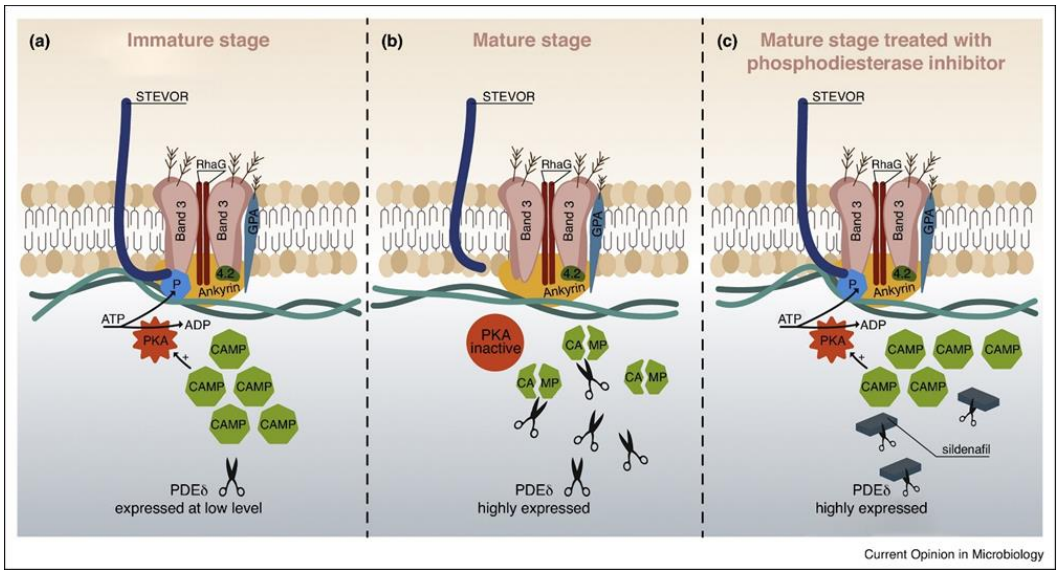


Figure 4. *Model of regulation of gametocyte-infected erythrocytes deformability (Lavazec C., 2017)*

A protein involved in the reduction of the infected erythrocytes deformability in stage V is STEVOR (Figure 4) which is expressed by both the asexual and the sexual stages of *P. falciparum* parasites. STEVOR is a transmembrane protein whose cytoplasmic domain can interact with the components of the ankyrin complex, such as α-spectrin, β- spectrin, ankyrin and band 3. This interaction is mediated by cAMP-dependent kinases (PKA) phosphorylation. PKA activity leads to the rigidity of immature gametocytes and the flexibility of mature forms: a recent study shows that these factors are linked. The cytoplasmic domain of STEVOR proteins can be phosphorylated by PKA, influencing the host erythrocyte deformability. STEVOR, by binding the components of the complex, could increase the vertical restraints between the skeleton and the lipid bilayer preventing the translational spread of transmembrane proteins. These interactions make the membrane become more rigid. The decrease in cAMP levels in mature gametocytes reduces STEVOR phosphorylation, preventing its

interaction with ankyrin. This leads to an increase in the deformability of infected erythrocytes (Lavazec, 2017; Ramdani et al., 2015).

1.1.4. Malaria Prevention

Vector Control

Vector control is an essential component of prevention. In areas of moderate or high transmission in Africa, deployment of pyrethroid-insecticide-treated mosquito nets and the use of indoor residual spraying are important components of malaria control (Mabaso et al., 2004; Chaccour et al., 2021). Wide-scale deployment of such nets has contributed substantially to the fall in malaria morbidity and mortality. Long-lasting insecticide treated nets that retain activity for the natural life of the net without retreatment have been developed. These insecticide-treated nets protect the community and kill anopheline mosquitoes (Moonen et al., 2010; Godfray H Charles J, 2013). However, the use of pyrethroids in agriculture and widespread deployment of insecticide-treated nets has put a tremendous selection pressure on anopheline mosquitoes and resistances have emerged. As a result, bed nets impregnated with two active insecticidal ingredients with different modes of action provide better malaria protection than single pyrethroid-only nets in areas of pre-existing mosquito pyrethroid resistance (Mosha et al., 2022; Accrombessi et al., 2023). In addition to chemical resistance, some species of *Anopheles* mosquitoes that typically bite at night and indoors are changing their behavior and showing resistance to traditional control measures as they also bite outdoors and during daylight hours (Sougoufara et al., 2017; Russell et al., 2013). In some of this cases, outdoor residual spraying has

shown promise as a potential means to combat this behavioral change (Mosha et al., 2022; Accrombessi et al., 2023).

In some parts of Asia, the main mosquito vectors, such as *Anopheles stephensi*, bite outdoors in the evening or early morning and therefore the protective effect of bednets and residual spray are limited. Recently, the spread of the *Anopheles stephensi* vector from Asia to the Horn of Africa and West Africa has raised many concerns. This vector was first reported in Djibouti in 2012 (Faulde et al, 2014). Then, its presence has been reported from Ethiopia in 2016 (Carter et al., 2018), Sri Lanka in 2017 (Gayan Dharmasiri et al., 2017), Sudan in 2016 (Ahmed et al., 2021), Somalia in 2019 (Ali et al., 2022), Nigeria in 2020, Yemen in 2021, in Kenya and Ghana 2022 (https://apps.who.int/malaria/maps/threats/; Afrane et al., 2023). In 2019, a WHO Vector Alert recognized the threat of emergence and spread of *An. stephensi* outside its native geographical range, calling for vigilance and actions (World Health Organization, 2019). *Anopheles stephensi* breeds mainly in water tanks, water storage containers, construction sites, desert coolers, wells and other human-made habitats (Sharma, 1995; Singh et al., 2021). It is a major malaria vector in urban settings in India and successfully sustains malaria transmission (Subbarao et al., 2019). For this reasons WHO in 2023 publicized an update called "WHO initiative to stop the spread of *Anopheles stephensi* in Africa" with the following aims: i) Encouraging collaboration among stakeholders for effective regional approaches; ii) Emphasizing entomological and human malaria case surveillance to target control measures; iii) Stressing the importance of sharing information at national and international levels; iv) Providing evidence-based guidance for national malaria control programs, with regular updates; v)

Prioritizing research to evaluate control interventions and find better ways to respond to *An. stephensi.*

The initiative aims to enhance coordination, surveillance, information sharing, guidance development, and research to address the threat of *An. stephensi* in Africa.

Vaccination

After more than four decades of basic research and clinical trials, in 2021 the World Health Organization (WHO) has recommended the first malaria vaccine for widespread use among children living in areas of moderate and high *P. falciparum* transmission. RTS,S/AS01 is a recombinant protein of the malaria vaccine that targets the circumsporozoite protein (CSP) of *Plasmodium falciparum*, expressed by the malaria parasite at the pre-erythrocytic stage, in which part of the circumsporozoite sequence is coexpressed with fused and free hepatitis B surface antigen and formulated with the AS01 adjuvant. CSP is located on the surface of the sporozoites and it is crucial for their development, for helping the parasite to invade salivary glands in mosquitoes, and is implicated in the binding of sporozoites to liver cells (Ménard et al., 1997; Frevert et al., 1993). In African phase 3 clinical trials (RTS,S Clinical Trials Partnership, 2015) three doses of the vaccine administered 1 month apart followed by a booster dose 18 months later reduced clinical malaria episodes over 3–4 years by 36% in young children first vaccinated between 5 months and 17 months of age and 26% in infants between 6 months and 12 weeks of age. Protection against severe malaria was 32% in young children and 26% in infants.

Furthermore in October 2023, WHO recommended a second malaria vaccine, R21/Matrix-M to prevent malaria in children living in areas of risk. This vaccine, also consists in a recombinant CSP protein but with a higher density. The vaccine showed good efficacy (66%) during the 12 months following the first three doses, when provided to children from 5 months of age. A fourth dose given a year after the third maintained efficacy (Stanisic and Good, 2023).

To date, other malaria vaccines are under development to achieve a protective efficacy of at least 75% by 2030.

1.1.5. Treatments of *P. falciparum* Malaria

Severe Malaria

Severe *P. falciparum* malaria is a medical emergency and necessitates intensive nursing care and careful management. This stage of the disease is defined by clinical or laboratory evidence of vital organ dysfunction. It may present with the following: impaired consciousness, acidosis, hypoglycaemia, hyperparasitaemia, severe anaemia, jaundice, significant bleeding and shock (WHO Guidelines for Malaria, 2023). Two classes of medicine are available for parenteral treatment of severe malaria: artemisinin derivatives (artesunate or artemether) and the cinchona alkaloids (quinine and quinidine). However, both a meta-analysis published in 2001 on individual patient data from several relevant studies (The Artemether-Quinine Meta-analysis Study Group, 2001), and two more recent randomized clinical trials conducted in severe falciparum malaria, showed a substantial reduction in mortality with intravenous or intramuscular Artesunate

compared to parenteral quinine. In the first trial, conducted on 1461 patients admitted to hospital with severe *falciparum* malaria in four Asian countries as Bangladesh, India, Indonesia and Myanmar between June 2003 and May 2005, Artesunate reduced mortality by 35% compared to quinine (Dondrop et al., 2005). The second trial was conducted in 11 centers in nine African countries (Mozambique, Gambia, Ghana, Kenya, Tanzania, Nigeria, Uganda, Rwanda and the Democratic Republic of Congo) in 5425 children hospitalized with severe malaria between October 3, 2005 and July 14, 2010. This study also highlighted a reduction in mortality of 22.5% in patients treated with Artesunate compared to those treated with Quinine (Dondrop A. M et al., 2010). As a result the World Health Organization recommended parenteral artesunate as the first choice anti-malarial treatment of severe malaria (Guideline WHO, 2022)

Uncomplicated Malaria

The treatment of malaria has improved substantially in the past 15 years, and morbidity and mortality have declined as a result (WHO Guidelines for Malaria, 2023). It depends on the severity of the infection, the patient's age, the degree of background immunity and the likely pattern of susceptibility to antimalarial drugs. For this reason, recommendations vary according to geographic region and should be under constant review. The major advance in antimalarial therapeutics has been the deployment of drugs derived from Artemisinin, dihydroartemisinin (DHA), artesunate and artemether, as first-line treatment of uncomplicated falciparum malaria in all areas in which malaria is endemic since the late 1990s (Lu et al., 2019). This compound is derived from the leaves of the plant *Artemesia annua.* Artemisinin and its derivatives are particularly fast-acting

against the intra-erythrocytic asexual blood-stage parasites, rendering them highly effective for the treatment of malaria. However, due to their very short in vivo half-lives, (~1 hr in humans) they are commonly co-administered with longer half-life partner drugs such as lumefantrine, amodiaquine, piperaquine, mefloquine or sulphadoxine-pyrimethamine in Artemisinin-based combination therapies (ACTs). They are the most rapidly acting of the available antimalarial drugs and they are very well tolered. However, just one year after ACTs were recommended as the first line treatment for uncomplicated *falciparum* malaria by the WHO, parasites with reduced sensitivity to Artemisinin were reported close to the Thailand-Cambodia border (Dondorp et al., 2009). Some reports documented that a subset of parasites were cleared from the blood more slowly than previously following ACTs treatment, and this phenotype has been currently used to categorise 'artemisinin resistant' parasites (Ferreira et al., 2013.; Lu et al., 2019). Since these first reports, the documented prevalence of Artemisinin resistance has expanded widely; the clinical phenotype have been documented across the Greater Mekong Sub-region of Southeast Asia, from the coast of Vietnam in the East to India in the West. There are also reports of parasites resistant to ACTs from China and, of particular concern, from Equatorial Guinea, Uganda Rwanda, Ethiopia, and Eritrea in Africa (Asua et al., 2021; Uwimana et al., 2021; Stoke et al., 2022; Tun et al., 2015; Das et al., 2018; Huang et al., 2015; Lu et al., 2017; Ikeda et al., 2018). Artemisinin resistance is characterized by a reduction in early ring stage parasite susceptibility and slow parasite clearance. Multiple studies have confirmed the association of polymorphisms in the *P. falciparum* Kelch 13 propeller protein with Artemisinin resistance, and parasites carrying various single nucleotide polymorphisms in the

gene encoding it (*PfK13*) display varying parasite clearance rate phenotypes (Mbengueet al., 2015; Mishra et al., 2015; Straimer et al., 2015). Depsite the resistant profiles, the ACTs continue to be the only widely deployable treatment option to treat uncomplicated *falciparum* malaria, even in the Asiatic context wherein the resistant parasites are highly prevalent. The WHO currently recommends five ACTs: artemether-lumefantrine, artesunate-mefloquine, dihydroartemisinin-piperaquine, artesunate– amodiaquine and artesunate–sulphadoxinepyrimethamine (WHO Guidelines for Malaria, 2023). The most recently licensed artemisinin-based combination therapy is Pyronaridine–artesunate. The current strategy to address confirmed ACT failure of more than 10% as assessed in therapeutic efficacy studies is to cycle through these ACTs based on the prevalent sensitivity of parasites to partner drugs.

1.1.6. The transmission-blocking activity targeting the human-to-mosquito transmission stage

Gametocytes are the only stages able to support transmission to mosquito. Depsite drugs are an important tool of malaria control strategies, gametocytocidal component of the treatment receive little attention. ACT treatment is very effective in killing the asexual blood stage malaria parasites and curing patients but as far as gametocytes concern it can remove only immature gametocytes and does not act against mature gametocytes (Kamya et al., 2007). This indicates that after treatment, despite being asymptomatic, an individual could carry gametocytes in the blood for days and possibly weeks, potentially available to continue the parasite cycle whether a competent mosquito bites this person (WWARN Gametocyte Study Group, 2016). To prevent transmission to

mosquitoes it is necessary to reduce the prevalence of the malaria parasite in endemic populations by targeting gametocyte reservoirs. This strategy can help also to slow the spread of resistance that is crucial, as drug-resistant parasites selected during the asexual blood stages proliferation phase can easily be transmitted. While developing dual-active compounds both on asexual and sexual blood stages may be considered easier, it may be a downside, as in the vast majority of the cases, it will block the same target in both the asexual and sexual-stage parasites, hence favoring the transmission of the resistance that might appear (Birkholtz et al., 2022). As a result, there is the necessity of exploring compounds specifically designed to block malaria transmission by targeting the unique biology of gametocytes. These compounds would remain inactive against the stages of the parasite's life cycle responsible for causing symptoms. The recommended approach involves combining these transmission-blocking compounds with two distinct antimalarials that act against the stages causing symptoms, forming a triple combination. This strategy aims to minimize the emergence of drug resistance (Graves et al., 2018).

To date, the only licensed drug recommended by WHO against the transmission stages is primaquine (PQ). It can effectively remove mature gametocytes from the blood. As a results World Health Organization (WHO) has recommended the addition of single low-dose PQ to ACTs for treating acute uncomplicated falciparum malaria in low-transmission areas, particularly where artemisinin-resistant *Plasmodium falciparum* is prevalent (White et al., 2012; WHO Malaria Policy Advisory Committee and Secretariat, 2012). Depsite recommendation of WHO, the use of PQ is not currently deployed anywhere in sub-Saharan African except for several low-endemicity countries, including Botswana, Eritrea,

Swaziland and Zimbabwe (Taylor et al., 2018; Chen et al., 2015) that have added the use of PQ to their national treatment guidelines. The main reasons cited by ministries of health for not enacting the WHO recommendation is the fear that PQ would cause acute haemolytic anaemia in Glucose-6 phosphate dehydrogenase deficient (G6PDd) individuals and the impossibility of widely deploying a suitable G6PDd test to exclude such individuals from receiving PQ (White et al., 2012). Indeed, in Africa, the average prevalence of G6PD deficiency is 7.5% and in some countries reaches 23- 39% (Nkhoma et al., 2009). Despite this, there is an increasing body of evidence that a single low dose (0.25 mg/kg) of PQ is tolerated well by both malaria patients with G6PDd and healthy individuals (Mwaiswelo et al., 2016; WHO and Global Malaria Programme: Updated WHO Policy Recommendation, October 2012; Stepniewska et al., 2022; van Beek et al., 2021).

A further problem consists in the fact that, in endemic countries there are substantial gametocyte carriages detected by molecular detection techniques amongst asymptomatic populations (Ouédraogo et al., 2009). More studies support the theory that asymptomatic, microscopically detected carriers of mature gametocytes drive a consistent percentage of transmission (Andolina et al., 2021; Schneider et al., 2007; Alves et al., 2005). Furthermore, in a study, some children with a gametocyte density below 1 gametocyte/μL were able to infect mosquitoes (Ouédraogo et al., 2009). This is surprising since a blood meal that is on average 2–3 μL, should contain at least one male and one female gametocyte to result in infection. The relative contribution to transmission per gametocyte carrier may be lower for submicroscopical gametocyte carriers but their relative abundance in a population may counterbalance this and makes them important

contributors to malaria transmission. For this reason, some countries are evaluating a low-dose primaquine drug mass administration campaigns in transmission hotspots (Raman, et al., 2019).

The efficiency with which *P. falciparum* gametocytes can infect mosquitoes means that transmission-reducing interventions, which reduce gametocyte density, will need to be highly effective in order to reduce human–mosquito transmission. As a result, with concerns about drug resistance, the need for additional drugs to prevent transmission, is evident (Burrows et al., 2013; Dechering et al., 2017). Over the past decade, there has been a renewed interest and progress in malaria drug discovery. Researchers have been exploring various inhibitors, such as ATP4 , PI4K, and AcCS inhibitors, in vitro (Paquet et al., 2017; Dechering et al., 2017; McNamara et al., 2013). These inhibitors have shown promise as novel antimalarials with transmission-blocking activity. Some of these compounds act by either killing or sterilizing gametocytes (referred to as 'anti-gametocyte') or by preventing parasite development in mosquitoes (referred to as 'sporontocidal'). To assess the effectiveness of these transmission-blocking drugs, reliable tools are necessary to measure their blocking properties. This likely involves developing assays or experimental methods that can accurately quantify the impact of the drugs on the transmission stages of the malaria parasite's life cycle. These tools are essential for evaluating the efficacy of potential antimalarial drugs in preventing the spread of the disease.

1.1.7. Gametocyte density and sex-ratio influence mosquito infectivity

The transmission of malaria from human to mosquito depends on the presence of gametocytes, sexual-stage *Plasmodium* parasites, in the peripheral blood. The

two factors that main influence the mosquito infectivity are the gametocyte density and their sex ratio. Understanding these two factors is crucial for assessing the human reservoir of infection and developing effective control strategies (Lin et al., 2016; Slater et al., 2015; Goncalves et al., 2017). Gametocyte density refers to the number of gametocytes (male and female) present in a volume of blood of an infected individual. Gametocyte sex ratio refers to the proportion of male gametocytes and is typically female biased in *Plasmodium* parasites (Bradley et al., 2018). The female biased sex ratio is explained as one male gametocyte produces up to eight male gametes (microgamete) and one female gametocyte produces one female gamete (macrogamete) (Read et al., 1992).

In human infections less than 10% of all asexual *P. falciparum* parasites undergo the commitment to form gametocytes (Collins et al., 2018; Reuling et al., 2018). Moreover, mature gametocytes typically constitute less than 5% of the total circulating parasite biomass in natural infections (Taylor et al., 1997). The rate of gametocyte production is influenced by both human factors (Carter et al., 2013) (haemoglobin level (Gbotosho G. O. et al., 2011; Drakeley et al., 1999), host immunity (Buckling et al., 2001; Ono et al., 1986) and anti-malarial drug (Talman et al., 2004)) and the parasite factors (genetic diversity of infection or mixed infection (Bousema et al., 2008), asexual stage densities (Ouédraogo et al., 2007)). The initiation of sexual commitment precedes the schizogony stage (Sinden RE, 1983; Poran et al., 2017; Bruce et al., 1990), where all the merozoites produced by a single sexually-committed schizont become either all male or all female gametocytes (Silvestrini et al., 2000). A higher percentage of schizonts giving rise to female gametocytes compared to those producing males accounts for the observed female-biased sex ratio.

Factors influencing gametocyte sex ratio have not yet been studied. In a study in which clones derived from a single isolate differed significantly, one clone maintained through fifteen subcultures showed a stable sex ratio, suggesting that the sex ratio may be clone specific (Burkot, Williams & Schneider, 1984). As an isolate of *P. falciparum* malaria from a natural infection is a mixture of genetically diverse parasite subpopulations, the balance between sexes may be influenced by the number of clones per individual (Read et al., 1992). However, other studies suggest the impact of some factors like the anemia on gametocyte sex ratio in rodent and avian *Plasmodium* parasites (Paul et al., 2000; Paul et al., 2002) and in *P. falciparum* infected children (Gbotosho et al., 2011; Robert et al., 2003). Other evidences indicate that the proportion of male gametocytes, thus the sex ratio, is negatively associated with total gametocyte density (Reece et al., 2008; Robert et al., 2003; Sowunmi et al., 2008).

It is unclear how these two factors, the density of gametocytes and the sex ratio influence infection to mosquitoes. However, prediction models have been developed over the years (Griffin et al., 2010; Smith et al., 2007). One of them (Churcher T. S. et al., 2013) established that there is a saturating relationship between gametocyte density and proportion of infected mosquito. This model predicts that beyond the level of 200 gametocytes per microlitre, infection rises to finally plateau at ~18% infected mosquitoes. A further implemented model, consider separately both male and female gametocytes (Bradley et al., 2018). In this model, the female gametocyte density is a primary predictor of infectivity and shows a rapid increase in infection, saturating at high levels (at a density of 200 per microlitre, approximately 30% of mosquitoes are infected). The model predicts that low male gametocyte densities (<50gametocytes/μl) reduce

transmission, with a further predicted 50% reduction in infected mosquitoes with a male gametocyte density < 10 per microlitre (Bradley et al., 2018) suggesting that male gametocyte density may become a limiting factor for transmission success at low gametocyte densities. It is therefore clear that the accurate measurement of both female and male gametocyte density is crucial for developing and deploying transmission-blocking strategies against malaria targeting reservoir responsible for maintaining parasite transmission from human hosts to *Anopheles* mosquitoes. This approach could be used to assess the infectiousness of human populations. In this context, investigating gametocyte sex ratios becomes crucial for comprehending *Plasmodium* biology, enhancing predictions of transmission potential in natural malaria infections, and estimating the probability of onward transmission to mosquitoes post anti-malarial drug treatment. This knowledge contributes to a more nuanced understanding of malaria dynamics and aids in optimizing treatment strategies to reduce transmission.

Furthermore, some studies indicate that anti-malarial drugs may exert distinct effects on male and female gametocytes (Delves et al., 2013; Dicko et al., 2018) with the hypothesis that mature male gametocytes are sensitive to a wider range of drugs than is the case for mature female gametocytes. During male gametocyte development, the cell undergoes several preparatory steps for gamete formation. These include reducing the endoplasmic reticulum and ribosome content, enlarging the nucleus, and aggregating kinetochores near a microtubule organizing center (Sinden RE, 1982; Sinden et al., 1978). These changes set the stage for rapid mitosis during exflagellation. Exflagellation is the explosive development phase during which the mature male gametocyte undergoes

significant changes within a short time span. This includes de novo synthesis of DNA (Raabe et al., 2009), three rounds of mitosis, assembly of eight axonemes, and ultimately escaping the red blood cell. The speed and coordination of male gametocyte development may make it more particularly sensitive to perturbation by antimalarial drugs than female gametocyte. Female gametocyte development, instead, require less stress. Female gametocytes held in translational repression controlled by DOZI approximately 370 transcripts, awaiting female gamete formation and fertilization (Mair et al., 2006). Upon induction, a female gamete must egress from the red blood cell, withdraw translational repression and commence translation of the repressed mRNA transcripts (van Dijk et al., 2001). It would seem, then, that the only biological target for antimalarial drug discovery could be general housekeeping functions, cell egress, and translation. Therefore, in the perspective of using of antimalarial compounds that act against these transmission stages of the parasite, these differences between male and female gametocytes need to be taken into account.

1.1.8. qPCR assays for male and female gametocytes quantification

Mosquitoes can become infected with gametocyte densities below the detection level of standard microscopy (Jeffery et al., 1955; Bonnet et al., 2000; Boudin et al., 1993; Graves PM, 1980). To overcome the limitations of microscopy, over the years the development of molecular techniques has represented a valuable additional tool for the detection and quantification of gametocytes. These technical allow specific detection of mRNA of genes that are expressed exclusively in sexual transmission stages of malaria parasites. Transcriptomic analysis revealed that RNA expression of most gametocyte-specific genes (i.e.

Pfs25, *Pfs48/45*, *Pfs230* and *Pfg377*) (Lasonder et al., 2016) peak after stage II-III of gametocytogenesis which occurs several days following the initiation of *P. falciparum* sexual differentiation (Sinden RE, 2004). These transcripts can be used for late stage gametocyte detection from *in vivo* samples, because only mature gametocytes circulate in the bloodstream. The first amplification-based protocol for detection of gametocytes detected abundant copies of *Pfs25* mRNA of *P. falciparum* in field samples and allowed detection of very low numbers of gametocytes (1–2 per microliter) in infected blood (Babiker et al., 1999). The Pfs25 protein is only expressed inside the mosquito vector, its transcripts are already present in stage V gametocytes that circulate in the host's blood stream (Babiker et al., 1999). As *Pfs25* mRNA even though female-specific marker, it was turned out to be a good candidate for the identification and quantification of total gametocytes, over the years, several protocols that involve this transcript have been developed (Wampfler et al., 2013; Schneider et al., 2004; Schneider et al., 2007) by Reverse Transcriptase quantitative PCR (RTqPCR) or Quantitative Nucleic Acid Sequence Based Amplification (qNASBA). Subsequently, sensitive qPCR assays based on detection and amplification of both gametocyte sex specific transcripts have been developed. In one of them (Stone W. et al., 2017) mRNA transcripts of *Pfs25* for female and *PfMGET* for male gametocytes were amplified. In this work, two randomized trials in Kenya and Mali, comparing dihydroartemisinin-piperaquine (DP) alone to DP with PQ were conducted and gametocyte sex ratio was examined in relation to time since treatment and infectivity to mosquitoes. Other sex specific transcripts were selected from the literature and used in RTqPCR assays in perspective of large-scale epidemiological surveys for detection of gametocyte: the female-specific pf

glycerol kinase (*pfGK*) (Santolamazza et al., 2017) and the male-specific *pfs230p*, *pf13* (Schneider et al., 2015 ; Santolamazza et al., 2017), *mssp* transcripts (PMID: 31437280) and *pfMGET* (Dicko A. et al., 2018).

In the work Meerstein-Kessel L. et al., 2018, a multiplex RTqPCR assay was developed to estimate the densities of female and male gametocytes amplifying female specific marker *pfCCp4* never used before, and *pfMGET* as male specific marker (Lasonder et al., 2016; Stone W. et al., 2017). The advantage of this protocol is in the use of intron-spanning primers allows simultaneous quantification of male and female-specific transcript levels in total nucleic acids without prior DNase I treatment. The new introduced *CCp4* transcript amplified is a member of the LCCL-domain containing adhesion protein family and translationally repressed in the gametocyte stage with protein expression occurring at the ookinete stage only (Saeed et al., 2013). This work aims to quantify the copy number of these sex specific transcripts per gametocyte. The male and female gametocyte quantification was estimated using transgenic line PfDynGFP/P47mCherry (Lasonder E. et al., 2016). This line expresses fluorescent proteins in a sex-specific manner: for the male-specific reporter line, the pDynGFP construct contains the *gfp* gene under control of the promoter region of dynein; instead, the female specific construct contains mCherry sequence under control of the promoter region of *Pf*P47 of the parasites. The sex specific reporters emit a different fluorescent signal that allows male and female gametocytes to be separated via FACS. The quantification was obtained by relating the cycle threshold (Ct) values obtained from RTqPCR on serial dilutions of both male and female gametocytes of the PfDynGFP/P47mCherry line with the Cts value from RTpPCR on serial dilutions of plasmid with the marker gene sequences,

expressed as the number of copies of the gene. That procedure assumes that the copy numbers of the two marker transcripts are identical in male and female gametocytes of the PfDynGFP/P47mCherry line and in male and female gametocytes of different laboratory lines of *P. falciparum* or natural isolates.

These two sex specific markers (*pfMGET* and *pfCCp4*) indicated in the aforementioned work are used in multiplex mode in this thesis work to measure their copy number per gametocyte in three laboratory lines, the monoclonal lines from natural infections NF54 (Delemarre-van H. A. and de Waal F.C., 1981) and HB3 sel 4 (Walliker et al., 1987), and the transgenic line PfDynGFP/P47mCherry. For female gametocytes quantification, the amplification of a further transcript was added, the *pfs25* (Wampfler et al., 2013). As it is impossible to physically separate male and female gametocytes, the experimental approach was to preliminarily determine the sex ratio of gametocytes from the three laboratory lines of *P. falciparum* with immunofluorescence assays (IFA) by reacting the gametocytes with antibodies against Pfg27 (Lobo et al., 1994) (which identifies all gametocytes) and Pfg377 (which identifies only female gametocytes) (Alano P. et al., 1995). This approach, which sees the comparison between IFA assay and molecular detection, was already use in a previous work (Gruenberg M. et al., 2019) to quantify female and male gametocytes and sex ratios in asymptomatic low-density malaria infections for assessing their transmission potential.

1.1.9. *In vitro* gametocyte drug screening assays

Several *in vitro* assays measuring transmission- blocking activity of compounds against mature stage V gametocytes have been developed. Most of these methods

often rely on indicators of metabolic viability. For example, one of them consists of detecting gametocyte viability using the dye MitoTracker Red CMXRos (MTR Red) which fluorescens at ~600 nm in parasites with intact mitochondrial membrane (Plouffe D.M. et al., 2018). The fluorescence of gametocyte cultures treated with MTR Red and exposed to the action of compounds are then related to those of untreated control sample. Other assays are based on the use of a reporter gene line, which expresses a green fluorescent protein (GFP)-luciferase fusion under the temporal control of a gametocyte-specific promoter (Lucantoni L. et al., 2016; D'Alessandro S. et al., 2016; Paonessa G. et al., 2022). In these assays, the luciferase activity revealed with the luciferin substrate formulation was compared after 24 and 48 hours of treatment of synchronous gametocytes with anti-gametocyte drugs. Another study describes a method to screen compounds for gametocytocidal activity using oxidoreduction indicator, alamarBlue, as a general measure of metabolic activity (Tanaka et al., 2011). Gametocytes emit fluorescent signal that increase linearly with alive gametocyte number by using the reducing power of living cells. However, the 'gold-standard' laboratory method for evaluating transmission blockade in malaria research are the standard membrane-feeding assay (SMFA). This assay involves cultivating gametocytes to maturity in vitro, introducing an intervention such as a drug, and then feeding the treated gametocytes to mosquitoes in a device that mimics body temperature and presents a skin-like membrane to the mosquito. After 7–10 days, mosquito infection is assessed by counting the oocysts in the midgut (Stone et al., 2014; Miura K. et al., 2013; Churcher T.S. et al., 2012). While the SMFA captures the complexity of parasite biology and mosquito interactions, it is low-throughput and costly. It also requires the training of expert personnel capable of

manipulating the arthropod and equipped laboratories with a containment level of 3.

However, none of these methods can differentiate between compound activities against male and female gametocytes. As male gametocytes seem to be more sensitive to antimalarial drugs than female gametocytes and as males may only represent ~20% or less of the total gametocyte population, these type of assays without a sex-specific readout are not likely to identify male-targeted transmission-blocking. However, since both sexes of gametocytes are required for transmission, a combined readout of male and female gametocytes is essential to maximize the screening potential of a gametocyte test. Currently, the only *in vitro* test predictive of transmission blocking activity on *P. falciparum* gametocytes and capable of differentiating between male and female is the Dual Gamete Formation Assay (Pf DGFA) (Delves M. et al., 2013; Ruecker A. et al., 2014). This assay is based on the ability of gametocytes to form gamete after treatment. As the presence of morphologically "normal" mature stage V gametocytes does not necessarily correlate with their ability to form gametes, this assay evaluates the formation of male and female gamete after 24 hours treatment and gametocyte activation. At 20 minutes after induction, exflagellation centers, indicative of male gametocyte activation, were captured with a camera and analyzed using specific automated software. The presence of female gamete is evaluated 24 hours after activation by live immunostaining using fluorescent antibody against the Pfs25 surface protein. Another automated algorithm captures and analyze the images. In this work the data obtained were validate in SMFA.

This thesis project aims to use a novel method for evaluating the *in vitro* transmission-blocking activity of compounds against both male and female

Plasmodium falciparum gametocytes by introducing an alternative approach to understanding the gender-specific responses to antimalarial compounds.

2. MATERIALS AND METHODS

2.1. *P. falciparum* cultures

The *Plasmodium falciparum* lines Pf2004/164-tdTom, (Brancucci et al. 2015), NF54-pfs47-pfs16-GFP (Bouyer et al. unpublished) and NF54 (Ponnudurai T. et al., 1981) were selected for this study.

The NF54 line was isolated from cases of malaria imported into the Netherlands and was established in culture.

The Pf2004/164-tdTom transgenic line carries a TdTomato red fluorescent protein whose expression is under the control of the gametocyte-specific promoter of gene *PF3D7_1016900*.

The *P. falciparum* NF54-pfs47-pfs16-GFP line expresses a Green Fluorescent Protein (GFP) under control of the gametocyte-specific promoter of gene *pfs16*; in this line, the reporter gene expression cassette is stably integrated into the *pfs47* locus.

2.1.1. Parasite culture medium

The incomplete culture medium was prepared from RPMI 1640 powder (Gibco) with 25 mM HEPES buffer (5.94 g/L) (Sigma), and 50 μg/ml hypoxanthine (Sigma). It was filtered through 0.22 μm Nalgene or Millipore filters and store at 4°C for up to 4 weeks. Prior to use, incomplete medium was implemented with 10% (v/v) naturally clotted heat-inactivated 0+ human serum (Interstate Blood Bank, Inc), 42 mL/L of freshly made up, sterile filtered, 5% $NaHCO_3$, and gentamycin (final concentration 50 μg/mL). Medium was supplemented with 4

nM WR99210 (Jacobus Pharamaceuticals) for the Pf2004 p164 Tomato Red strain. Complete medium was stored at 4°C and used within 2 weeks; it was warmed to 37°C before using.

2.1.2. Red blood cells (RBC)

Fresh human blood, group 0, rhesus group positive, in CPD (citrate phosphate dextrose)-adenine packs was obtained about once a week from the Blood Transfusion Center of the Policlinico Umberto I, Roma from volunteers after written consent. Blood was washed 3 times in incomplete medium by centrifugation at 2000 rpm for 10 min, removing the white blood cells by aspiration of the buffy coat. At the end of the last centrifugation the blood pellet was resuspended in equal volume of complete medium to give a 50% hematocrit.

2.1.3. Thawing and maintenance of P. falciparum cultures

The cryo-preserved *P. falciparum* strains were thawed by the stepwise addition of two thawing solutions: (A) 12% NaCl and (B) 1,6% NaCl. Ampoules (Nunc cryotube) with the strains were removed from liquid N_2 and thawed quickly in water bath at 37°C. The volume of the parasite material (usually 0.5 mL) was measured and 0.2 vol of thawing solution A were added drop by drop with gently and continuous mixing. Then, 10 vol of solution B were added drop by drop, mixing gently and slowly, then the RBCs were pelleted by centrifugation at 1500 rpm for 5 min at room temperature. Supernatant was removed and 5 ml of complete medium were added, and parasite were again centrifuged at 1500 rpm for 5 minutes. After supernatant removal parasites were resuspended in 5 ml of

complete medium containing washed RBCs at final 5% haematocrit in 25 cm^2 base (50 mL) cell culture flask. The cultures were maintained in the flask at 37°C in a standard gas mixture consisting of 5% O_2, 5% CO_2 and 90% N_2.

The parasite populations were kept between 0.1 and 10% parasitaemia, defined as number of infected erythrocytes / 100 erythrocytes. Every day or two day, the medium in the flasks was removed under hood with the aspirator and a drop of blood was removed with a Pasteur pipette and smeared on a glass slide to evaluate the cultures parasitaemia (number of infected RBCs/total number of RBCs). When the parasitaemia reached 5-6%, the culture was diluted by adding fresh erythrocytes to reduce parasitaemia to around 0,5-1%.

2.1.4. Blood smears

The smears are made by initially placing a drop of blood on a slide. On the slide, next to the drop of blood, a second slide leans with an inclination of 40. The second slide is pulled towards the drop, so that the latter spreads by capillarity along the contact line of the two slides. The second slide is pushed towards the opposite end, with constant, rapid and light movement, to obtain the smear (Figure 1). Smear is air- dried, and then dipped into 100% methanol to fix the smear. Then it is stained for 20 minutes with 10% Giemsa solution in buffered dH_2O (pH 7.2) [Weiss Buffer] filtered with 0,45 μm filters.

The stained smears were observed by optical microscopy at a magnification of 40 or 100 times.

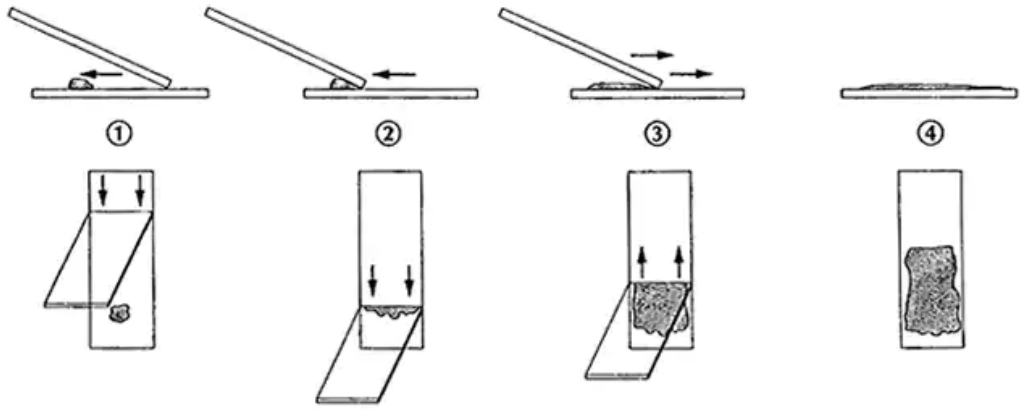


Figure 5. Procedure to make a blood smear

2.1.5. Liquid N_2 preservation of parasites

To preserve parasites strains in liquid N_2, cultures were grown to a parasitemia of 5%. A high proportion of these parasites MUST be at the early ring stages, as these are the only ones able to survive to freezing passages. The cultures were pellet by centrifugation at 2000 rpm for 5 min and then Resuspend gently with continuous mixing in 2 vol of deep-freezing solution. It was made with 28% glycerol, 3% sorbitol, 0.65% NaCl in double distilled water, sterilized through 0.22 µm filter and stored at 4°C. The cultures with deep-freezing solution were aliquoted 0.5 mL in a sterile cryotube (Nunc) and placed in isopropanol container to allow a slow freezing (1°C per min) at -80°C, overnight. The following morning, they were placed in liquid N_2.

2.1.6. Gametocyte culture protocols

Cultures of gametocytes can be obtained by two protocols, whose main difference is presence or absence of the compound N-acetylglucosamine:

- Gametocytes were obtained from a culture at high parasitaemia (10- 13 %) whose maintenance medium has been supplemented with N-acetylglucosamine 10X (0.5 M) (NAG; Sigma A3286, Molecular Weight: 221.21) in order to clear asexual parasites and to obtain a virtually pure gametocyte culture. The medium had to be changed every day at the same time of day, if it was possible and after that, the flasks were gassed. It is very important to keep the cultures as warm as possible at all the times.
- Gametocytes were also obtained by starting a culture with a parasitaemia oft 1% ring-stages and 5% hematocrit (day 0). The medium is replaced daily for 14 subsequent days without the addition of fresh erythrocytes, by which time most of the gametocytes have reached maturity (stage V). During this culture period, parasites undergo several phases of development, which possess different metabolic states. Between days 1 and 4 after culture induction, the asexual parasitemia increases rapidly to a peak. Stress thought to be naturally produced by the high parasitemia induces gametocyte formation. By day 7, as the asexual population wanes, early-stage gametocytes are observable. By day 8, gametocyte maturation progresses, and by day 14 morphologically distinguishable mature stage V male and female gametocytes are present (Delves M. J. et al., 2016).

2.1.7. Sorbitol Treatment to Synchronize Parasites

This synchronization method is based on the differential permeability of the infected erythrocyte membranes. At the trophozoite or at the schizont stages, the host membrane becomes permeable to substrates such as sorbitol. Parasite incubation in a 5% sorbitol solution followed by incubation in parasite culture

medium leads to osmotic hemolysis of the stages, whereas the non permeable rings survive at the sorbitol treatment.

To synchronize the culture to obtain only rings, removing schizonts and old trophozoites, most of the used medium was removed from the flask and the remaining culture was transferred into a 15 mL tube. The culture was centrifuged at 1800 rpm and pellet volume was annotated. Five volumes (1 volume = RBCs pellet volume) of prewarmed solution of 5% D-sorbitol (Sigma) were added to the culture pellet and cellular suspension was incubated for 5 min at 37°C. After this incubation two washing step in incomplete medium were performed. Finally, parasites were resuspended in complete medium at 5% haematocrit.

2.1.8. Enrichment of early sexual stage infected erythrocytes by 60% Percoll centrifugation

In order to obtain a gametocyte *P. falciparum* culture cleaned from uninfected erythrocytes, 5 ml of a *P. falciparum* asynchronous sexual culture in a 15 ml tube were centrifuged, resuspended in 5 ml of incomplete medium and carefully placed on top of 5 ml of a 60% Percoll solution in incomplete medium (v/v). The tube was centrifuged at 2000 rpm for 10 min and it was stopped with slow deceleration. The top layer containing early gametocytes was recovered with a pipette and washed twice with complete medium for 5 minutes by centrifugation at 2000 rpm. The purified gametocytes were be suspended in complete medium and let them grown until stage V.

2.1.9. Purification of trophozoites, schizonts and gametocytes by magnetic cell sorting (MACS)

To purify *P. falciparum* gametocytes and trophozoites from uninfected RBCs, it is possible to exploit the paramagnetic properties of the pigment contained in the parasites, which allow to retain them in the metal mesh of a CS magnetic column (MACS- Miltenyi Biotec).

MACS separation columns ¨CS¨ (Figure 2) were placed into the vario MACS magnetic support containing the magnet and equilibrated by adding 4 ml of pre-warmed (37°C) incomplete medium. Parasite cultures were centrifuged at 2000 rpm for 5 minutes, resuspended in 4 ml of incomplete culture medium and loaded on the top of the column. A low flow rate was used to pass the culture through the column. The flow-through containing the uninfected human RBCs and the ring stage parasites (that have no pigment) were discarded while mature trophozoites, schizont and gametocytes were retained into the matrix. Columns removed from the magnet and were washed to elute the trophozoites or gametocytes infected erythrocyte forms using 20 ml of pre-warmed incomplete medium. The eluted material was centrifugated at 1500 rpm for 5 minutes and pellet was resuspended in complete medium.

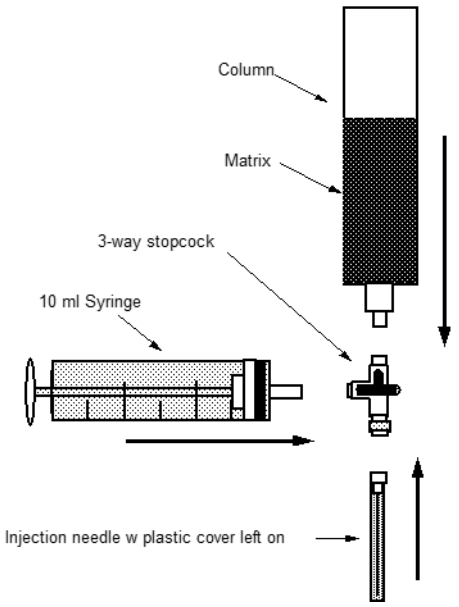


Figure 6 *Assembly of the MACS column (Uhlemann A. et al., 2000).*

Purified infected RBCs were counted using a Neubauer chamber and haematocrit and parasitaemia were adjusted with fresh erythrocytes.

2.1.10. Counts of parasites with Neubauer chamber

10 µl of culture were taken after adequate dilution for counting uninfected and infected red blood cells and were placed in counting chamber. For the count the large central square of the chamber is used (Figure 7). In the chamber a grid is visible, which is divided into 25 squares with sides of 0.2 mm. Each of these squares contains 16 smaller squares with sides of 0.05 mm each with an area of 0.0025 mm^2.The number of cells/ml is obtained by multiplying the average number of cells counted in each mini- square of the chamber x 250 x 1000 x the nb

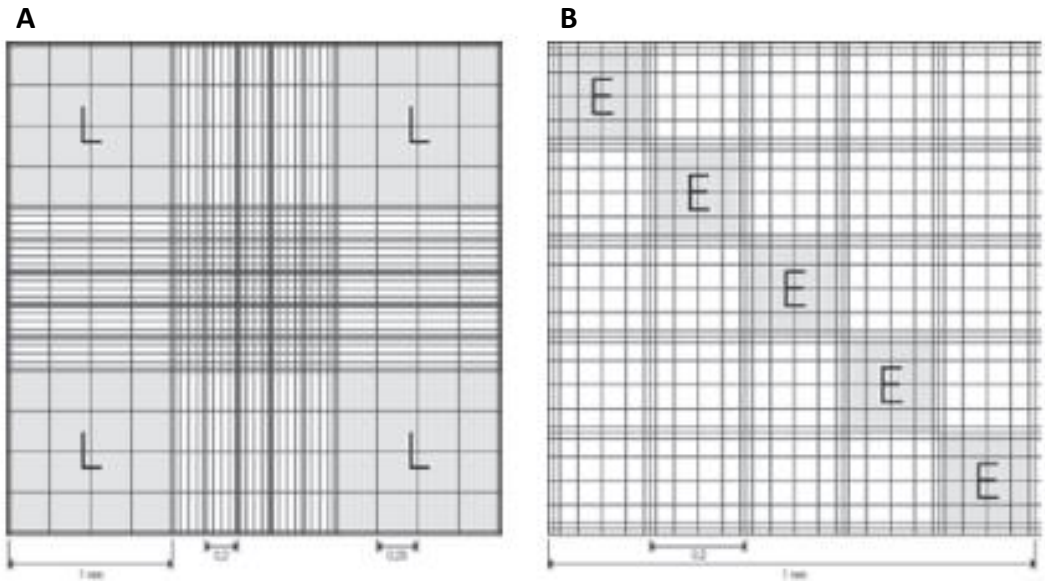


Figure 7*. Neubauer chamber. A. The 4 large squares at the corners (indicated by an "L") are divided into another 16 squares, with sides of 0.25 mm. They are used for leukocyte counting. The large central square is divided into 25 squares. B. Enlargement of the central square divided into 25 squares with 0.2 mm sides each of which is further divided into 16 mini squares*

2.2 Evaluation of gametocyte infectivity in mosquito

2.2.1. Study area and recruitment of *P. falciparum* gametocyte carriers

The current study includes field activities with *ex vivo* assessments in Burkina Faso. The field activities were conducted in Saponé Health district in the province of Bazèga, located 50 km southwest of Ouagadougou, the capital city of Burkina Faso. Two surveys were conducted at schools to recruit *P. falciparum* gametocyte carriers among 5-15 year old children at the end of the malaria transmission season, i.e., from September to December 2019 and September to December 2020. Every child was clinically examined for the presence of chronic diseases, acute infections other than malaria and signs of severe malaria. Finger-prick blood was collected and used for the preparation of thick smears. Samples were considered negative if no parasites were detected in 100 microscopic fields. Both asexual and gametocyte densities were simultaneously assessed by counting against 500 leukocytes in the thick smear. Parasite counts were converted to numbers of parasites per µl by assuming a standard count of 8000 leukocytes/µL of blood. Asymptomatic malaria-infected individuals with *P. falciparum* gametocytemia ≥ 32 gametocytes/µl, were selected as blood donors for direct membrane feeding assays (DMFA), with blood collected within 24 hours of gametocyte detection. Of a total of 945 children screened, 36 met the inclusion criteria. From each of them 9 ml of whole blood was collected in lithium heparin tubes.

These samples were used to measure the infectivity of *P. falciparum* gametocytes by direct membrane feeding assays (DMFA) and to extract RNA to perform RTqPCR assays.

The *Anopheles coluzzii* strain raised in the insectarium of the Centre National de Recherche et de Formation sur le Paludisme (CNRFP), was used to measure the infectivity of gametocytes collected from study subjects using the DMFA technique.

2.2.2. Antimalarial compounds

A set of 11 compounds, being dihydroartemisin (DHA), methylene blue (MB), MMV390048 (MMV048), MMV693183, SJ773, Atovaquone, Ferroquine, Pyronaridine, DDD107498, Lumefantrine and P218, was provided by Malaria Medicine Venture for testing (MMV, Geneva, Switzerland). These compounds were dissolved in DMSO and kept in a stock solution of 10mM at -20°C. Three different concentrations equivalent to roughly 0.1x, 1x, and 10x of the mean IC50 values were tested. These IC50 values were previously determined by Standard Membrane Feeding Assay with cultured gametocytes (Henry et al., 2023). Compounds were prepared in DMSO and then complete medium to achieve a final DMSO concentration of 0.1%. Each concentration was tested in duplicate. The diluting agent for all test drugs, DMSO (Dimethylsulfoxide) (Sigma-Aldrich no. D4540), was used as a negative control (referred to as "no-drug control"). Atovaquone was used as positive drug control for transmission-blockade.

2.2.3. Preparation of blood for the transmission-blocking DMFA

To allow for a 24 hours incubation with compounds of interest, donor plasma was replaced with RPMI 1640 supplemented with 25 mM sodium bicarbonate (Sigma S8761) and 10% malaria-naïve European Serum A. A 360 µL aliquot of each

sample was added to 40 µL of 10x Concentrated Compound Solution in RPMI 1640 and incubated for 24 hours at 37°C. Subsequently, the tubes were centrifuged for 20 seconds at 14000 rpm and RPMI 1640 was carefully removed and replaced by an equivalent volume of 2x concentrated compound in malaria-naïve European Serum A. The tubes were mixed before feeding the mosquitoes.

2.2.4. Artificial infection of mosquitoes

The DMFA was performed using female mosquitoes from an *Anopheles gambiae* colony established from field mosquitoes at Center National de Recherche et de Formation sur le Paludisme (CNRFP) ten years ago and previously successfully used for transmission assays. Mosquitoes are maintained on 25 ± 2 °C and 80 ± 10% relative humidity and fed ad libitum with a 5% glucose solution. For DMFA, 2-3 days old female mosquitoes were starved for ≥ 6 hours; 40 mosquitoes per cup were fed during 15-20 min via an artificial membrane (Parafilm) attached to a water-jacketed glass feeder to maintain the temperature at 37ºC (Figure 8). After feeding, unfed mosquitoes were removed; engorged mosquitoes were kept at a temperature range from 26 to 28ºC with permanent access to a glucose solution without further blood meals.

***Figure 8**. Experimental device for artificial infection of mosquitoes at the CNRFP.*

2.2.5. Dissection of mosquitoes and determination of infectivity

On the eighth day after artificial infection, the mosquitoes were dissected. This dissection was carried out under a binocular microscope, using Dumont N° 5 forceps. The mosquito held by a clamp at the level of the thorax and the abdomen is removed using a second clamp placed at the level of the 7th abdominal segment, the exoskeleton tearing frees the intestine. This is delicately detached at both ends and placed in 50 µl of PBS (Phosphate Buffer Saline). The mosquito carcass is preserved in a cryotube containing an "oocyst lysis buffer" lysis solution for subsequent molecular analyses. The intestine is placed in a drop of 0.40% mercurochrome solution on a slide then observed under a microscope (X20) for

the search and quantification of oocysts (Figure 9). The presence or not of oocysts in the mosquito's stomach allowed evaluating the capacity of gametocytes to infect the mosquito. The number of oocysts in the mosquito midgut was recorded to determine mosquito infection prevalence.

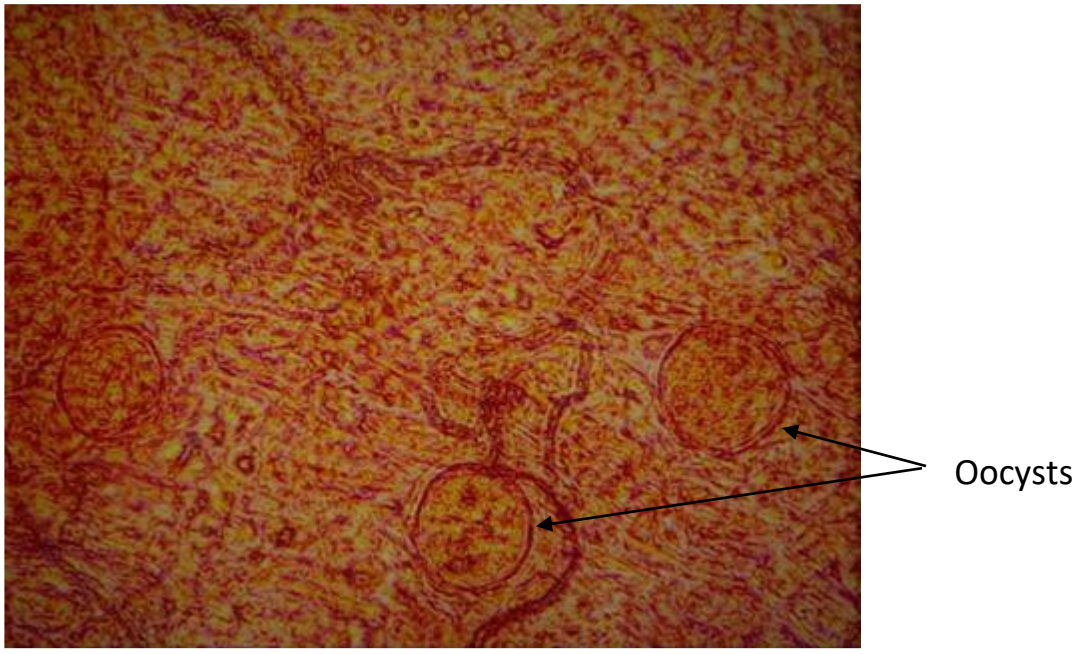


Figure 9. *Anopheles stomach showing mercurochrome-stained oocysts (Oocyst photo: Sermé, 2017)*

2.3. Molecular methods

2.3.1. Blood sample storage

All gametocyte-infected blood samples obtained from the laboratory line were suspended in ten volumes of TRIzol™ Reagent (Invitrogen) to lyse blood cells.

The samples were then promptly stored at -80°C until the RNA extraction procedures.

For gametocyte-infected blood samples from naturally infected individuals, they were resuspended in five volumes of RNAlater™ Stabilization Solution (Invitrogen) to preserve the blood samples. This precaution was taken in case it was not possible to maintain a temperature of -80 degrees during the shipment from Ouagadougou, Burkina Faso, to Istituto Superiore di Sanità in Rome.

2.3.2. RNA extraction

Before performing the extraction, RNAlater™ Stabilization Solution had to be removed from samples obtained from naturally infected individuals by 5000 x g centrifugation. The remaining blood pellet was then resuspended in ten volumes of TRIzol™ Reagent.

For the lysis process, 0.2 ml of Chloroform (Sigma-Aldrich, 34854) was added per 1 mL of TRIzol™ Reagent. The solution was thoroughly mixed by shaking and incubated for 2–3 minutes. After centrifugation of the sample for 15 minutes at 12,000 × g at 4°C, the mixture separated into a lower phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out.

RNA purification was performed using the automatic extractor MagPurix® EVO 12- Zinexts Life Science Corp with the MagPurix® Total RNA Extraction Kit

(ZP02019), selecting the appropriate program RNA- Total RNA 2015. The elution volume of RNA selected was 50 µl.

The obtained RNA was quantified with NanoPhotometer NP80-Implen, and the quality parameters were evaluated, establishing both 260/280 and 260/230 ratios close to 2. Then, it was stored at -80°C.

2.3.3. Generation of synthetic mRNA targets

To generate the synthetic copies of the *pfCCp4* (PF3D7_0903800), *pfs25* (PF3D7_1031000) and *pfMGET* (PF3D7_1469900) genes, the following portions of the gene sequences were cloned into the pEX-A128 plasmid (see Figure 12):

CCp4

GGGGGAAAATtaatacgactcactatagggagattgtcgagcaagcttcaaagaatatt
tatctaataagaaaatatttttaaaatataatggtacctattgtttataccctgaaaat
gttatagaaaatgatataattgaaatagccaccgatgaatgtgataaagtagcacatga
atatgagaataaaattgtttggttttcgttcgataatggaagattaagaagcaacaacg
gtatgtgccttaaaacgtacaaaaatattttagtgctttccacatgttcgcctaataaa
aatgaaaagtcagaaatgtggaagatagatgaagaaacacatttaaaaaatgaaaacaa
caattgtgctcaaatagctgggaataaattattttcttttacatgcaataatttatcaa
ctaaagaatttgaacaatccatattttcttcagacgaattatatttaatgaaaacaaga
tattc

477 bp dsDNA ; 450 nt ssRNA

PfMGET

GGGGGAAAATtaatacgactcactatagggagaatgagccgcataaaggacaaaagcac
aacactttcaaaaaataaaaaattcggtccaaatataaaatcctgttcagaagaaaaaa
aaagtatcctggtaaaaaacagctccagcattaaaaacacaaaaagggatttttaatt
gatgaagataatataattaacaaaataaaagagctaaatataattctgttacaaaataa
agacgaaatagataatctgaagcaagaaaatgaatcatacgttacacaataaccaatt
ttatgaataagtgcaaagaactacaaaatatatgtaacgataaagatattatacttttc
aaatataaacaagaagaaaagaatcttttacaattaatagattcttataaaaaagaaaa
ggaagaattacaaaatgaaatagaaaaactagaaaataacatccaatctacaaaaggac
aattgttggtacaaaataatgaaatagatatattaaagaaagaaataaaagaaaa

527 bp dsDNA; 500 nt ssRNA

Pfs25

GGGGGAAAATtaatacgactcactata*gggaga*catgtggagattttccaaatgtatt
*aaaatagatgg*aaatcccgtttcatacgcttgtaa*atgtaatcttggatatgatatggt*
aaataatgtttgtataccaaatgaatgtaagaatgtaacttgtggtaacggtaaatgta
*tattagatacaagcaatcctg*ttaaaactggagtttgctcatgtaatataggcaaagtt
cccaatgtac

246 bp dsDNA; 219 nt ssRNA

In green: T7 promotor sequence
In yellow: qPCR amplicon
In italic: RNA transcript
Underlined: qPCR primers

The resulting plasmids were amplified by transforming competent One Shot™ TOP10 Chemically Competent E. coli bacteria (Invitrogen) following the manufacturer's instructions. After transformation, the bacteria were plated on LB-agar Petri dishes supplemented with the antibiotic ampicillin and incubated overnight at 37°C.

On the following day, bacterial colonies were selected from the plates and grown overnight at 37°C in LB medium containing the selection antibiotic. Plasmids were then extracted using the QIAprep Spin Miniprep Kit (Qiagen).

After DNA extraction, the plasmids were digested with the restriction enzyme EcoRI, with recognition sites inserted upstream and downstream of the gene sequences. DNA fragments containing the target sequences were purified by extraction from a 1% agarose gel following electrophoretic separation.

For transcription, 10-50 nM of dsDNA template, following the manufacturer's instructions, was transcribed using the MEGAshortscript kit (Invitrogen™). T7 RNA polymerase with the specific promoter sequence inserted upstream of the genes was employed. RNA was purified using RNeasy Mini Kit columns (Qiagen), and quantification was performed on the Qubit 2 Fluorometer. Purity was estimated from the ratio of absorbance readings at 260 and 280 nm, falling in the range of 1.7-2.1. Copy number was calculated based on the sequence length using the formula:

$$copy\ number\ of\ RNA = \frac{\text{weight (ng) x } 6{,}0221 \text{ x } 10^{23}}{\text{lenght x } 330 \text{ x } 1 \text{ x } 10^{9}}$$

RNA samples were stored at -80°C until their use.

2.3.4. RTqPCR protocol amplifying sex specific genes

The sequences of primers and probes used for amplification reactions are the following:

pfMGET Forward primer 5′- cggtccaaatataaaatcctg -3′ (Eurofins Genomics)
pfMGET Reverse primer 5′- tgtgtaacgtatgattcatttc-3′ (Eurofins Genomics)
pfMGET Probe: 5′-6FAM-cagctccagcattaaaaacac-MGB-3′ (HPLC - Thermo fisher Scientific)

pfCCp4 Forward primer 5′- cacatgaatatgagaataaaattg-3′ (Eurofins Genomics)

pfCCp4 Reverse primer 5′- taggcgaacatgtggaaag-3′ (Eurofins Genomics)
pfCCp4 Probe: 5′-ABY -agcaacaacggtatgtgccttaaaacg-MGB -3′ (HPLC Thermo fisher Scientific)

Pfs25 Forward primer 5′- aaatcccgtttcatacgcttgtaa-3′ (Eurofins Genomics)
Pfs25 Reverse primer 5′- cagttttaacaggattgcttgtatctaatatac-3′ (Eurofins Genomics)
Pfs25 Probe: 5′- JOE-accaaatgaatgtaagaatgtaacttgtggtaacggt- [BHQ1]-3′ (HPLC-Eurofins Genomics)

hGAPDH: TaqMan™ GAPDH Assay, JUN™ dye/QSY™ probe (Cat. Number: 4485712) (Applied Biosystems™). This product consist in a pre-formulated assay for quantitating human GAPDH. This assay enables relative gene expression quantification in RNA samples when used with other gene expression assays. The assay consists of a JUN™ dye-labeled QSY™ probe plus sequence-specific forward and reverse primers, and it can be used for multiplex or singleplex PCR reactions.

The RTqPCR reactions were performed using TaqPath™ 1-Step Multiplex Master Mix (No ROX) (Applied Biosystems) in a final reaction volume of 20 μL.
The concentrations of the primers and probes used at the following final concentration:
pfCCp4 primers: 900 nM
pfCCp4 probe: 200 nM
pfMGET primers: 250 nM

pfMGET probe: 200 nM

pfs25 primers: 400 nM

pfs25 probe: 200 nM

GAPDH probe and primers: following the manufacturer's concentration

The volume of RNA used in each RTqPCR reaction is 5 µl. Each reaction was performed in triplicate replica.

The amplification reactions were performed using QuantStudio 5 real-time PCR systems (Thermo Fisher Scientific) setting the following program:

55°C 15 min > 95°C 1 min > 95°C 10 sec, 60°C 1 min, 44 cycles.

2.3.5. ΔΔCt method

ΔΔCt method describes the change in expression of the target gene relative to some reference group such as an untreated control. This method compares the difference in expression (ΔCt) between the gene of interest (***pfCCP4***, ***pfs25*** or ***pfMGET***) and the reference gene (***GADPH***) under the experimental conditions and separately in the untreated control sample with the following formula:

ΔCt (sample) = Ct(*pfCCP4*/*pfMGET*/*pfs25* gene of interest) - Ct(*GAPDH* reference gene)

Then compare the difference between the compound-treated experimental sample and the DMSO-treated control sample from the same time point:

ΔΔCt = ΔCt (treated sample) - ΔCt (DMSO control)

Using the formula $\mathbf{2}^{-\Delta\Delta Ct}$ it is possible to calculate the relative changes in gene expression between the treated sample and an untreated sample, where 2 is the efficiency fixed at 100%. In this work, this method was used in an attempt to test the activity of compounds that may act differently on male and female gametocytes after 24 hours of treatment.

2.3.6. Data analysis software

The QuantStudio Design & Analysis Software was used to set up RTqPCR experiments, send experiments to the instrument, collect data, and analyze the collected data all in an integrated and streamlined manner.

Excel program (Microsoft window) was used as spreadsheet.

Graphs and statistical analyses were made with GraphPad Prism (version 5.0.3).

2.4. Immunofluorescent Assays

The slides on which the gametocyte cultures of each replicate of each line had been smeared were fixed with acetone chilled at −20°C for 2 min. The smears were blocked with 3% BSA in PBS for 30 min.

Female gametocytes were stained in a 1:200 dilution in PBS of rabbit- α-Pfg377 serum (produced in Pietro Alano laboratory). All gametocytes were stained using 1:500 diluted mouse IgG1 mAb α-Pfs16 (kind gift from Robert Sauerwein). Three washes were given maintaining gentle agitation.

Secondary antibodies, Alexa Fluor 488-conjugated goat-α-mouse-IgG1 (Life Science Technology) and Alexa Fluor 568-conjugated goat-α-rabbit-IgG1 (Life

Science Technology) were diluted 1:1000. Three washes were given maintaining gentle agitation. The cover slips were mounted on glass slides using Vectashield Antifade Mounting Medium (Vector Laboratories, Inc).

Images were taken at 40-fold magnification using THUNDER Imaging Systems microscopy (Leica)

3. AIMS OF THE THESIS

The focus of this thesis work is on the development and use of multiplex *Reverse Transcriptase quantitative Polymerase Chain Reaction* (RTqPCR) assays, specifically TaqMan assays, for the identification and quantification of male and female gametocytes of *Plasmodium falciparum*. The assays are designed to amplify the sex-specific transcripts, *pfCCp4* and *pfs25*, expressed by female gametocytes, and *pfMGET*, by male gametocytes. These assays will be used to:

1. Measure the sex ratio of gametocytes of wild type and transgenic laboratory lines of the parasite by different analytical approaches, one based on the DCt method and the other on the transcript copy number determination.
2. Measure the effect of a panel of antimalarial compounds on gametocytes from asymptomatic individuals, subsequently analyzed for efficiency of transmission through mosquitoes, by measuring levels of the sex specific mRNAs by the DeltaDeltaCt (ΔΔCt) method.

4. RESULTS

4.1. Development of a multiplex RTqPCR assay to identify male and female gametocytes and measure sex ratio in three *Plasmodium falciparum* laboratory lines

Althrought sex ratio is defined as proportion of male gametocytes, in these results I refer to sex ratio as number of male gametocytes over number of female gametocytes.

4.1.1. Gametocyte Sex Ratio Estimates by IFA

The laboratory lines NF54, HB3 sel 4 and PfDynGFP/P47mCherry were used to develop a multiplex RTqPCR assay to gain a comprehensive understanding in sex ratio of *Plasmodium falciparum* gametocytes because preliminary data indicated that they had three different sex ratios. A preliminary experiment produced values of morphological sex ratio to be compared with those derived from the RTqPCR assays. Since the male and female gametocytes of these lines, except for those produced by line PfDynGFP/P47mCherry, cannot be physically separated to determine the transcript copy number per male/female gametocytes, the values of sex ratio were estimated morphologically by immunofluorescence (IFA) by reacting the gametocytes with an antibody against protein Pfg27, which identifies all gametocytes, and protein Pfg377, specific for female gametocytes. For each line, three different biological replicas were performed. The results obtained from each replica are reported in Table 1 and a representative image of typical IFA results is shown in Figure 10.

The morphological sex ratio value is calculated using the formula:

$$\frac{Pfg27\ positive\ gametocytes - \text{Pfg377 positive gametocytes (}\mathbf{male\ gametocytes}\text{)}}{Pfg377\ positive\ gametocytes\ (\boldsymbol{female\ gametocytes})}$$

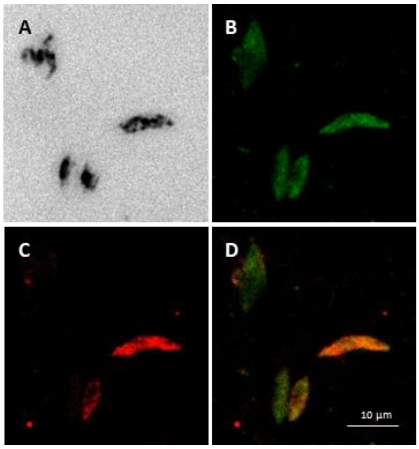


***Figure 10**. Immunofluorescence assay of gametocytes hybridized with B) antibody (primary from rat) anti Pfg27 (in green) and C) antibody (primary from rabbit) anti Pfg377 (in red) and D) the overlay of the images. Scale bar: 10 µM*

		REPLICA 1	REPLICA 2	REPLICA 3	
NF54	Pfg377 positive	400	443	275	
	Pfg27 positive	444	495	304	
SEX RATIO		**0.11**	0.114	0.105	**0.11** ± 0.005
HB3 sel 4	Pfg377 positive	142	136	159	
	Pfg27 positive	178	170	198	
SEX RATIO		**0.253**	**0.25**	**0,245**	**0.25** ± 0.004
PfDynGFP/ P47mCherry	Pfg377 positive	216	147	93	
	Pfg27 positive	390	264	168	
SEX RATIO		**0.805**	**0.795**	**0,806**	**0.80** ± 0.006

***Table 1**. Number of antibody anti Pfg27-positive gametocytes (which identify all gametocytes) and of antibody anti-Pfg377 positive gametocytes (which identify female gametocytes) for each replica of the laboratory lines NF54, HB3 sel 4 and PfDynGFP/ P47mCherry. In each replica the morphological sex ratio value are reported.*

The result of this experiment was that the sex ratio values are similar within the replicas of each laboratory line: **0.11**±0.005 for NF54, **0.25**±0,004 for HB3 sel 4 and **0.80**±0.006 for PfDynGFP/P47mCherry line.

4.1.2. Determination of the quality parameters of the Multiplex RTqPCR assay to identify male and female gametocytes

Aliquots of the gametocyte samples from lines NF54, HB3 sel 4 and PfDynGFP/P47mCherry used for IFA were employed to develop the RTqPCR assays. Gametocytes from the three lines were obtained in culture and purified from uninfected erythrocytes through centrifugation through a 60% Percoll cushion, resulting in a 90% purification of the gametocyte preparation. The purified parasites were quantified and 10^7 gametocytes were resuspended in 50 microliters of whole blood, i.e. preserving presence of leukocytes. To preserve the integrity of the RNA, samples were resuspended in 10 volumes of TRIzol and stored at –80°C for subsequent RNA extraction. Serial dilutions of the RNA preparations from the three lines were obtained, starting with a maximum concentration of 10^6 gametocyte equivalents and spanning eight tenfold dilutions. Each dilution was subjected to perform two RTqPCR assays, employing TaqMan probes in multiplex mode. One assay was set to amplify the sex-specific *pfCCP4* and *pfMGET* transcripts, while the other targeted the *pfs25* and *pfMGET* transcripts. Importantly, both assays were consistently conducted in biological triplicate replicas for each experiment.

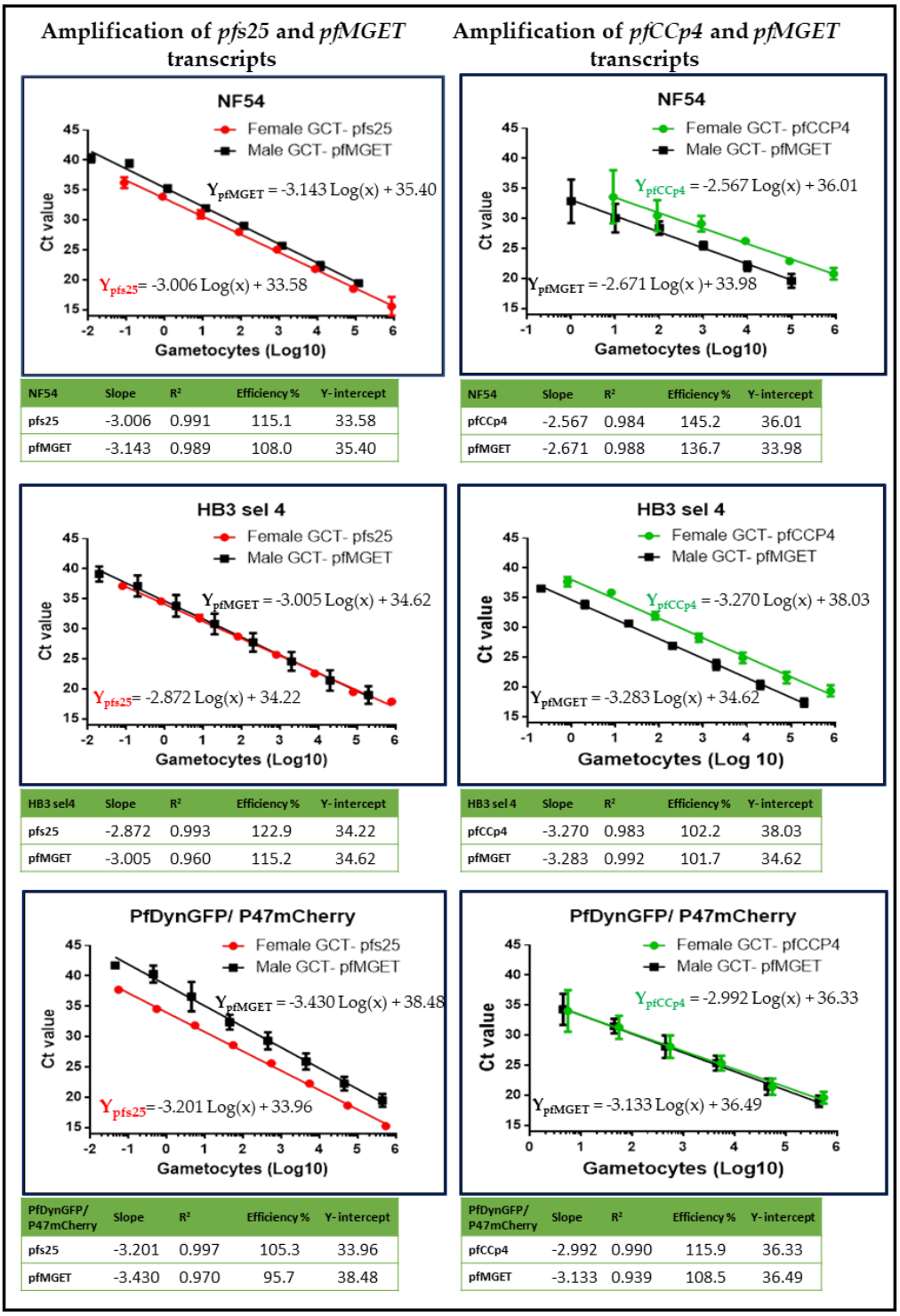
The slopes obtained in the multiplex RTqPCR assays are presented in Figure 11. Each panel includes equations for each curve, expressed by the general formula:

$$y=m*Log(x)+q$$

Here, **m** represents the slope of the line, **q** the y-intercept and **x** the number of gametocyte equivalents in each sample, associated with the corresponding Ct value (y).

The values of R^2, all > 0.9, the linearity of the slopes, and the amplification efficiency across all experiments indicate the robust reproducibility of the assay.

Based on the quality of the assay parameters, two methods were used to determine the sex ratio of gametocytes from the aforementioned parasite lines.



NF54	Slope	R^2	Efficiency %	Y-intercept
pfs25	-3.006	0.991	115.1	33.58
pfMGET	-3.143	0.989	108.0	35.40

NF54	Slope	R^2	Efficiency %	Y-intercept
pfCCp4	-2.567	0.984	145.2	36.01
pfMGET	-2.671	0.988	136.7	33.98

HB3 sel4	Slope	R^2	Efficiency %	Y-intercept
pfs25	-2.872	0.993	122.9	34.22
pfMGET	-3.005	0.960	115.2	34.62

HB3 sel 4	Slope	R^2	Efficiency %	Y-intercept
pfCCp4	-3.270	0.983	102.2	38.03
pfMGET	-3.283	0.992	101.7	34.62

PfDynGFP/ P47mCherry	Slope	R^2	Efficiency %	Y-intercept
pfs25	-3.201	0.997	105.3	33.96
pfMGET	-3.430	0.970	95.7	38.48

PfDynGFP/ P47mCherry	Slope	R^2	Efficiency %	Y-intercept
pfCCp4	-2.992	0.990	115.9	36.33
pfMGET	-3.133	0.939	108.5	36.49

Figure 11. *In the left column the multiplex amplification pfs25 and pfMGET, in the right column the multiplex amplification of pfCCp4 and pfMGET. N= 3 in each experiment.* LOD_{pfCCP4}*=1 gct per µl;* LOD_{pfMGET}*=0.01 gct per µl;* LOD_{pfs25}*=0.1 gct per µl.*

4.1.3. Comparison between the sex ratio values obtained by IFA and by DCt determination in RTqPCR on gametocytes from the NF54, HB3 sel 4 and PfDynGFP/P47mCherry lines

The first method involved measuring the sex ratio of a gametocyte population through the parameter **DCt** derived from RTqPCR reactions. In these experiments, the DCt parameter is defined as the algebric difference between the Cycle threshold (Ct) of the female-specific target (*pfCCp4* or *pfs25*) and the Ct values of the male-specific target (*pfMGET*). As two female specific markers have been used, a DCt1 parameter was calculated as *pfCCp4* – *pfMGET*, and a second one, DCt2, as *pfs25* - *pfMGET*.

The Ct values obtained from the RTqPCR assays, along with the corresponding counts of male and female gametocytes obtain by IFA, are shown in Tables 2 and 3. For the three lines, the average DCt value for each clone was then compared with the sex ratio value obtained by IFA. Results, shown also in the Table 4, are the following:

- For NF54 line: **DCt1 = 0.79** ± 0.80. **DCt2= -4.28** ± 0.68
- For HB3 sel 4 line: **DCt1 = 1.48** ± 0.39. **DCt2= -1.94** ± 0.99
- For PfDynGFP/P47mCherry line: **DCt1 = -0.18** ± 0.80. **DCt2= -3.46** ± 0.79

	Male GCT	CT *pfMGET*	Female GCT	CT *pfs25*
NF54	1.E+05	**19.4** ± 0.53	9.E+05	**15.5** ± 1.58
	1.E+04	**22.3** ± 0.67	9.E+04	**18.4** ± 0.08
	1.E+03	**25.7** ± 0.57	9.E+03	**21.8** ± 0.10
	1.E+02	**28.9** ± 0.50	9.E+02	**25.0** ± 0.15
	1.E+01	**31.9** ± 0.25	9.E+01	**27.9** ± 0.39
	1.E+00	**35.3** ± 0.60	9.E+00	**30.9** ± 0.68
	1.E-01	**39.5** ± 0.61	9.E-01	**33.9** ± 0.55
	1.E-02	**40.3** ± 0.66	9.E-02	**36.2** ± 0.90
HB3 sel 4	2.E+05	**18.9** ± 1.48	8.E+05	**17.8** ± 0.14
	2.E+04	**21.4** ± 1.71	8.E+04	**19.4** ± 0.31
	2.E+03	**24.5** ± 1.56	8.E+03	**22.5** ± 0.31
	2.E+02	**27.7** ± 1.54	8.E+02	**25.7** ± 0.38
	2.E+01	**30.8** ± 1.73	8.E+01	**28.7** ± 0.60
	2.E+00	**33.8** ± 1.79	8.E+00	**31.7** ± 0.61
	2.E-01	**37.1** ± 1.76	8.E-01	**34.6** ± 0.50
PfDynGFP/ P47mCherry	5.E+05	**19.8** ± 1.18	6.E+05	**15.2** ± 0.26
	5.E+04	**22.7** ± 0.91	6.E+04	**18.7** ± 0.41
	5.E+03	**26.5** ± 1.03	6.E+03	**22.4** ± 0.28
	5.E+02	**29.9** ± 1.25	6.E+02	**25.8** ± 0.22
	5.E+01	**32.9** ± 0.98	6.E+01	**28.7** ± 0.23
	5.E+00	**37.5** ± 2.55	6.E+00	**31.9** ± 0.02
	5.E-01	**40.9** ± 1.22	6.E-01	**34.2** ± 0.31
	5.E-02	**41.7** ± #	6.E-02	**37.6** ± 0.36

Table 2. *Values of Cts of the targets pfMGET and pfs25 in NF54, HB3 and PfDynGFP/ P47mCherry laboratory lines and number of equivalent male and female gametocytes obtain by IFA*

	Male GCT	CT *pfMGET*	Female GCT	CT *pfCCp4*
NF54	1.E+05	**19.6** ± 1.14	9.E+05	**20.8** ± 0.95
	1.E+04	**22.0** ± 0.78	9.E+04	**22.8** ± 0.51
	1.E+03	**25.5** ± 0.62	9.E+03	**26.2** ± 0.47
	1.E+02	**28.4** ± 1.11	9.E+02	**29.1** ± 1.30
	1.E+01	**30.0** ± 2.39	9.E+01	**30.5** ± 2.56
	1.E+00	**32.8** ± 3.63	9.E+00	**33.6** ± 4.42
HB3 sel 4	2.E+05	**17.4** ± 0.68	8.E+05	**19.3** ± 0.94
	2.E+04	**20.3** ± 0.70	8.E+04	**21.5** ± 0.96
	2.E+03	**23.7** ± 0.81	8.E+03	**24.9** ± 0.88
	2.E+02	**26.9** ± 0.56	8.E+02	**28.2** ± 0.70
	2.E+01	**30.6** ± 0.38	8.E+01	**31.9** ± 0.65
	2.E+00	**33.8** ± 0.62	8.E+00	**35.9** ± 0.55
	2.E-01	**36.5** ± 0.20	8.E-01	**37.7** ± 0.79
PfDynGFP/ P47mCherry	5.E+05	**18.9** ± 0.94	6.E+05	**19.6** ± 0.96
	5.E+04	**21.7** ± 0.78	6.E+04	**21.4** ± 1.35
	5.E+03	**25.8** ± 0.67	6.E+03	**25.3** ± 1.23
	5.E+02	**28.5** ± 1.16	6.E+02	**28.1** ± 1.87
	5.E+01	**31.5** ± 1.29	6.E+01	**31.3** ± 1.92
	5.E+00	**34.3** ± 2.60	6.E+00	**34.0** ± 3.43

Table 3. *Values of Cts of the targets pfMGET and pfCCp4 in NF54, HB3 and PfDynGFP/ P47mCherry laboratory lines and number of equivalent male and female gametocytes obtain by IFA*

The results show that in NF54 and HB3 sel 4 both the DCt1 and the DCt2 values show an increase which is consistent with the increase of the sex ratio values as determined by IFA. However, in the pfDynGFP/P47mCherry transgenic line, in which the sex ratio determined by IFA is higher than those of the other lines, the DCt values are lower (DCt1) and intermediate (DCt2) with respect to those obtained in NF54 and HB3 sel 4. In conclusion, although the three lines used here were chosen for the markedly different sex ratios, the results from the pfDynGFP/P47mCherry transgenic line made it impossible to establish a positive correlation across all lines between the values of DCt and those of the IFA-based sex ratios.

4.1.4. Calculation of transcript sex ratio by using Ct values from RTqPCR on gametocytes from the NF54, HB3 sel 4 and pfDynGFP/P47mCherry lines

The second approach proposes a calculation of a parameter called **transcript sex ratio ($T_{SEX\ RATIO}$)** in each *P. falciparum* line. This strategy would predict the ratio between the numbers of copies of sex-specific transcripts in each gametocyte population. The numbers of copies were obtained by interpolating the Ct values from RTqPCR reactions performed on serial dilutions of gametocytes in the standard curves obtained from serial dilutions of synthetic gene sequences, expressed as gene copy number.

To generate the synthetic copies of the *pfCCp4*, *pfs25* and *pfMGET* genes, portions of the gene sequences were cloned into the pEX-A128 plasmid (see Figure 12).

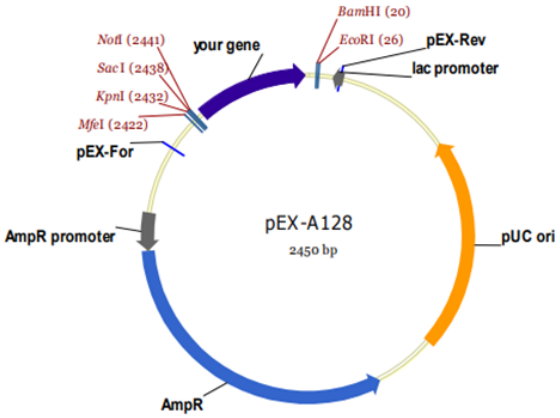


Figure 12. *Plasmid map pEX-A128 containing portions of the P. falciparum pfCCp4 (489 bp), pfMGET (539 bp), and pfs25 (213 bp) genes*

The resulting plasmids were amplified by transforming competent bacteria of the One shot TOP 10 strain. Following DNA extraction, the plasmids were digested with the restriction enzyme EcoRI, whose recognition sites were inserted upstream and downstream the gene sequences. The DNA fragments containing the target sequences were purified by extraction from agarose gel after electrophoretic separation and transcribed using the MEGAshortscript kit, employing T7 RNA polymerase with the specific promoter sequence inserted upstream of the genes. The resulting RNA was then quantified on the Qubit 2 Fluorometer and the copy number was calculated based on the sequence length using the formula:

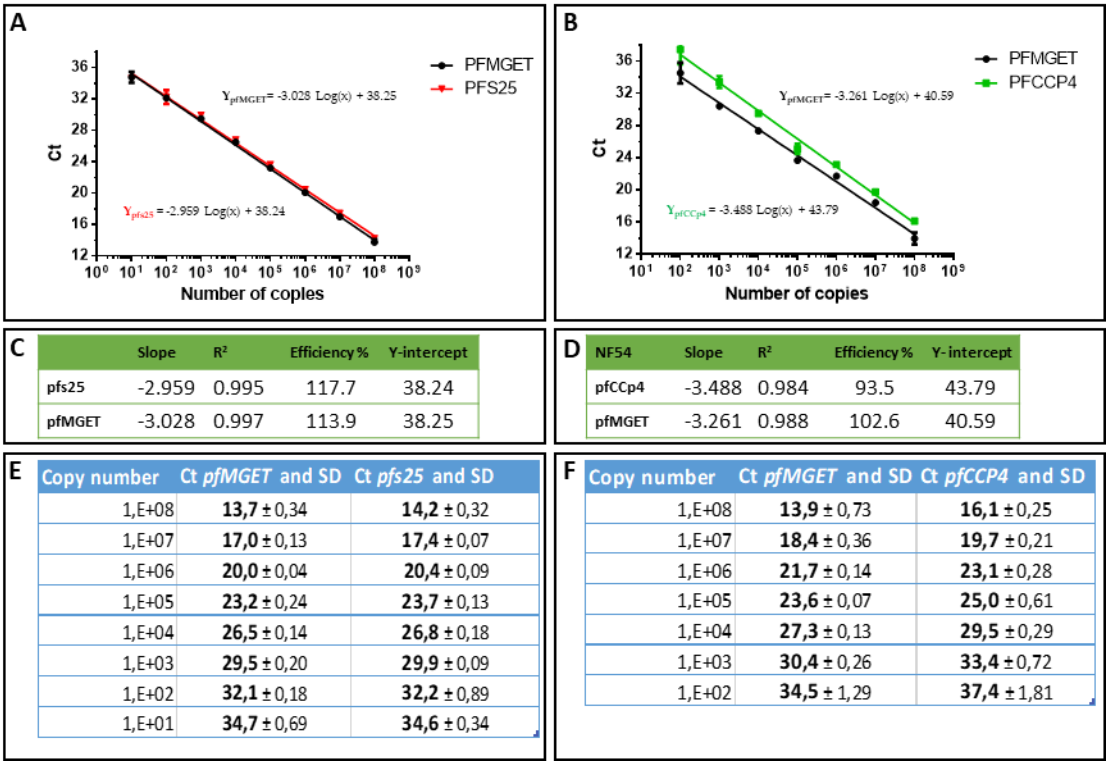
$$copy\ number\ of\ RNA = \frac{\text{weight (ng) x } 6{,}0221 \text{ x } 10^{23}}{\text{lenght x } 330 \text{ x } 1 \text{ x } 10^{9}}$$

To determine the copy number of the *pfCCP4*, *pfs25* and *pfMGET* transcripts, standard curves were obtained through tenfold serial dilutions of the three

synthetic genes, starting with 10^8 copies, to be used to calculate the number of copies of each target mRNA per gametocyte dilution in the three *P. falciparum* lines.

As with the RTqPCR assays described above, two different multiplex assays were performed, in which the male marker *pfMGET* was paired with one of the two female markers, *pfs25* or *pfCCp4*.

The curves generated by the synthetic gene constructs and the equations that describe them, are shown in Figure 13.



C

	Slope	R^2	Efficiency %	Y-intercept
pfs25	-2.959	0.995	117.7	38.24
pfMGET	-3.028	0.997	113.9	38.25

D

NF54	Slope	R^2	Efficiency %	Y-intercept
pfCCp4	-3.488	0.984	93.5	43.79
pfMGET	-3.261	0.988	102.6	40.59

E

Copy number	Ct *pfMGET* and SD	Ct *pfs25* and SD
1,E+08	**13,7** ± 0,34	**14,2** ± 0,32
1,E+07	**17,0** ± 0,13	**17,4** ± 0,07
1,E+06	**20,0** ± 0,04	**20,4** ± 0,09
1,E+05	**23,2** ± 0,24	**23,7** ± 0,13
1,E+04	**26,5** ± 0,14	**26,8** ± 0,18
1,E+03	**29,5** ± 0,20	**29,9** ± 0,09
1,E+02	**32,1** ± 0,18	**32,2** ± 0,89
1,E+01	**34,7** ± 0,69	**34,6** ± 0,34

F

Copy number	Ct *pfMGET* and SD	Ct *pfCCP4* and SD
1,E+08	**13,9** ± 0,73	**16,1** ± 0,25
1,E+07	**18,4** ± 0,36	**19,7** ± 0,21
1,E+06	**21,7** ± 0,14	**23,1** ± 0,28
1,E+05	**23,6** ± 0,07	**25,0** ± 0,61
1,E+04	**27,3** ± 0,13	**29,5** ± 0,29
1,E+03	**30,4** ± 0,26	**33,4** ± 0,72
1,E+02	**34,5** ± 1,29	**37,4** ± 1,81

Figure 13**. Standard curves of:* **A** *pfs25 and pfMGET and* **B** *pfCCp4 and pfMGET synthetic targets in Real time PCR reactions. N=3. Data on Slope, R^2 and amplification efficiency value of the two RTqPCr assays are reported in* ***C *and* ***D***. *Ct value and standard deviation (SD) of the target gene pfs25 and pfMGET in* C *and pfCCp4 and pfMGET in* **E** and **F**

The process for obtaining the transcript copy numbers in each dilution involves the comparison of these synthetic gene constructs with the data obtained in the same assays conducted on serial dilutions of gametocyte, with the Ct values shown in Tables 2 and 3 and the slopes in Figure 11.

The procedure is the following:

1. **Obtaining gene copy numbers**: Utilize the Ct values acquired from the RTqPCR assay conducted on serial dilutions of gametocytes in the equations of the standard curves of the corresponding synthetic genes. Calculate the gene copy numbers (x) by applying the inverse formula:

$$x\ (gene\ copy\ numbers\ ) = 10^{(\frac{Ct}{Slope} - \frac{intercept}{Slope})}$$

   Interpolate all the Ct values obtained from gametocyte dilutions amplified in RTqPCR into the equation, using the Slope and Intercept values specific to the corresponding synthetic target. This process yields the number of copies of the gene amplified in each gametocyte sample dilution.

2. **Linearity Assessment**: To evaluate the linearity of dilutions for the transcripts within the gametocyte dilutions in each RTqPCR assay, a fitted linear regression for each one of the two transcript was performed using the total number of gametocytes and the copy number of transcripts in each dilution respectively as abscissae and ordinate whit the slope constrain to 1. The fitted linear regression is shown in the Figure 14.

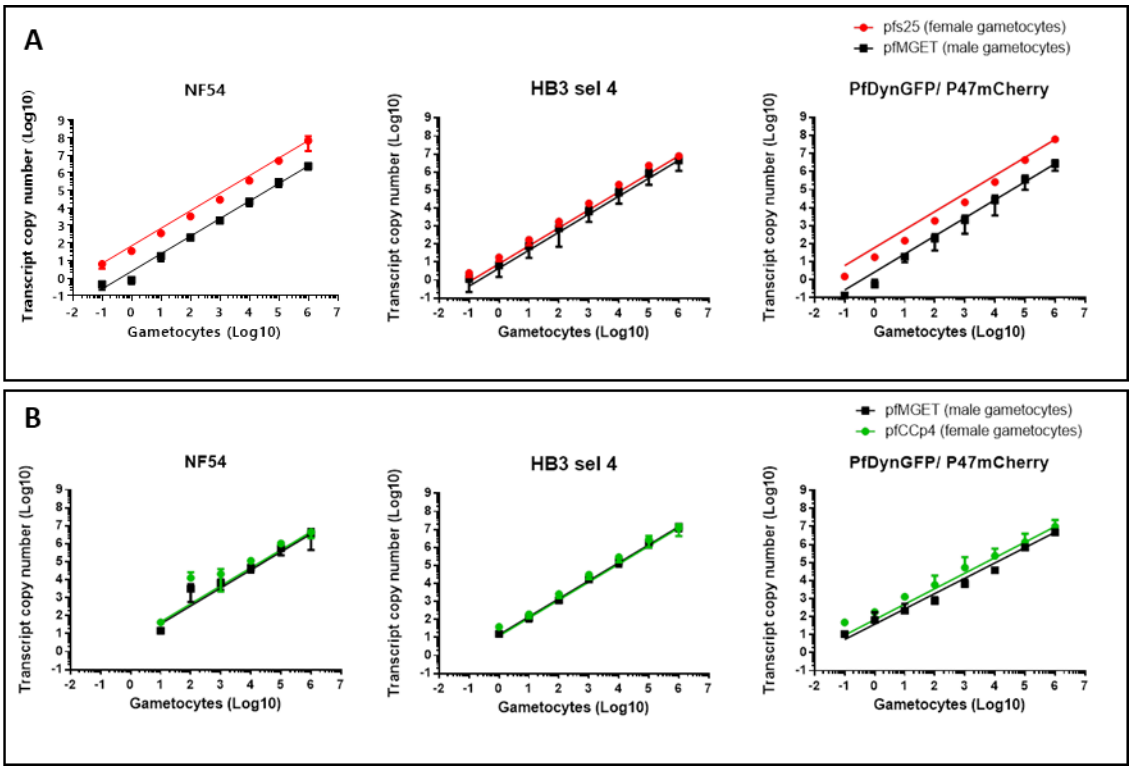


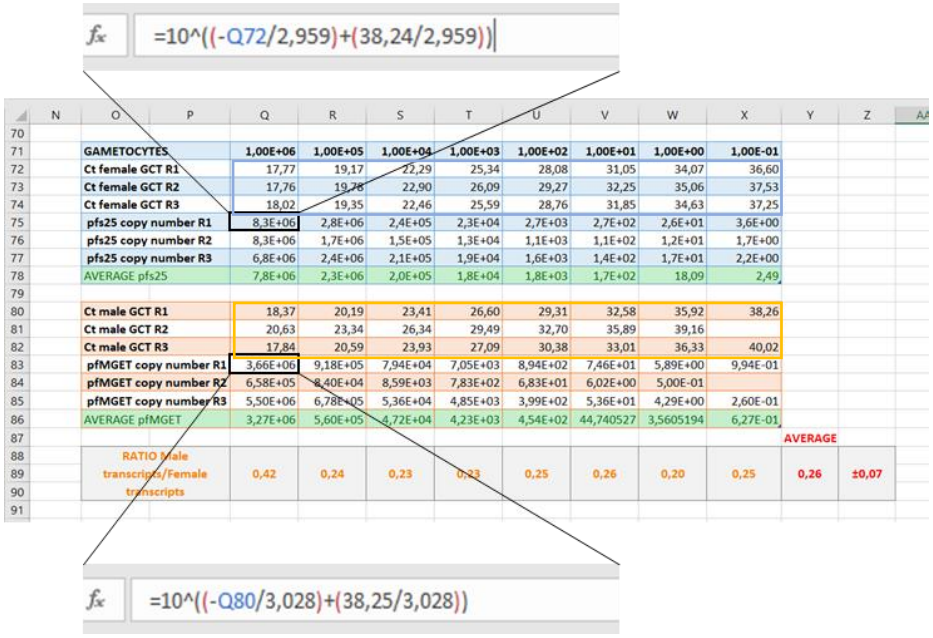
***Figure 14**. Fitted linear regression of RTqPCR amplifying the couple of transcripts pfs25/pfMGET in panel **A** and pfCCp4/pfMGET in the panel **B**. These curves were performed with gametocytes used for each dilution as ordinate and transcript copy number as abscissae. They allow to assess the linearity of the dilution with the quantity of transcripts.*

3. **Obtaining transcript sex ratio**: As the sex ratio is defined as the number of male over the number of female gametocytes, the number of copies obtained from the interpolation of Ct values in the equation of the synthetic target was used to obtain the transcript sex ratio by using the following formula:

$$\mathbf{T}_{\text{SEX RATIO}} = \frac{\text{Copy number } pfMGET}{\text{Copy number } pfCCp4/pfs25}$$

As two female specific markers have been used, a $T_{SEX\ RATIO}1$ parameter was calculated as *pfMGET/pfCCp4*, and a second one, $T_{SEX\ RATIO}2$, as *pfMGET/pfs25*.

In Figure 15, a detailed illustration demonstrating the process of Ct interpolation was provide, showcasing how $T_{SEX\ RATIO}2$ values are derived. This specific example focuses on the HB3 sel 4 line. Ct values from three replicas of gametocytes amplified in RTqPCR assays using *pfs25* and *pfMGET* primers are interpolated in the equations of correspondent synthetic targets. This procedure yields the corresponding number of copies for each gene in every gametocyte sample dilution. $T_{SEX\ RATIO}$ values are obtained by dividing the copy number of *pfMGET* by the copy number of *pfs25* in each dilution.



GAMETOCYTES	1,00E+06	1,00E+05	1,00E+04	1,00E+03	1,00E+02	1,00E+01	1,00E+00	1,00E-01		
Ct female GCT R1	17,77	19,17	22,29	25,34	28,08	31,05	34,07	36,60		
Ct female GCT R2	17,76	19,78	22,90	26,09	29,27	32,25	35,06	37,53		
Ct female GCT R3	18,02	19,35	22,46	25,59	28,76	31,85	34,63	37,25		
pfs25 copy number R1	8,3E+06	2,8E+06	2,4E+05	2,3E+04	2,7E+03	2,7E+02	2,6E+01	3,6E+00		
pfs25 copy number R2	8,3E+06	1,7E+06	1,5E+05	1,3E+04	1,1E+03	1,1E+02	1,2E+01	1,7E+00		
pfs25 copy number R3	6,8E+06	2,4E+06	2,1E+05	1,9E+04	1,6E+03	1,4E+02	1,7E+01	2,2E+00		
AVERAGE pfs25	7,8E+06	2,3E+06	2,0E+05	1,8E+04	1,8E+03	1,7E+02	18,09	2,49		
Ct male GCT R1	18,37	20,19	23,41	26,60	29,31	32,58	35,92	38,26		
Ct male GCT R2	20,63	23,34	26,34	29,49	32,70	35,89	39,16			
Ct male GCT R3	17,84	20,59	23,93	27,09	30,38	33,01	36,33	40,02		
pfMGET copy number R1	3,66E+06	9,18E+05	7,94E+04	7,05E+03	8,94E+02	7,46E+01	5,89E+00	9,94E-01		
pfMGET copy number R2	6,58E+05	8,40E+04	8,59E+03	7,83E+02	6,83E+01	6,02E+00	5,00E-01			
pfMGET copy number R3	5,50E+06	6,78E+05	5,36E+04	4,85E+03	3,99E+02	5,36E+01	4,29E+00	2,60E-01		
AVERAGE pfMGET	3,27E+06	5,60E+05	4,72E+04	4,23E+03	4,54E+02	44,740527	3,5605194	6,27E-01		
									AVERAGE	
RATIO Male transcripts/Female transcripts	0,42	0,24	0,23	0,23	0,25	0,26	0,20	0,25	0,26	±0,07

Figure 15. Procedure for obtaining transcript sex ratio values (T SEX RATIO). *The Ct values obtained from each RTqPCR replica in which gametocyte dilutions were amplified whit pfs25 and pfMGET primers (cells Q72-X74 for pfs25 and cells Q80-X83 for pfMGET), were interpolated in the equations of the respective synthetic genes (see Figure 13 A). The average values were calculated for each gametocyte dilution.* ***T SEX RATIO*** *values were calculated by dividing average pfMGET transcripts with average pfs25 transcripts in each dilution. Finally a unique average T SEX RATIO with standard deviation value was obtained.*

T SEX RATIO values obtained with the above shown procedure are the following:

- **NF54**: TSEX RATIO 1: **0.408** ± 0.22; TSEX RATIO 2: **0.033** ± 0.01.
- **HB3**: TSEX RATIO 1: **0.620** ± 0.282; TSEX RATIO 2: **0.260** ± 0.07.
- **pfDynGFP/P47mCherry**: TSEX RATIO 1: **0.200** ± 0.13; TSEX RATIO 2: **0.051** ± 0.02

In Table 4, a summary of the results obtained from DCt calculation, TSEX RATIO and IFA assay are reported.

	NF54 (n=3)	HB3 sel 4 (n=3)	Dyn-GFP (n=3)
DCt1 (Ct_{pfCCp4}-Ct_{pfMGET})	**0.79** ± 0.80	**1.48** ± 0.39	**-0.18** ± 0.80
DCt2 (Ct_{pfs25}-Ct_{pfMGET})	**-4.28** ± 0.68	**-1.94** ± 0.99	**-3.46** ± 0.79
$T_{SEX\ RATIO}$ 1 (pfMGET/pfCCp4)	**0.408** ± 0.22	**0.620** ± 0.282	**0.200** ± 0.13
$T_{SEX\ RATIO}$ 2 (pfMGET/pfs25)	**0.033** ± 0.01	**0.260** ± 0.07	**0.051** ± 0.02
Sex ratio by IFA	**0.11** ± 0.005	**0.25** ± 0.004	**0.80** ± 0.006

Table 4. *Summary table with the results obtained from DCt calculation and T SEX RATIO from both RTqPCR assay performed on dilutions of gametocytes of the laboratory lines NF54, HB3 sel 4 and pfDynGFP/P47mCherry.*

In summary also whit this approach, the values of TSEX RATIO across the different lines and assays, mirror the increase of the sex ratio values determined by IFA between NF54 and HB3 sel 4. However, in the pfDynGFP/P47mCherry transgenic line, in which the sex ratio determined by IFA is higher than those of the other lines, TSEX RATIO values are lower (TSEX RATIO1) and intermediate (TSEX RATIO2) with respect to those obtained in NF54 and HB3 sel 4 . In conclusion, although the three lines used here were chosen for the markedly different sex ratios, the results from the pfDynGFP/P47mCherry transgenic line made it impossible to establish a positive correlation across all lines between the values of sex specific transcripts and those of the IFA-based sex ratios.

With the aim to understand the sex ratio dynamics across the above cited *P. falciparum* laboratory lines, further studies were done.

4.1.5. Calculation of the copy number of *pfCCp4*, *pfs25* and *pfMGET* transcripts for female and male gametocytes using Ct values obtained in RTqPCR

A further approach to understand the sex ratio dynamics across the above cited *P. falciparum* laboratory lines, consisted in determining the copy number of sex-specific transcripts for male and female gametocytes. The copy number was obtained by relating the Ct values from RTqPCR reactions on serial dilutions of gametocytes, whose number of males and females had been preliminarily estimated by IFA, with the Ct values from RTqPCR reactions on serial dilutions of the synthetic gene sequences shown in the Figure 13. The process for obtaining the transcript copy number per gametocyte involving the comparison of these synthetic gene constructs with the data obtained in the same assays conducted on serial dilutions of gametocyte equivalents, with the Ct values shown in Tables 2 and 3 and the slopes in Figure 11. The procedure is the following:

1. **Ct selection**: Choose several Ct values within the range of Ct of the standard curves to compared (4-5 values)
2. **Ct values interpolation**: Replace the chosen Ct values in both the equations of the standard curves for gametocytes amplified with sex-specific transcripts and in those of the corresponding synthetic genes. Utilize the same inverse formula mentioned in the previous paragraph to

calculate the gene copy numbers (in the first case) or the gametocyte number values (in the second case):

$$x\ (gene\ copy\ number\ or\ gametocyte\ number) = 10^{(\frac{Ct}{Slope} - \frac{intercept}{Slope})}$$

Interpolating a selected Ct values in the equation with the Slope and Intercept values of syntetic target and in the equation with the Slope and Intercept values of the gametocyte curve, a corresponding number of copies of the synthetic target and a corresponding number of gametocytes were obtained, respectively.

3. **Interpolation Result**: The x values obtained from the interpolation of Ct values both in the equation of the synthetic target and in the equations of the three replicas of each gametocyte line, were used respectively as abscissae and ordinate to perform a fitted linear regression whit the slope constrained to 1 with the aim to calculate the conversion factor (copy numbers per gametocyte).

An illustrative example of Ct interpolation is presented in Figures 16 and 17, showcasing the interpolation of the female-specific target *pfs25* and female gametocytes of the HB3 sel 4 line. The same Ct values (19, 22, 25, 28, 31) were interpolated in both the equation of the synthetic target *pfs25* and in the equations of each three HB3 sel 4 replicas where *pfs25* transcripts were amplified.

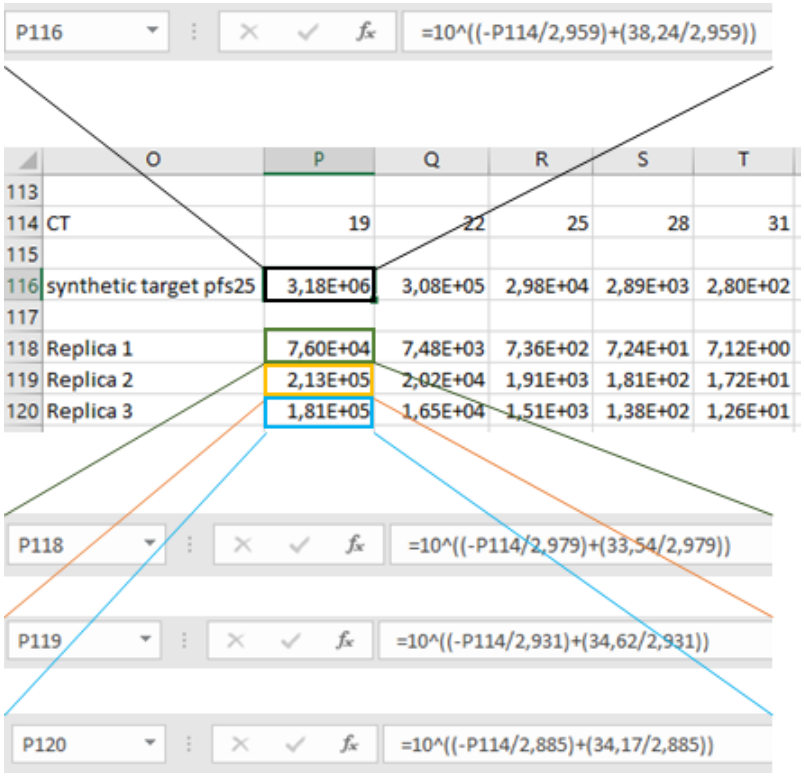


Figure 16 *Procedure for interpolation of the same Ct values (reported in the line 114) both in the curve of synthetic target gene pfs25 (line 116) and in those of three replicas of HB3 sel 4 gametocytes amplified with pfs25 primers and probe (118, 119, 120). For each replica, the different equations used were reported.*

The x values obtained from interpolation of Ct in the equation of synthetic target *pfs25* and those obtained from the interpolation in the equations of the three HB3 sel 4 replicas where *pfs25* transcripts were amplified, were used respectively as abscissae and ordinate to develop a fitted linear regression. For the calculation of the conversion factor (copy numbers per gametocyte), linear regression was performed with the slope constrained to 1. The linear regression is reported in the Figure 17.

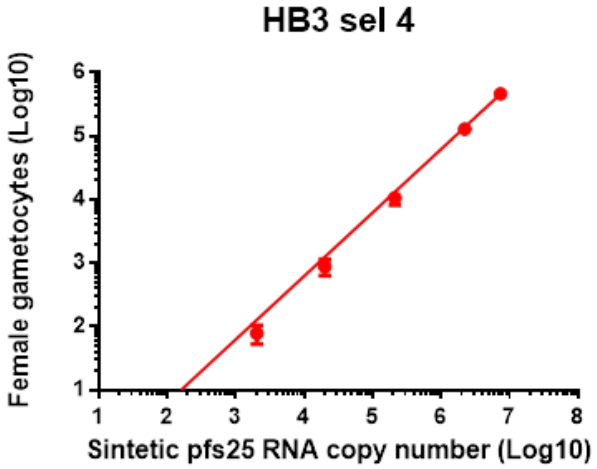


***Figure 17**. Quantification of pfs25 transcripts per female gametocyte in the line HB3 sel 4. Pfs25 transcript copies for female gametocyte are 20.30 (95% CI 17,48- 24,25).*

The error bar is given by the data of the three replicas. The calculated *pfs25* copies for female gametocytes are 20.30 (95% CI 17,48- 24,25).

The same procedure was used for the other transcripts in all the three laboratory lines and the results are presented in the Figure 18.

For the extrapolation of a copy number of *pfMGET,* in each line, the equations of both RTqPCR experiments (*pfMGET-pfs25* and *pfMGET- pfCCp4*) were used.

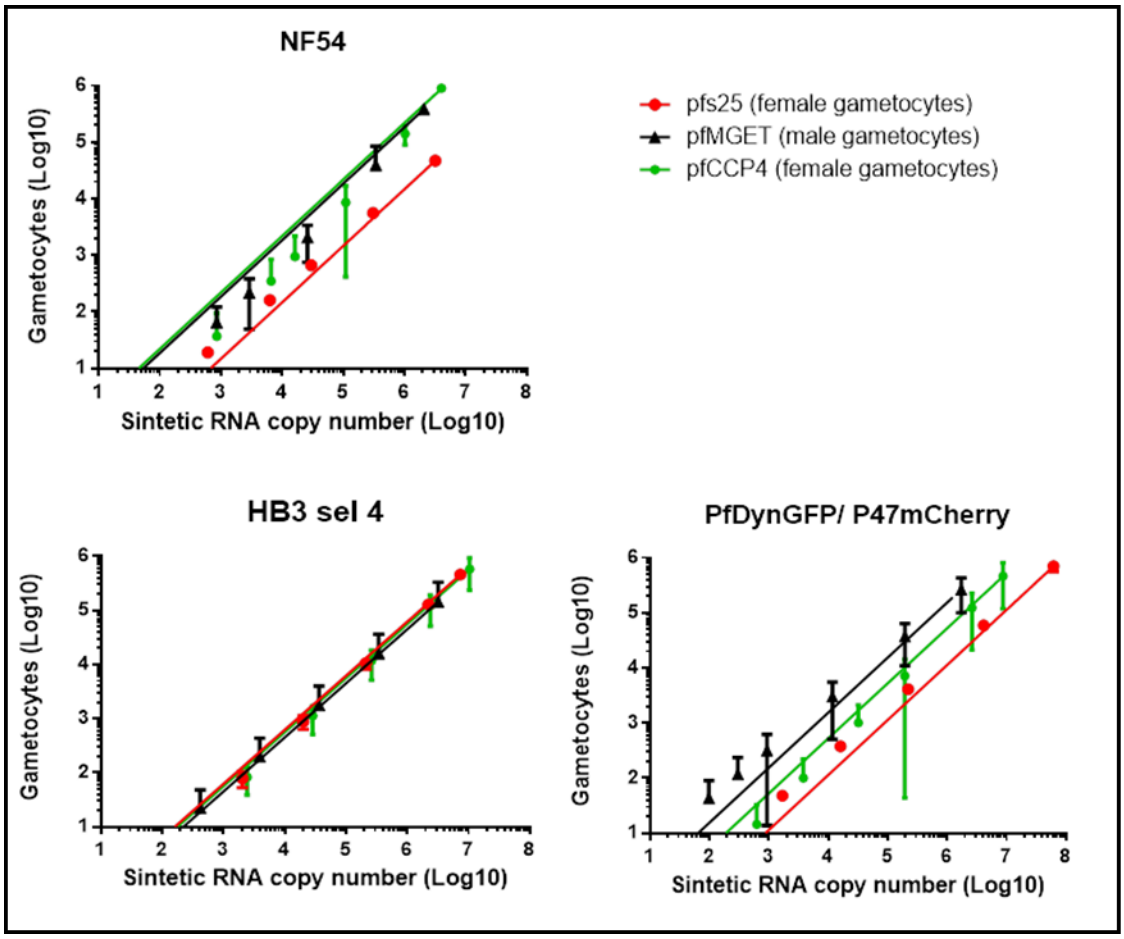


***Figure 18**. Fitted linear regression for sex-sorted gametocytes and calculated copy numbers for pfCCp4, PfMGET and Pfs25 in the laboratory lines NF54, HB3 sel 4 and PfDynGFP/P47mCherry*

The data obtained from the interpolations are as follow:

- **NF54**: for female gametocyte, **67.71** (95% CI 62.57 – 73.47) transcript copies of *pfs25* and **4.52** (95% CI 4.26 – 4.88) of *pfCCp4* were estimated. For male gametocyte **5.5** (95% CI 3.64 – 10.39) copies of *pfMGET*.
- **HB3 sel 4**: for female gametocyte, **20.30** (95% CI 17.48 – 24.25) transcript copies of *pfs25* and **17.94** (95% CI 14.24 – 24.21) of *pfCCp4* were estimated. For male gametocyte **22.18** (95% CI 16.04 – 35.91) copies of *pfMGET*.

- **PfDynGFP/P47mCherry**: for female gametocyte, **87.89** (95% CI 81.89 – 94.79) transcript copies of *pfs25* and **18.93** (95% CI 14.39 – 27.60) of *pfCCp4* were estimated. For male gametocyte **6.48** (95% CI 5.46 – 7.98) copies of pfMGET.

Analysis of the data reveals variations in the number of copies of sex-specific transcripts among different P. falciparum laboratory lines. Consequently, the molecular sex ratios, determined by the copy numbers of synthetic transcripts, differ across these lines and assays as follows:

- **NF54**: **0.009** using *pfMGET/pfs25* amplification; **0.135** using *pfMGET/pfCCP4* amplification.

- **HB3 sel 4**: **0.273** using pfMGET/pfs25 amplification; **0.309** using pfMGET/pfCCP4 amplification.

- **PfDynGFP/P47mCherry**: **0.06** using pfMGET/pfs25 amplification. **0.280** using pfMGET/pfCCP4 amplification.

4.2. Analysis of RTqPCR assays with the ΔΔCT method to measure levels of sex specific transcripts in gametocytes treated with antimalarial compounds to reveal effects on sex ratio.

To test the activity of antimalarial compounds on *Plasmodium falciparum* gametocytes and to evaluate possible sex specific effects, the above multiplex RTqPCR assays amplifying sex specific transcripts were used. In these experiments, the data obtained were analyzed using the DeltaDeltaCt (ΔΔCt) method, which has been described in detail in the Methods section.

As this method requires, each data point necessitates the normalization of marker transcript levels with those of a reference transcript. To achieve this, RTqPCR reactions were employed to amplify the human *GAPDH* gene (E7EUT5_HUMAN) in each experiment. This gene is expressed in human leukocytes present in the whole blood in which gametocytes are present, both in the control samples and those exposed to treatment, as detailed below.

In the relative quantification method defined as ΔΔCt, the quantitative level of a given target is calculated by determining for each sample the difference between the level of the target transcript and that of the reference transcript, whose quantity is not affected by the treatment. This difference is then compared to an untreated control sample. The procedure for the ΔΔCt calculation is illustrated in the Figure 19.

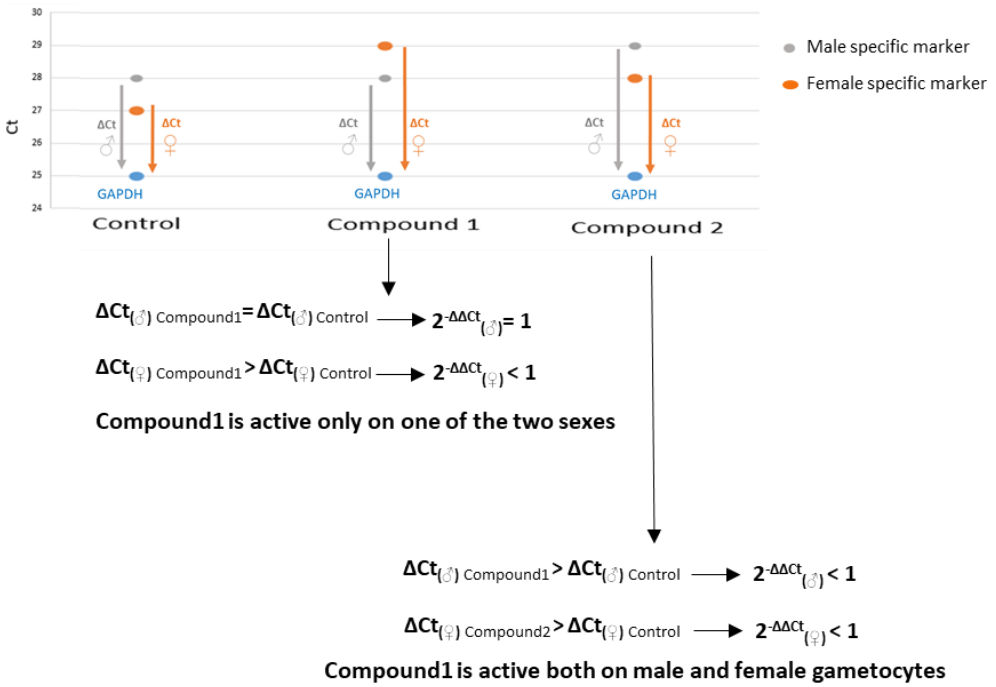


Figure 19. *The image represents a scheme for ΔΔCt calculation procedure.*

Data from experiments evaluated with the ΔΔCt method are analyzed using the $2^{-\Delta\Delta Ct}$ parameter. ΔCt values, representing the difference in Ct values between the target and reference genes, play a crucial role in this analysis. When ΔCt values are identical in both the experimental and control groups, the $2^{-\Delta\Delta Ct}$ value = 1, indicating no change in the target transcript gene levels between the two groups.

Furthermore, if the $2^{-\Delta\Delta Ct}$ value is < 1, it signifies an increase in ΔCt, suggesting a decrease in the level of the mRNA of interest compared to the control. For instance, in Figure 19, in the case of the sample treated with Compound 1, the ΔCt of the male-specific marker and the reference gene GAPDH is the same as the control sample, indicating no change in the transcript level. However, the ΔCt

of the female-specific marker increases in the treated sample compared to the control, revealing a decrease in transcript level. This example illustrates a compound that acts selectively against one of the two sexes.

In contrast, in the sample treated with Compound 2, ΔCt values of both sex-specific markers increase compared to the control sample. This increase in ΔCt values indicates a decrease in the transcript level of both genes, resulting in $2^{-\Delta\Delta Ct}$ values < 1.

In summary, the ΔΔCt method is a methodological approach based here on multiplex RTqPCR assays used to evaluate the effects of antimalarial compounds on *Plasmodium falciparum* gametocytes. The inclusion of a reference transcript (*GAPDH*) facilitates data normalization, and the $2^{-\Delta\Delta Ct}$ parameter helps quantify and compare changes in target transcript levels induced by treatment.

4.2.1. Validation of the method

To validate the ΔΔCt method it was necessary to compare the amplification efficiency of all target sequences through the construction of preliminary standard curves generated from the serial dilutions of RNA extracted from gametocyte samples and amplified by RTqPCR.

To achieve this, two RTqPCR experiments were conducted using 1.4 x 10^5 NF54 gametocytes suspended in 100 microliters of whole blood. In the first experiment, 100 ng of RNA extracted from these gametocytes was used to obtain five 1:5 serial dilutions, used to amplify in multiplex mode the sex specific transcript *pfs25* and *pfMGET* and the reference gene *GAPDH* . In the second experiment, 85 ng of RNA were used to produce the same 1:5 serial dilutions, used for amplifying the sex

specific transcripts *pfCCp4* and *pfMGET*, as well as *GAPDH*. Amplification curves and the value of the Slope, R^2 and amplification efficiency are reported in Figure 20.

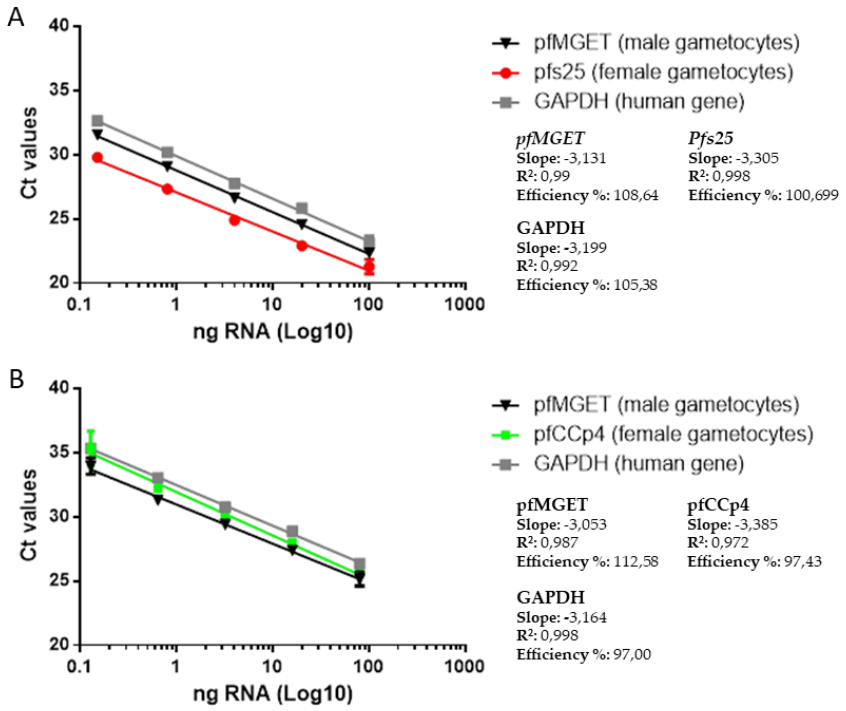


***Figure 20**. Amplification curves of **A)** pfs25, pfMGET and GAPDH transcripts **B)** pfCCp4, pfMGET and GAPDH transcripts in serial dilutions of RNA extracted from gametocytes in whole blood with the values of the Slope, R^2 and amplification efficiency of each target. N=3*

These standard curves serve to assess the efficiency of the RTqPCR reaction for each target. For ΔΔCt method calculation to be used, the amplification efficiency of the target and reference genes must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency consists in the following steps:

4. **ΔCt Calculation**: Calculate ΔCt for each dilution by subtracting the Ct value of the reference gene (GAPDH) from the Ct value of each target gene.

5. **Logarithmic Plot**: Plot the Log of the RNA dilution against ΔCt for each dilution.
6. **Slope Calculation**: Calculate the slope of the resulting straight line. The slope is indicative of the relationship between RNA dilution and ΔCt. If the slope value is close to zero, it suggests that the efficiencies of the target and reference genes are similar.

Figure 21 shows the results were Log of the RNA dilution samples used to performed standard curves of the Figure 20 were plotted against ΔCt (Ct *target gene* – Ct *GAPDH*).

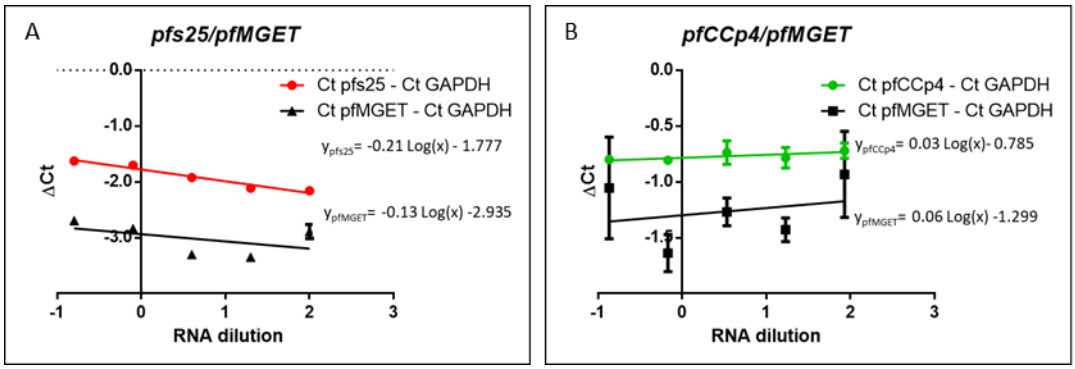


***Figure 21**. Validation of the ΔΔCT method. ΔCt (Ct target gene- Ct GAPDH) was plotted against the logarithm of the template amount to calculate the slope of the resulting straight*

As shown in the Figure 19, the slopes of the lines obtaine range from -0.2 to 0.027. The therefore, the assumption holds and the ΔΔCT method may be used to analyze the data.

4.2.2. Validation of the assay

The above results indicated that the ΔΔCt assay can be appropriately used as a relative quantification method to measure level of gametocyte sex specific

transcripts, and therefore that its use can be explored to test effects of compounds on gametocytes of *Plasmodium falciparum* to possibly reveal specific activity on male and female gametocytes.

To preliminarily validate the ΔΔCt assay, purified mature gametocytes of the *P. falciparum* NF54 line were resuspended in 50 microliters of whole blood, resulting in a final concentration of $3x10^3$ gametocytes per microliters. The samples were incubated with two compounds, Methylene blue (1 μM) and Epoxomycin (1 μM) for 24 hours. In the untreated control samples, gametocytes were resuspended in DMSO 0.1% in RPMI, the same organic compound in which the test compounds were dissolved. The experiment was conducted in duplicate. At the end of the treatment, the samples were resuspended in ten volume of TRIzol™ reagent and stored at -80 °C until RNA extraction. RNA was amplified using the two RTqPCR assays *pfMGET/pfCCp4* and *pfMGET/pfs25* and the data obtained were analyzed with the ΔΔCt method.

The results shown in Figure 22 indicate a reduction in the transcript levels of the three targets when compared to the untreated sample in both treatments. In the case of Epoxomycin, levels of both female transcripts are more reduced compared to the extent of reduction of the level of the male transcript. This is not observed in the case of gametocytes treated with MB, where transcript levels of all se specific markers are comparably reduced.

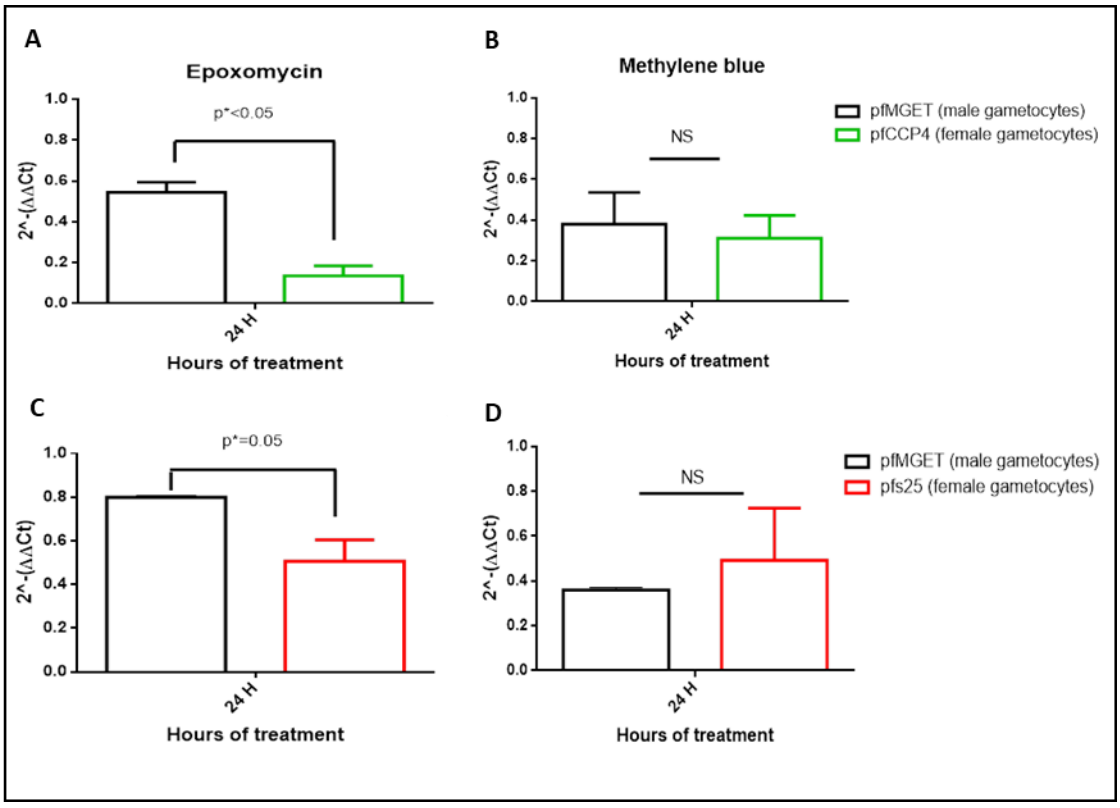


***Figure* 22**. *$2^{-\Delta\Delta Ct}$ values of gametocytes treated for 24 hours with the compounds Epoxomycin 1 µM and Methylene blue 1 µM. The RNA extracted from this gametocytes was used in two different RTqPCR experiments: in one for the amplification of the transcripts pfMGET and pfs25 (A,B), in the other the sequences pfMGET and pfCCP4 (C,D).*

These results affirm the effectiveness of the assay in evaluating the impact of compounds on gametocytes. Moreover, they suggest that the assay has the capability to assess potential sex-specific activity of compounds.

4.2.3. Use of the ΔΔCt method in assays on gametocytes from natural infections after treatment with compounds decreasing parasite transmission through mosquitoes.

With the aim to assess the effectiveness of various antimalarial compounds on *P. falciparum* gametocytes, in this study a unique approach of parallel utilizing the

RTqPCR with ΔΔCt method and the Direct Membrane Feeding Assay (DMFA) was used on gametocytes from natural infection after treatment with compounds. This innovative strategy, made possible through collaboration with Dr. Noëlie Bere Henry in Burkina Faso, allowed for an analysis linking molecular changes (evaluated through the ΔΔCt method) with the infectivity potential of gametocytes in mosquitoes, measured by the DMFA. By correlating the molecular responses, as indicated by changes in sex-specific transcripts, with the outcomes of mosquito infectivity, the study tried to find measurable factors that could forecast the efficacy of antimalarial treatments on gametocytes. The unique aspect of the study was our collaborator's ability to collect gametocytes from individuals and maintain their viability in culture for up to 24 hours.

In collaboration with Dr. Noëlie Bere Henry of the Center National de Recherche et de Formation sur le Paludisme (CNRFP – Ouagadougou, Burkina Faso), *P. falciparum* gametocyte-infected blood samples were collected from 15 naturally infected asymptomatic individuals from the Saponé Health district in the province of Bazèga, located 50 km southwest of Ouagadougou, the capital city of Burkina Faso. The gametocytemia of the samples, previously evaluated by microscopy, ranged from 32 to 286 gametocytes per microliter of blood.

The samples were exposed to the action of nine compounds (Atovaquone, DHA, Methylene blue, MMV390048, DDD107498, P218, Pyronaridine, Ferroquine, and Lumefantrine) and the vehicle control for compounds, 0.1% DMSO in RPMI, was used as a negative control. Compounds were tested at different concentrations, based on prior assessments using *in vitro* cultivated gametocytes (Henry B. N. et al., 2023), namely 0.1xIC50, 1xIC50, and 10xIC50.

The details of the infected blood samples and their use in the different treatment are shown in Table 5.

Compounds → / ID individuals and GCT per µl ↓	MMV048 2 µM	MMV048 0.2 µM	MMV06931 83 0.5µM	METHYLENE BLUE 5 µM	METHYLENE BLUE 0.5 µM	SJ733 10 µM	SJ733 1 µM	DHA 1 µM
EV 113 - 78 GCT	✓	✓	✓					
EV 114 - 48 GCT	✓	✓	✓					
EV 111 – 286 GCT	✓	✓	✓					
EV 112 - 199 GCT	✓	✓	✓					
EV 107 - 92 GCT				✓	✓	✓	✓	
EV 115 - 54 GCT								
EV 108 - 32 GCT				✓		✓	✓	
EV 109 - 36 GCT				✓	✓	✓	✓	
EV 110 - 66 GCT				✓	✓	✓	✓	
EV 101- 32 GCT								✓
EV 102 -32 GCT								✓
EV 103 - 103 GCT								✓

***Table 5**. List of individuals with the number of gametocytes per µL of blood detected microscopically and treatment employed.*

A diagram of the entire experimental procedure, from blood sampling to RT-qPCR and mosquito infectivity analysis (DMFA) is shown in Figure 23.

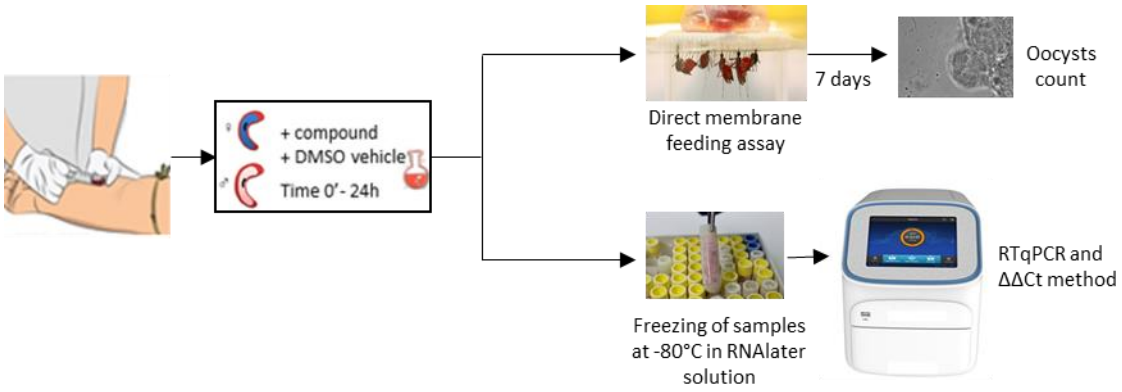


***Figure 23**. Procedure of the experiment. Gametocyte infected blood samples were collected from individuals and used to perform in parallel RTqPCR assays with ΔΔCt method and DMFA to connect changes in transcript level with the infectivity potential of gametocytes in mosquitoes.*

The samples taken at time 0 and at time 24h for all treatments, were divided into two aliquots of 50 µl of blood each. For each sample, one aliquot was treated for use in RT-qPCR and shipped to ISS, and the other identical aliquot was used for artificial infection with the Direct Membrane Feeding Assay (DMFA) to assess the infectivity of gametocytes in mosquitoes after the 24 hour treatment. The DMFA involves feeding infected blood from *P. falciparum* gametocytes to female Anopheles mosquitoes and measuring the level of human-to-mosquito transmission to estimate infectivity, i.e. the ability of the gametocytes to undergo fertilization and develop through sporogonia in the insect to continue their cycle. Infectivity levels are determined by counting the percent of infected mosquitoes over those that fed on the gametocyte sample (prevalence of infection) and the number of oocysts that have developed in the infected mosquitoes seven days following the blood meal (intensity of mosquito infection). The results of DMFA, reporting infectivity as the prevalence of infected mosquitoes (Figure 24), showed

that all compounds tested, with the exception of DHA and Ferroquine, reduce mosquito infection rates in a dose dependent manner.

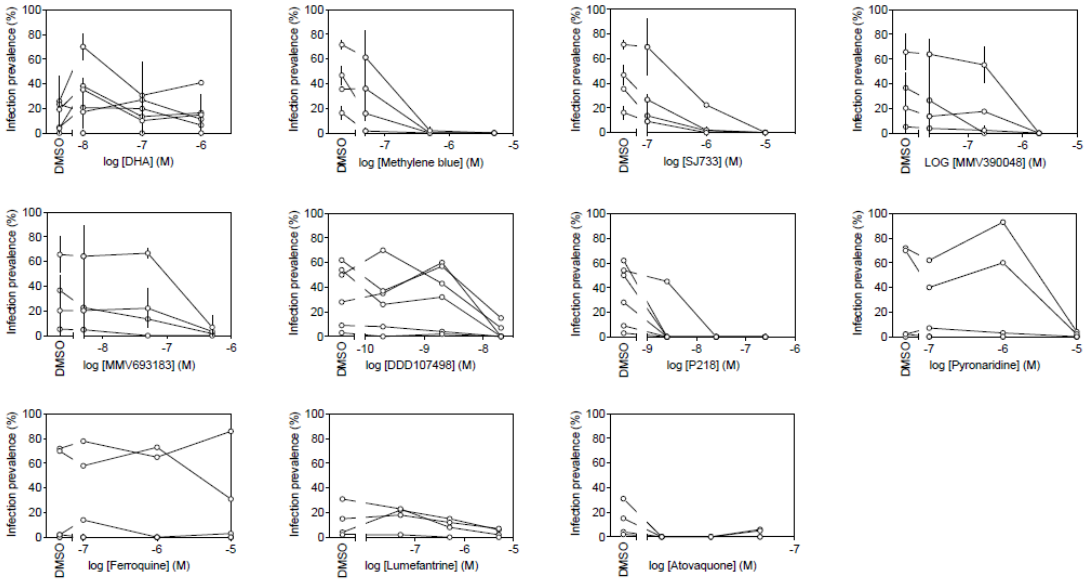
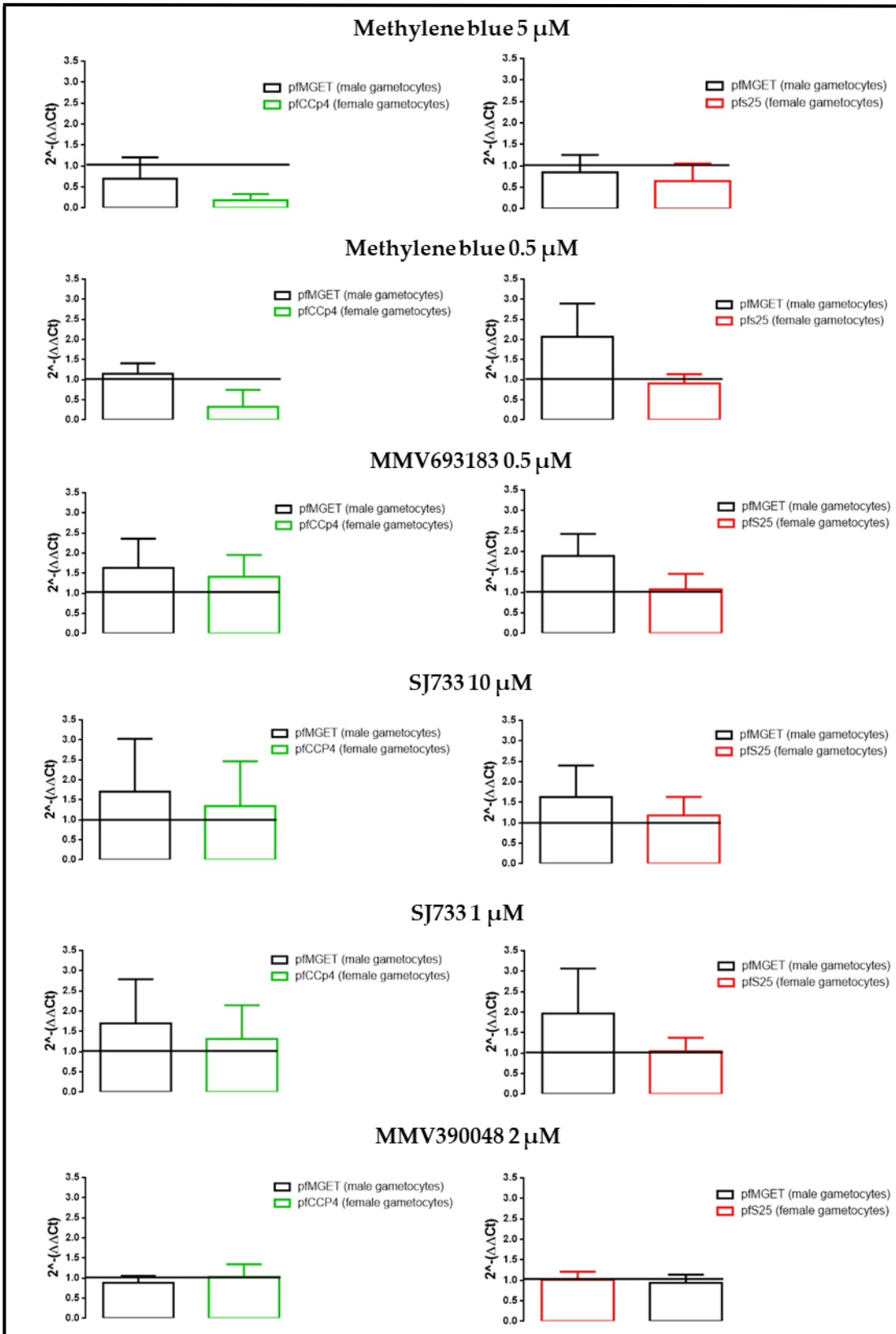


Figure 24. *Transmission-blocking effects of compounds when incubated with Plasmodium falciparum gametocyte field isolates (Henry B. N. et al., 2023). Serial dilutions of compounds (DHA, Methylene Blue, SJ733, MMV390048, MMV693183, DDD107498, P218, Pyronaridine, Ferroquine, Lumefantrine and Atovaquone) were added to gametocytes, incubated for 24 hours, and fed to mosquitoes. The plot illustrates their impact on mosquito infection prevalence. DMSO served as a negative control. Symbols denote individual donors, with connecting lines indicating different incubation conditions for the same donor (Henry B. N. et al., 2023).*

The aliquot shipped to ISS was used for RNA extraction and to perform the RTqPCR assays amplifying sex-specific transcripts *pfCCP4*, *pfs25*, and *pfMGET* and for analysis with the ΔΔCt method as described earlier. Based on the DMFA results, only samples in which treatment resulted in inhibition of mosquito infectivity were selected for further analysis, with the aim to explore if results of the molecular analysis could be correlated, and predictive, of the outcome of the mosquito infectivity experiments.


Methylene blue 5 µM
pfMGET (male gametocytes)
pfCCp4 (female gametocytes)
pfMGET (male gametocytes)
pfs25 (female gametocytes)
2^-(ΔΔCt)
Methylene blue 0.5 µM
pfMGET (male gametocytes)
pfCCp4 (female gametocytes)
pfMGET (male gametocytes)
pfs25 (female gametocytes)
MMV693183 0.5 µM
pfMGET (male gametocytes)
pfCCp4 (female gametocytes)
pfMGET (male gametocytes)
pfS25 (female gametocytes)
SJ733 10 µM
pfMGET (male gametocytes)
pfCCP4 (female gametocytes)
pfMGET (male gametocytes)
pfS25 (female gametocytes)
SJ733 1 µM
pfMGET (male gametocytes)
pfCCp4 (female gametocytes)
pfMGET (male gametocytes)
pfS25 (female gametocytes)
MMV390048 2 µM
pfMGET (male gametocytes)
pfCCP4 (female gametocytes)
pfMGET (male gametocytes)
pfS25 (female gametocytes)

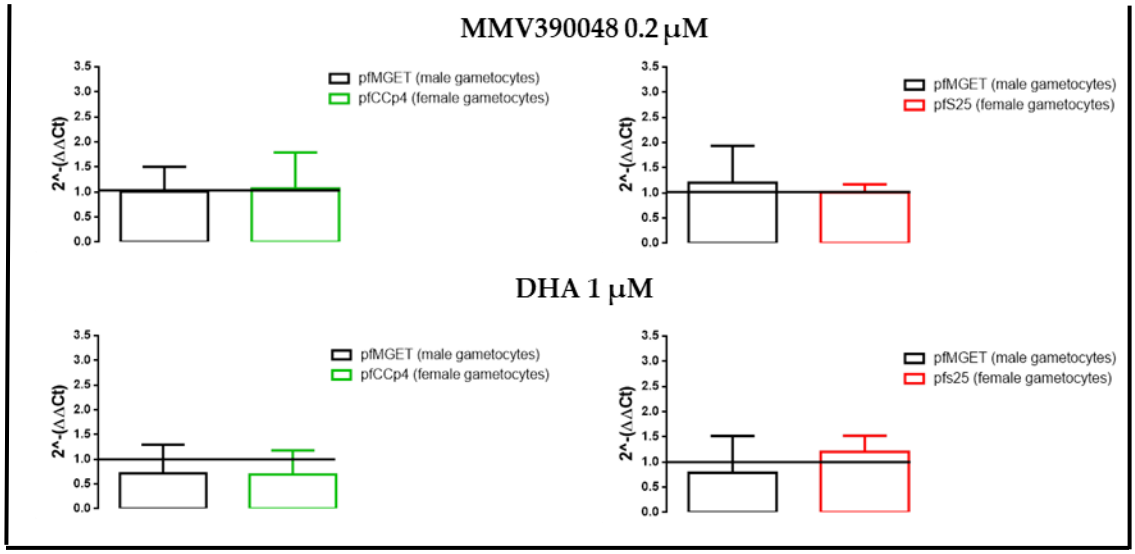


Figure 25. *$2^{-\Delta\Delta Ct}$ data of gametocyte sample collected from individuals and treated in vitro for 24 hour with compounds. Value of $2^{-\Delta\Delta CT}$ equal to 1 are signed with a horizontal line. The results of the t-test assessing the statistical significance between the actions of compounds on male and female gametocytes indicate a lack of significance for each of the compounds.*

The selected treatments included MMV390048 at 2 µM and 0.2 µM, MMV0693183 at 0.5 µM, Methylene Blue at 5 µM and 0.5 µM and SJ733 at 10 µM and 1 µM. Furthermore, also DHA at 1 µM was selected. For each individual, gametocytes resuspended in 0.1% DMSO in RPMI were used as a negative control.

The results of the ΔΔCt analysis are shown in Figure 25 and indicate that, with the exception of 5 µM Methylene Blue, none of the 24 hour treatments with the compounds analysed led to a decrease in the level of the sex-specific transcripts *pfMGET*, *pfCCp4*, and *pfs25*.

The discrepancy between molecular analysis and mosquito infectivity raised questions about the temporal changes of sex-specific transcript levels. Therefore, an experiment was conducted on in *vitro* cultivated gametocytes to explore how

the transcript level evolves over a 72 hour period following a 24 hour treatment with specific antimalarial compounds, providing insights into prolonged effects.

4.2.4. RTqPCR assays and ΔΔCt analysis to measure sex-specific transcript levels in a time course experiment on compound treated *in vitro* gametocytes.

To investigate the temporal changes in the levels of sex-specific transcripts in treated gametocytes, the RTqPCR assays were conducted on samples containing 1.5 x 10^5 gametocytes in 50 microliters of whole blood from the NF54 laboratory line.

Gametocytes were subjected to a 24 hour incubation with some of compounds employed in the treatment of gametocytes from natural infections described in the previous paragraph. The treatments selected are MMV048 2 μM and 0.2 μM, MMV0693183 0.5 μM and SJ733 10 μM. The experiment was conducted in duplicate.

For each antimalarial compound tested, a dual sample approach was employed. In the first sample, the treated gametocytes were resuspended in Trizol immediately after the 24 hour treatment and promptly frozen. In contrast, the second treated sample underwent a different path. After the 24 hour treatment, these gametocytes were washed to remove the compound, then maintained in culture for an extended period of up to 72 hours. Following this prolonged incubation, the gametocytes were processed for analysis. This strategy enabled a comparison between the immediate post-treatment state and the effects observed after an extended period of incubation, providing insights into the temporal

dynamics of the impact of antimalarial compounds on gametocyte gene expression. The results of these assay are reported in the Figure 26.

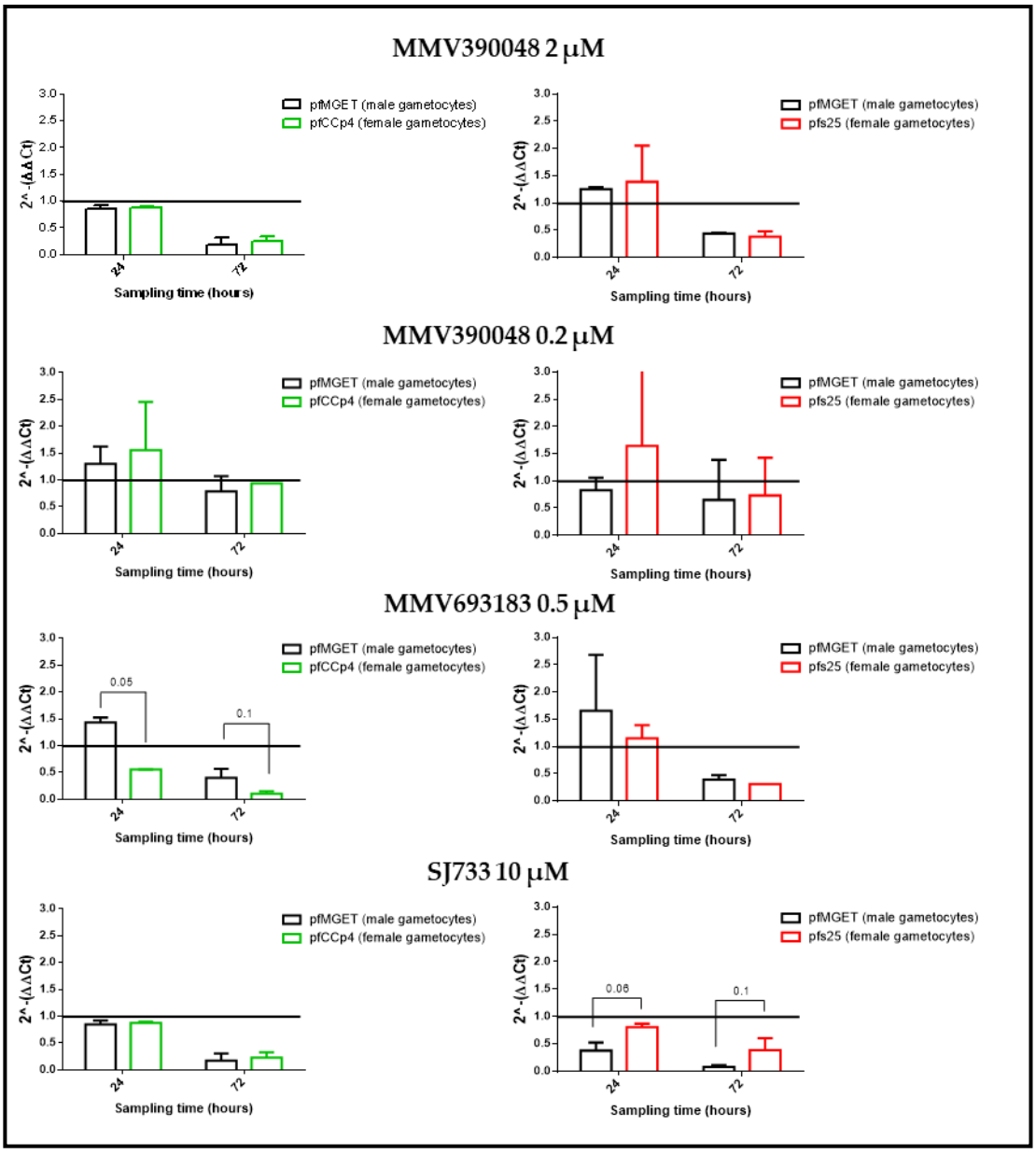


***Figure 26**. Anti-gametocyte activity of the antimalarial compounds MMV048 2 µM and 0.2 µM, MMV0693183 0.5 µM and SJ733 10 µM against Plasmodium falciparum laboratory line NF54 evaluated at the sampling time of 24 hours and 72 hours. N=2. The t-test results for the actions of these compounds against male and female gametocytes indicate statistical significance only in the cases indicated.*

Methylene blue, as mentioned in the "Validation of the assay" paragraph, was also tested *in vitro* on NF54 gametocytes and showed a decrease in the level of sex-specific transcripts after 24 hours of treatment.

The NF54 laboratory line gametocytes, when treated for 24 hours, did not show a decrease in sex-specific gene transcript levels. However, after keeping the gametocytes in culture for up to 72 hours, a decrease in transcript levels was observed, except for MMV048 at 0.2 μM. Specifically:

- **MMV048 2 μM** samples: *pfMGET* decrease between 56 and 61%, *pfCCp4* between 56 and 80% and of *pfs25* decrease between 55 and 69%
- **MMV693183 0.5 μM** samples: *pfMGET* decrease between 59 and 61%, the level of *pfCCp4* between 48 and 71% and *pfs25* decrease between 61 and 93%
- **SJ733 10 μM** samples: *pfMGET* decrease between 73 and 90%, *pfCCp4* between 70 and 86% and *pfs25* decrease between 46 and 77%

Additionally, with the same procedure, the compounds DDD107498 at 0.2 μM and P218 at 0.25 μM and 0.025 μM were tested. These two compounds showed transmission-blocking activity in mosquitoes, and the results of these experiments are shown in Figure 27. In this series of experiments, DDD107498 at a concentration of 0.2 μM demonstrated a reduction in sex-specific transcript levels after just 24 hours of treatment. Specifically, in the RTqPCR assay amplifying *pfMGET*/*pfCCp4* transcripts, this compound exhibited a significant

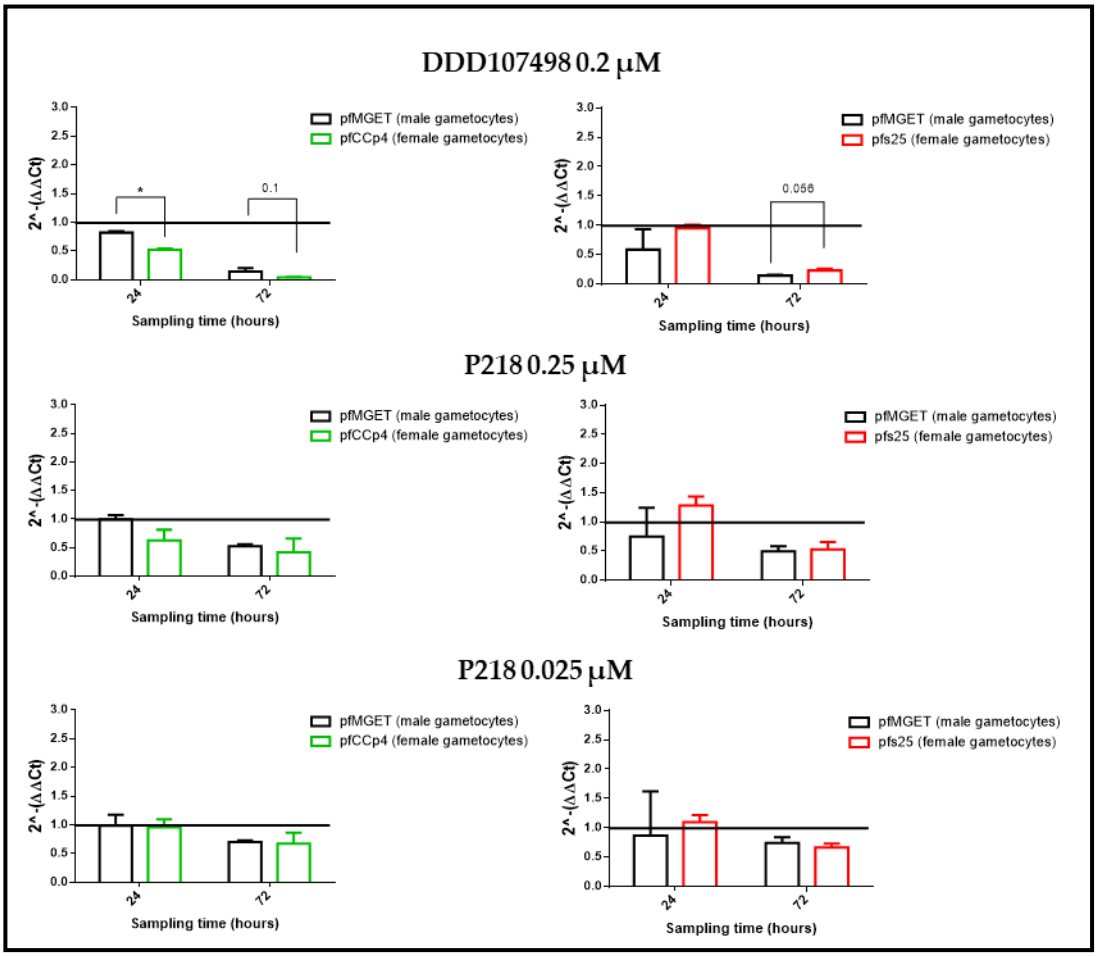


***Figure 27**. Anti-gametocyte activity of the antimalarial compounds DDD107498 0.2 µM and P218 0.25 and 0.025 µM against Plasmodium falciparum laboratory line NF54 evaluated at the sampling time of 24 hours and 72 hours. N=2. The t-test results for the actions of these compounds against male and female gametocytes indicate statistical significance only in the cases indicated.*

decrease in female-specific transcript levels *pfCCp4* relative to *pfMGET*. However, this observed pattern is not confirmed in the *pfMGET*/*pfs25* assay in which both sex-specific transcripts decrease equally.

In all the samples of gametocytes kept in culture for up to 72 hours, the transcript levels decreased. In particular:

- **DDD107498 0.2 µM** samples: the level of *pfMGET* decrease by about 85%, the level of *pfCCp4* by 95% and the level of *pfs25* decrease by 80%.

- **P218 0.25 µM** samples**:** the level of *pfMGET* decrease between 91 and 98%, the level of pfCCp4 between 41 and 75% and the level of *pfs25* decrease between 48 and 66%
- **P218 0.025 µM** samples**:** the level of *pfMGET* decrease between 26 and 30%, the level of *pfCCp4* between 19 and 46% and the level of *pfs25* decrease between 29 and 48%

Below is a summary table with the list of compounds tested in DMFA and in RTqPCR on gametocytes from natural infections (*ex vivo*) and *in vitro:*

COMPOUND	DMFA TESTED	*EX VIVO* TESTED	*IN VITRO* TESTED
Methylene Blue 5 µM	✓	✓	✓
Methylene Blue 0.5 µM	✓	✓	
MMV048 2 µM	✓	✓	✓
MMV048 0.2 µM	✓	✓	✓
MMV0693183 0.5 µM	✓	✓	✓
DDD107498 0.5 µM	✓		✓
P218 0.25 µM	✓		✓
P218 0.025 µM	✓		✓
SJ733 10 µM	✓	✓	✓
SJ733 1µ nM	✓	✓	
DHA 1 µM	✓	✓	

Table 7*. Summary table with the list of compounds tested*

5. DISCUSSION

Malaria is one of the most lethal and widespread parasitic disease in the world caused by a parasite belonging to the genus *Plasmodium*. Between the species able to infect the human being, *Plasmodium falciparum* is the responsible for the majority of deaths. Its life cycle occurs between a human host and an *Anopheles* mosquito vector.

In the human host, the intra-erythrocytic blood stage asexual parasites are responsible for the symptoms of the disease. However, periodically a little subset of these parasites differentiate into male and female gametocytes, the sexual forms responsible for the transmission of the parasite from human host to mosquito. The maturation process of *P. falciparum* gametocytes unfolds over a span of 10 to 12 days within the human host, during which the immature gametocytes are sequestered in internal organs, particularly the bone marrow. Once mature, stage V gametocytes are released back into circulation, poised to be picked up by a mosquito during its blood meal. Successful transmission hinges on the mosquito ingesting at least one male and one female gametocyte, which transform into male and female gametes, triggering subsequent stages of fertilization, zygote maturation, and the development of mosquito-specific phases.

The current therapeutic regimen employed to cure malaria symptoms involves the administration of combined therapies with Artemisinin-based Combination Therapy (ACT). ACT is highly effective in clearing asexual blood stage parasites and eliminating immature gametocytes. However, a notable limitation is its

ineffectiveness against mature gametocytes, which can persist in the bloodstream for over a week even after the asexual parasites have been eradicated.

This persistence of circulating mature gametocytes is of concern because individuals, also when asymptomatic, may continue to carry these transmission-capable forms of the parasite. As a result, there is a risk of continued transmission to mosquitoes, perpetuating the life cycle of malaria. For this reason, developing compounds specifically designed to block malaria transmission by targeting gametocytes is needed. Moreover, there is a pressing need to promote the development of reliable analytical tools and experimental methods capable of accurately measuring the blocking properties of compounds designed to impede transmission. The ability to quantify the impact of these drugs on the transmission stages of the malaria parasite's life cycle is essential for evaluating their effectiveness in preventing the spread of the disease. The development of such tools represents a crucial step in the ongoing efforts to enhance the fight against malaria and curb the transmission of the parasite between human host and mosquito vector.

In the pursuit of developing transmission-blocking strategies for malaria, two crucial factors influencing the transmission from human to mosquito must be taken into consideration: gametocyte density and gametocyte sex ratio. Gametocyte density is defined as the quantity of gametocytes, both male and female, present in a volume of blood of an infected individual. The number of gametocytes significantly affects the likelihood of successful transmission to mosquitoes. Beyond a certain threshold gametocyte density, the proportion of infected mosquitoes reaches a plateau, indicating a saturating relationship. On the other hand, when gametocytemia is low, the probability of successful

transmission tends to decrease. Gametocyte sex ratio is defined as the proportion of male gametocytes over female gametocytes in an infection. Typically, *Plasmodium* parasites exhibit a female-biased sex ratio. Research, such as the study conducted by Bradley et al. (2018), suggests that low male gametocyte densities (<50 gametocytes/µl) are associated with reduced transmission, particularly at lower gametocyte densities. This implies that male gametocyte density may act as a limiting factor for transmission success when density of female gametes is low.

Some anti-malarial drugs are described to have distinct effects on gametocytes of different sex, with mature male gametocytes being sensitive to a wider range of drugs than mature female gametocytes. This indicates that these differences need to be taken into account in transmission blocking interventions development.

The accurate measurement of female and male gametocyte density is therefore crucial for the development and implementation of transmission-blocking strategies against malaria. This measurement approach could be possibly used to assess the infectiousness of human populations, providing valuable insights into the potential for transmission to *Anopheles* mosquitoes.

In summary, the understanding of the interplay between gametocyte density and sex ratio may be a useful information to determine malaria transmission dynamics. Developing strategies to control transmission requires a nuanced understanding of these parameters, allowing for the targeted reduction of the human reservoir of infection and, consequently, the spread of the parasites to mosquito vectors.

In this perspective, in the lasts years several works aimed to assess gametocyte sex ratio were published. In one of them, Meerstein-Kessel et al, 2018 used RTqPCR assay amplifying *pfMGET* male- and *pfCCp4* female-specific markers of *P. falciparum* gametocytes and proposes sex-ratio values after calculating the number of male and female gametocytes of pfDynGFP/P47mCherry transgenic laboratory line. By relating the Ct values obtained by RTqPCR with the Ct values obtained with the same primers on serial dilutions of synthetic RNA target genes, the number of copies of each target per gametocyte was determined. This estimate was developed in perspective to be used to assess gametocyte sex ratio in field studies. Another work, Stone et al. (2017) investigated the hypothesis that Primaquine (PQ) might disproportionately affect male gametocytes, leading to sterilization of infections while female gametocyte densities remain stable. To investigate this hypothesis, that work analysed data from two randomized controlled trials of PQ in combination with dihydroartemisinin-piperaquine (DP) conducted in Kenya and Mali. The trials assessed the effect of PQ on gametocyte sex ratio using RTqPCR amplyfing the sex-specific RNA markers pfs25 and *pfMGET* from infected blood samples taken by individuals under different treatments. In the RTqPCR assays, the PfDynGFP/P47mCherry reporter line served as a tool as its gametocytes express sex-specific fluorescent markers, i.e. a chromosomally integrated GFP gene is under the control of the male specific dynein heavy chain gene promoter and the episomal *mCherry* gene is controlled by the female specific *p47* gene promoter. In this line, male and female gametocytes can be physically separated by fluorescence-activated cell sorting (FACS) to produce standard curves of the sex specific markers from known quantities of sorted male and female gametocytes. In the blood samples from the

clinical trials, male and female gametocytes of the treated individuals were quantified and sex ratio values were determined, by extrapolating the number of male and female gametocytes from the standard curve of sorted male and female gametocytes of the PfDynGFP/P47mCherry line. This was done on the assumption that transcript copy number of the genes were invariant in the PfDynGFP/P47mCherry gametocytes and in the wild gametocytes present in the infected blood samples.

In the third work, Gruenberg et al., 2020, female and male gametocytes were quantified in *Plasmodium falciparum* infections from a survey in Papua New Guinea. RNA from infected blood was amplified by RTqPCR using female-specific *pfs25*, male-specific *pfMGET* and another male specific gametocyte marker, the *mssp* (meiosis-specific nuclear structural protein 1) expressed in male 50-200-fold higher compared to female gametocytes. The number of male and female gametocytes was calculated based on standard curves of known number dilutions of female and male sorted gametocytes of the transgenic laboratory line pfDynGFP/P47mCherry. Gametocyte sex ratios obtained from RTqPCR were compared with those calculated after counting gametocytes from immunofluorescence assays (IFA) with an anti-Pf16 antibody that identifies all gametocytes and an anti-Pfg377 antibody to identify only female gametocytes. The comparison of the results of the RTqPCR assay and the IFA reveals that sex ratio estimates overall correlated well, although differences of 10%- 15% were observed. Based of these data, the probability of *P. falciparum* infections to be efficient in the onward parasite transmission to mosquitoes was estimated to be achieved with as few as 1 female and 1 male gametocyte per 2.5 µL blood.

These above works used the results from the RTqPCR assays on FACS sorted gametocytes of the pfDynGFP/P47mCherry transgenic laboratory line as a standard to assess gametocyte sex ratios in infected blood samples, assuming that sex-specific transcript abundance are identical in male and female gametocytes of this line and in male and female gametocytes of natural isolates of *P. falciparum*.

Referring to the above works, this doctoral thesis explores the reliability and applicability of these assumptions in assays conducted on gametocytes of different parasite lines. To do this, this work developed two multiplex Reverse Transcriptase quantitative Polymerase Chain Reaction (RTqPCR) TaqMan assays designed to amplify in multiplex mode the sex-specific transcripts *pfCCp4* and *pfs25* for female and *pfMGET* for male gametocytes.

In assay n.1, the amplification of the *pfMGET* transcript is coupled with that of *pfs25*, in assay n.2 it is coupled with that of *pfCCp4*. In experiments on three laboratory lines of the parasite, the two female markers exhibited different sensitivity in the detection of female gametocytes. Higher sensitivity of the RTqPCR amplifying *pfs25* mRNA compared to that amplifying pfCCp4 could be attributed to the estimated lower number of *CCp4* transcripts per female gametocyte compared to those of *pfs25*. The choice to use two female-specific markers contributes to increasing of the robustness of the experiments by reducing the likelihood of false positives and allows flexibility in choosing the most suitable one for a particular experimental context or parasite line. Furthermore, comparative analysis of results obtained from using two markers can reveal possible variability of marker expression across different conditions or lines. In assays evaluating the impact of antimalarial compounds on gametocytes,

for example, using two markers can provide a more comprehensive assessment of compound effects. Changes in expression levels of both markers can be compared to evaluate the compound's impact on different aspects of gametocytes.

In the first part of work, (Chapters 4.1.1. to 4.1.5.), these assays were used to measure the sex ratio of gametocytes in three different parasite laboratory lines, the two clonal lines NF54 and HB3 sel 4 and the transgenic pfDynGFP/P47mCherry line. The choice was motivated by the fact that previous report indicated that these lines have markedly different sex ratios and that the pfDynGFP/P47mCherry line is used as a reference for sex ratio determinations, as described above.
Two analytical approaches were used to obtain the sex ratio values from the RTqPCR data.

The first approach involves obtaining a measurement of the sex ratio of a population of gametocytes through a parameter called DCt, which is the difference between the Cycle threshold (Ct) of the female specific target, either *pfCCp4* or *pfs25*, and the Ct of the male target *pfMGET*. This is motivated as follows. As I show that the amplification efficiencies of the target transcripts in all the RTqPCR are very similar, I can consider that at each PCR amplification cycle the amount of target-derived DNA doubles. For this reason, the DCt parameter can then be used to estimate the difference in expression of the two sex specific markers (a low Ct indicates that the target transcript is present in larger quantity compared to that of the other transcript whose Ct is higher).

The second approach proposes the use a parameter, the transcript sex ratio ($T_{SEX\ RATIO}$), defined as the ratio between the copies of the male-specific transcript *pfMGET* over those of the female-specific transcript (either *pfCCp4* or *pfs25*, respectively indicated as $T_{SEX\ RATIO}$ 1 and $T_{SEX\ RATIO}$ 2). This approach involves determining, in each *P. falciparum* laboratory line, the number of copies of the sex-specific transcripts in a gametocyte population, by conducting RTqPCRs on serially diluted samples of purified gametocytes. As detailed in Chapter 4.1.4, for each sex specific marker, the transcript copy number is derived by correlating the Ct values obtained from RTqPCR reactions on serially diluted gametocytes with the Ct values obtained in RTqPCR assays on serial dilutions of the synthetic sequences of the target gene.

Both of these approaches were performed on gametocytes of the above three lines. Published data indicated that gametocytes of these lines had different sex ratios. To directly confirm this information and to independently measure the gametocyte sex ratio values, the same gametocyte preparations used in the RTqPCR assays were used to count male and female gametocytes by IFA with specific antibodies. The experiment was designed to compare the DCt and the $T_{SEX\ RATIO}$ parameters described above with the sex ratio values obtained from IFA with the goal to see if the two RTqPCR parameters could be correlated with the IFA derived sex ratio values.

The result was that the DCt value (both DCt1 and DCt2) and the $T_{SEX\ RATIO}$ value (both $T_{SEX\ RATIO}$ 1 and $T_{SEX\ RATIO}$ 2) were higher in HB3 sel 4 compared to those obtained in NF54. This is in line with the IFA result showing that the number of

male gametocytes in HB3 sel 4 is the double of those of NF54. However, the results from the same experiments on the pfDynGFP/P47mCherry line showed that the DCt and $T_{SEX\ RATIO}$ values are lower (DCt1 and $T_{SEX\ RATIO}$ 1) and intermediate (DCt2 and $T_{SEX\ RATIO}$ 2) with respect to those determined in the above parasite lines, despite the fact that the transgenic line showed a higher sex ratio (i.e. a higher fraction of male gametocytes) by IFA. This result made it impossible to establish a positive correlation across all lines between the DCt and the $T_{SEX\ RATIO}$ values and the IFA-based sex ratios.

The lack of a linear relationship between the "morphological" and "molecular" parameters used to measure the gametocyte sex ratios in the lines used here could be attributed to various factors.

One possible explanation could be provided by the hypothesis that the transgenic modifications introduced in this line may have affected the expression patterns of the mRNAs used as markers to obtain the DCt and $T_{SEX\ RATIO}$ values. The transgenic line, therefore, may exhibit unique characteristics or behaviours that differ from the monoclonal lines (NF54 and HB3 sel 4). For instance, the transgenic modifications even if unrelated to sex determination might have led to alter the copy number of the sex specific transcripts produced in gametocytes of the different sex.

In summary, this analysis suggests that there may be significant differences between the pfDynGFP/P47mCherry transgenic line and those of wild type lines in the expression of sex specific transcripts. This is relevant as the RTqPCR parameters obtained with male and female gametocytes of this transgenic line, as they can be physically separated and purified, are used as the reference for

molecular determination of gametocyte sex ratio in studies on wild type laboratory lines or natural isolates of P. falciparum. In this context, exploring the correlation between DCt and $T_{SEX\ RATIO}$ values and morphological sex ratios in other *Plasmodium falciparum* lines that are not transgenic would be a valuable avenue for future experiments. Performing the analysis conducted here on additional non-transgenic lines can investigate the possible correlation of sex ratio values obtained by molecular parameters and by morphology across different parasite lines. This would contribute to a more comprehensive understanding if and how well the data from use of molecular markers align with those from the morphological observations on sex ratios in various parasite populations.

A further approach to better understand the factors that influence the molecular determination of sex ratios was explored. One of these is the copy number of sex specific transcripts per gametocyte, which is important for the following reason. Assuming that a sex specific mRNA, used as target, is expressed only in gametocytes of that sex (or that its aspecific expression in gametocytes of the other sex is negligible) the molecular determination of the number of copies of that transcript in a population can be used to calculate the number of gametocytes of that sex present in that sample, from which the male to female sex ratio can be obtained. For instance, this has been performed in the work by Meerstein-Kessel et al, 2018, in which the copy number of markers *pfCCp4* and *pfMGET* were obtained by RTqPCR on sorted male and female gametocytes of the pfDynGFP/P47mCherry line and the values were then used to analyse the

RTqPCR data from gametocyte containing blood samples from infected individuals.

In this thesis, the approach on this issue involved determining the number of copies per gametocyte of sex-specific transcripts in each of the above *P. falciparum* laboratory lines. The copy number of a given transcript per gametocyte is derived by correlating the Ct values from the specific RTqPCR reactions on serial dilutions of gametocytes, whose male and female numbers were estimated by IFA, with the Ct values from RTqPCR reactions on serial dilutions of synthetic copy of that gene. This analysis was performed on the above parasite lines and results, presented in Chapter 4.1.5. , showed that the copy number per gametocyte of the three target genes (*pfCCp4*, *pfs25*, and *pfMGET*) exhibit variations between the three parasite lines.

The sex ratios calculated based on these copy number values were using *pfMGET/pfs25* amplification 0.09 in NF54, 0.27 in HBE sel 4 and 0.06 in pfDynGFP/P47mCherry. Instead, using *pfMGET/pfCCp4* primer the sex ratio values were 0.135 in NF54, 0.309 in HB3 sel 4 and 0.280 in pfDynGFP/P47mCherry. These values not only differed from the sex ratios determined by IFA but also exhibit differences according to the specific female gene used for the amplification reaction.

This further result reinforces the assumption according to which caution should be exercised when a singular reference line was used to quantify male and female gametocytes in field samples.

In light of these results, the thesis challenges the conventional belief that gametocytes from a sorted transgenic line can serve as a universal reference for quantifying field gametocytes and estimating their sex ratio. Moreover, the thesis posits that there may be different molecular parameters to measure sex ratios in diverse contexts and conditions. Among these, this thesis proposes the utilization of synthetic gene curves using a parameter here defined as $T_{SEX\ RATIO}$ for tracking the copy number of sex-specific transcripts in a gametocyte population as a way of offering indirect insights into how the proportion of male and female gametocytes evolves over time and under diverse experimental conditions. In practical terms, in this approach, the standard curves set for each transcript are used to compare mRNA quantities within a given experiment to measure changes in sex-specific targets within the gametocyte population.

In the second part of the study (Chapters 4.2.1-4.2.4), the RTqPCR assays described above were employed to assess the effects of compounds on the viability of *P. falciparum* gametocytes, aiming to evaluate potential sex-specific impacts. To appreciate the innovation of this new method, it is crucial to contextualize it among existing gametocyte cell- based assays documented in the literature.

Various methods used to evaluate compound activity on gametocytes assess parasite metabolic viability, using the MitoTracker Red CMXRos (MTR Red) dye or relying on the oxidoreduction indicator AlamarBlue. Another assay uses a transgenic line, where the gene encoding a GFP-luciferase fusion protein is fused with a promoter sequence to be activated during gametocytogenesis as a reporter. The green fluorescence of the GFP-luciferase fusion serves to visualize the

transgenic gametocytes due to the gametocyte-specific promoter activity, while changes in luciferase activity when exposed to anti-gametocyte drugs offer quantitative insights into the impact of the compounds on gametocyte viability. However, the outputs of gametocyte cell based assays may not reliably indicate if the compound under analysis effectively impair viability. For this reason, relevant information is provided by phenotypic assays.

Among these, the Standard Membrane-Feeding Assay (SMFA) is considered the 'gold standard'. This involves cultivating gametocytes in vitro, treating them with anti-malarial drugs and feeding them to mosquitoes in a controlled environment. However, the SMFA is low throughput and has high costs, requiring specialized facilities, equipment, and skilled personnel.

Relevant to the work of this thesis, existing assays generally do not distinguish activity against male and female gametocytes, a crucial factor in understanding transmission dynamics. One exception is the Dual Gamete Formation Assay (pfDGFA) which is an in vitro phenotypic test with a sex-specific readout, as it measures the functional viability of Stage V male and female gametocytes based on their ability to form gametes after incubation with treatments. This assay evaluates the activity of compounds against male and female gametocytes at different times. Male gametes are evaluated 15 minutes after gametocyte activation, observing the formation of exflagellation centres, while female gametocytes are assessed 24 hours later, distinguishing them through a reaction with the anti-Pfs25 antibody. The criticality of this assay lies in the fact that two different readouts are compared, one phenotypic and the other immunological.

The work in this thesis introduced the use of the previously described multiplex RTqPCR assays as another tool for evaluating the responses of *P. falciparum*

gametocytes to antimalarial compounds. The DeltaDeltaCt (ΔΔCt) method to analyse the RTqPCR results was employed for this purpose. In this method of relative quantification, the expression levels of a given transcript in parasites exposed to different treatments are calculated by determining, for each sample, the difference between the quantity of the target transcript and that of a reference transcript whose expression is not influenced by the treatment. This difference is then compared to the same difference obtained in an untreated control sample. As both male and female specific transcripts are amplified in the same reaction, this method is able to distinguish possible sex specific effect of the compounds.

To enable the normalization of the measurements of marker transcripts across treated and untreated samples, the amplification of a reference transcript, the human *GAPDH* gene (E7EUT5_HUMAN), was introduced.

The validation of the ΔΔCt method involves the introduction of standard curves to assess the efficiency of the RTqPCR reactions. These standard curves, generated from serial dilutions of RNA, serve as a foundational step to ensure the comparability of target and reference gene amplifications. The examination of the logarithmic plot and slope calculation confirmed that the quality of the RTqPCR was adequate to apply the method for analysing the data.

Preliminary validation experiments on NF54 gametocytes with known antimalarial compounds, Methylene Blue and Epoxomycin, were able to reveal the reduction in sex-specific transcript levels in the treated gametocytes, demonstrating the efficacy of the RTqPCR assay in capturingcompound-specific effects on male and female gametocytes.

In this thesis, the RTqPCR assay with the ΔΔCT method was used to study the impact of a panel of compounds on gametocytes from natural malaria infections thanks to collaboration with Dr. Noëlie Bere Henry in the Center National de Recherche et de Formation sur le Paludisme (CNRFP), Ouagadougou, Burkina Faso. Importantly, this assay was combined with the Direct Membrane Feeding Assay (DMFA), aiming to link the results on alterations in sex-specific transcripts with the infectivity of gametocytes in mosquitoes, measured by the DMFA. Another important aspect of this work is that it is based on the possibility to maintain in culture, and therefore treating, gametocytes present in natural infections for 24h.

In the study described in Chapter 4.2.3, blood samples infected with P. falciparum gametocytes from 15 naturally infected asymptomatic individuals in Burkina Faso were exposed in duplicate to nine different compounds at various concentrations and a DMSO control for 24 hours. After treatment, one aliquot of each sample was used for the DMFA while the other was promptly frozen for subsequent RNA extraction and molecular analysis. Results from the DMFA demonstrated that, except for DHA and Ferroquine, all tested compounds reduced mosquito infection rates in a dose-dependent manner.

For the RTqPCR experiments to evaluate sex-specific transcripts (*pfCCP4*, *pfs25*, and *pfMGET*) using the ΔΔCt method, specific treatments were selected with compounds that were able to impede transmission to mosquitoes in the DMFA. These were MMV390048, MMV0693183, Methylene Blue, and SJ733, to which DHA was added.

Unexpectedly, the results of the molecular analysis were not consistent with the DMFA outcomes, as among the compounds used only the treatment with

Methylene Blue was associated to a decrease in sex-specific transcript levels in gametocytes.

This discrepancy prompted a deeper exploration to investigate whether the sex-specific transcript levels required a period longer than 24 hours after treatment to decrease in an appreciable way. To answer this question, a time course experiment was designed to with in vitro NF54 cultivated gametocytes that were treated with some of selected antimalarial compounds and then incubated for additional 48 hours following the 24-hour treatment.

The time course experiment tested the effect of MMV048, MMV0693183 and SJ733 and confirmed the lack of decreased transcript abundance in treated gametocytes sampled after 24 hours. However, a notable decrease in sex-specific transcript levels was observed after 72 hours, i.e. 48 hours after the end of the treatment, for all tested compounds. Remarkably, the RTqPCR assays demonstrated similar results on both female-specific transcripts *pfCCp4* and *pfs25*.

In this set of time course experiments, the analysis of the treatment with compound DDD107498 produced intriguing results. In the RTqPCR assay amplifying *pfCCp4* and *pfMGET* the compound displayed a female sex-specific effect after just 24 hours, a pattern consistently observed also after 72 hours. However, this sex-specific effect did not appear as a result of the RTqPCR amplifying *pfs25* and *pfMGET*, highlighting the complexity of the observed molecular responses.

The results obtained from the RTqPCR assays in this study raise intriguing questions about the temporal aspects of molecular changes induced by antimalarial compounds on *P. falciparum* gametocytes. The findings suggest that

the effects on transcript abundance may not be manifest shortly (few hours) after treatment but a decrease in mRNA quantities, likely due to the unhealthy condition of the affected parasite, become measurable only over an extended cultivation period after treatment. This emphasizes the importance of sampling treated gametocytes at extended time points to ensure to reveal possible decrease in mRNA levels and accurately assess compound efficacy.

In summary, the study has demonstrated promising advances in developing assays able to evaluate the activity of antimalarial compounds against gametocytes, while also highlighting the complexity of molecular responses in P. falciparum gametocytes to these compounds. The identified challenges now serve as the baseline for future research, providing valuable insights for further investigations in this field.

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Declaration

I declare that I have personally carried out all the experiments described in this thesis except for the collection of samples from individuals infected with malaria in Burkina Faso and for Direct Membrane Feeding Assays (DMFA) in mosquitoes to evaluate their infectivity.