Characterization of *Plasmodium falciparum* mature gametocytes: lifespan, immunogenicity and susceptibility to novel compounds

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Zusammenfassung

Malaria ist eine Infektionskrankheit von globaler Bedeutung an der jährlich etwa eine halbe Millionen Menschen sterben. Die sexuellen Stadien (Gametozyten) sind verantwortlich für die Transmission des Parasiten von dem humanen Zwischenwirt auf den Mückenvektor. Trotz ihrer Bedeutung für die Transmission und damit für die Aufrechterhaltung des Zyklus ist weder ein sicheres und hocheffektives Medikament noch ein transmissionblockierender Impfstoff gegen plasmodiale Gametozyten erhältlich, auch nicht gegen *Plasmodium falciparum* - die medizinisch bedeutenste Plasmodienspezies. Darüber hinaus ist nur sehr wenig bekannt über die Biologie dieses Parasitenstadiums, seine Medikamentensensitivität, seine Langlebigkeit und die Erkennung durch das Immunsystem.

Im Rahmen dieser Arbeit wurden die Lebensdauer von *P. falciparum* Gametozyten und die natürliche Antikörperantwort auf reife Gametozyten systematisch untersucht. Zusätzlich wurde die Aktivität von neuen Substanzen auf reife Gametozyten evaluiert. Bisher wurde die Lebensdauer von *P. falciparum* Gametozyten *in vivo* nur geschätzt, meistens durch mathematische Modellierungen. Hier wurde zum ersten Mal die Lebensdauer von Gametozyten *in vitro* durch das Messen der Viabilität von Parasiten in der Gametozytenkultur für einen längeren Zeitraum gemessen. Es konnte mit verschiedenen Viabilitätsassays beobachtet werden, dass reife Gametozyten für ein Maximum von 16 bis 32 Tagen ab dem Tag der Reife (*in vivo* Zirkulationszeit) mit einer durchschnittlichen Halbwertszeit von 2,6 bis 6,5 Tage überleben. Zusätzlich konnte beobachtet werden, dass reife männliche Gametozyten für 2 Wochen exflagellieren konnten, dies zeigt ihre Viabilität und ihre nach wie vor vorhandene potentielle Fähigkeit Mücken zu infizieren.

Die natürlich erworbene Antikörperantwort gegenüber Gametozyten wurde ebenfalls in einem klinischen Umfeld untersucht. Wie erwartet war die Antikörpererkennung nach Fixierung und Permeabilisierung der mit Gametozyten infizierten Erythrozyten erhöht. Die Erkennung des Laborstamms NF54 und des klinischen Isolats JH013 durch Antikörper war ähnlich. Erwachsene zeigten eine stärkere Antikörperantwort

und höhere Seroprävalenz verglichen mit Kindern. Eine signifikant schwächere Antikörperantwort wurde in Erwachsenen (nicht aber in Kindern), die mit dem Malariaimpfstoff-Kandidaten GMZ2 geimpft wurden, beobachtet, wenn Tag 84 mit Baseline verglichen wurde. Die Untersuchung der Zusammenhänge zwischen den Antikörpern gegen Gametozyten und der Antikörperantwort gegen alle asexuellen Antigene zeigte eine positive Korrelation in Erwachsenen; diese Korrelation war bei Kindern nicht nachweisbar. Die Ergebnisse zeigten auch, dass Kinder, die mit intestinalen Helminthen (Ascaris) infiziert waren, eine signifikant höhere Antikörperantwort gegen Gametozyten aufwiesen als nicht infizierte Kinder.

Zusätzlich wurde die Sensitivität reifer Gametozyten gegen neue Substanzen untersucht. Hierfür wurde die Aktivität von synthetisch hergestellten Farbstoffen gegen reife Gametozyten getestet. Fünf Fluoreszenzfarbstoffe zeigten eine Aktivität bei einer submikromolaren Konzentration gegen reife *P. falciparum* Gametozyten des kulturadaptierten 3D7 Stamms und gegen zwei klinische Isolate. 3D7 war sensitiver als die klinischen Isolate. Drei mitochondriale Farbstoffe (MitoRed, DiOC6 und Rhodamine B) zeigten mit der 50% inhibitorischer Konzentration (IC50) von unter 200 nM sogar eine noch höhere Aktivität als der Kontrollfarbstoff Methylenblau (770 nM), von dem bekannt ist, dass er antigametozytär wirkt. Unter den drei Farbstoffen war MitoRed am aktivsten sowohl gegen 3D7 als auch gegen die klinischen Isolate, mit einer 3-fachen höheren Inhibition nach einer Inkubationszeit von 48 h verglichen mit 24 h. Der Effekt der Inkubationszeit auf die Aktivität von Medikamenten wurde auch bei den Kontrollmedikamenten Epoxomicin und Artesunate beobachtet.

In einem weiteren Projekt, wurde der antigametozytäre Effekt von einer neuen Substanz aus Myxobakterien, Chlorotonil A untersucht. Reife Gametozyten reagierten sensitiv auf Chlorotonil A bei einer geringen nanomolaren Konzentration, ähnlich der Konzentration die Aktivität gegen asexuellen Blutparasitenstadien zeigt, und ist damit besser als die meisten anderen Malariamedikamente, die momentan in der Entwicklung sind. Dies unterstreicht das Potential von Chlorotonil A als einen Kandidaten für die weitere Medikamentenentwicklung.

Allgemein wurde in dieser PhD Arbeit gezeigt, dass reife *P. falciparum* Gametozyten 2-4 Wochen *in vitro* überleben. Dies ermöglicht eine Einschätzung der *in vivo* Zirkulationszeit, welche für die Modellierung der Transmission und den Effekt von transmissionsblockierenden Maßnahmen verwendet werden kann. Diese Maßnahmen könnten entweder immunologisch oder medikamentenbasiert sein. Im zweiten Teil der Untersuchungen wurde gezeigt, dass die natürlich erworbenen Antikörper gegen die Oberflächenantigene von Erythrozyten, die mit Gametozyten infiziert sind, beeinflusst werden durch Impfung und Koinfektion mit Helminthen. In einem dritten und vierten Teilprojekt, zeigte das Screening von Substanzen, dass mitochondriale Fluoreszenzfarbstoffe und Chlorotonil A eine hohe inhibitorische Aktivität gegen reife *P. falciparum* Gametozyten haben und damit interessant für die klinische Entwickung sind.

Summary

Malaria is an infectious disease of global health importance with approximately half a million global annual deaths. The sexual forms (gametocytes) are responsible for transmission of the malaria parasite *Plasmodium* from the human host to the mosquito vector. Despite their importance for transmission and therefore maintenance of the cycle, no safe and highly effective drug nor a transmission blocking vaccine against *Plasmodium* gametocytes including *P. falciparum*, the most important plasmodial species, are available. In addition, only little is known about the biology, susceptibility, longevity and recognition by the immune system of these essential stages. In this PhD work, the lifespan of *P. falciparum* gametocytes and natural antibody response to late-stage gametocytes were systematically investigated. Additionally, susceptibility of mature gametocytes to new compounds was evaluated.

Previously*, P. falciparum* gametocyte lifespan was estimated *in vivo*; mostly by mathematical modelling. Here, longevity of gametocytes was measured for the first time *in vitro* by measuring parasite viability in gametocyte culture for an extended period. It was observed that mature gametocytes can survive a maximum of 16-32 days from the day of maturation (representing the *in vivo* circulation time) with an average half-life of 2.6 to 6.5 days as evaluated by the different viability assays. In addition, mature male gametocytes were found to be able to exflagellate for two weeks, which shows their viability and sustained potential capability to infect mosquitoes.

In line with this, the naturally acquired antibody (Ab) response towards gametocytes was also explored in clinical settings. As expected, Ab recognition increased after fixation and permeabilization of gametocyte-infected erythrocytes. Ab recognition between the laboratory strain (NF54) and a clinical isolate (JH013) was similar. Adults showed a higher Ab response and sero-prevalence compared to children. A significant lower level of Ab response was observed in adults vaccinated with the malaria vaccine candidate GMZ2 (but not in children) after 84 days compared to

baseline. When testing the association between the anti-gametocyte Ab response to the Ab response to whole asexual antigens, a positive correlation was observed in adults but not in children. The evaluation of the effect of intestinal helminths infection showed that children infected with *Ascaris* had a significantly higher anti-gametocyte Ab response compared to uninfected children.

Additionally, to evaluate the sensitivity of mature gametocytes to new compounds, activity of synthetic dyes was tested against mature gametocytes. Five fluorescent dyes showed activity at submicromolar concentration against *P. falciparum* mature gametocytes of the culture-adapted 3D7 clone and two clinical isolates. 3D7 was observed to be more sensitive than the clinical isolates. Three mitochondrial dyes (MitoRed, DiOC6, and rhodamine B) were even more active, with 50% inhibitory concentrations (IC_{50}s) below 200nM, than the control dye methylene blue (770nM) that is known to have gametocidal activity. Among the three, MitoRed was most active against 3D7 and clinical isolates, with a 3-fold higher inhibition when incubated for 48h than for 24h. The effect of incubation time on the activity of drugs was also pronounced with the control drugs, epoxomicin and artesunate.

In a different project, the gametocidal effect of a new myxobacterial compound, chlorotonil A, was investigated. Mature gametocytes were sensitive to chlorotonil A at a low nanomolar concentration, which is similar to its activity against asexual blood stage parasites and superior to most other antimalarials in development. This underlines the potential of chlorotonil A as a drug development candidate.

Altogether in this PhD work, it was shown that *P. falciparum* gametocytes survive *in vitro* 2-4 weeks after maturation. This provides an estimate of the *in vivo* circulation time that can be used to model transmission and the effect of transmission-blocking interventions. These interventions could be immunological or drug-mediated. The second set of investigations showed that naturally acquired antibodies against gametocyte-infected erythrocyte surface antigens were affected by vaccination and helminth co-infections. In the third and fourth subproject, screening of compounds

showed that mitochondrial fluorescent dyes and chlorotonil A had high inhibitory activity against *P. falciparum* mature gametocytes and may be developed further.

List of papers

This dissertation is based on the four original papers listed below:

1. **Tamirat Gebru**, Albert Lalremruata, Peter G. Kremsner, Benjamin Mordmüller, Jana Held. Life-span of *in vitro* differentiated *Plasmodium falciparum* gametocytes. *Malaria Journal*. 2017;16 (1): 330
2. **Tamirat Gebru**, Anthony Ajua, Michael Theisen, Meral Esen, Ulysse Ateba Ngoa, Saadou Issifou, Ayola A. Adegnika, Peter G. Kremsner, Benjamin Mordmüller, and Jana Held. Recognition of *Plasmodium falciparum* mature gametocyte-infected erythrocytes by antibodies of semi-immune adults and malaria-exposed children from Gabon. *Malaria Journal*. 2017; 16(1):176.
3. **Tamirat Gebru**, Benjamin Mordmüller, Jana Held. Effect of fluorescent dyes on *in-vitro* differentiated, late-stage *Plasmodium falciparum* gametocytes. *Antimicrob. Agents Chemother.* 2014 58(12):7398-404.
4. Jana Held, **Tamirat Gebru**, Markus Kalesse, Rolf Jansen, Klaus Gerth, Rolf Müller and Benjamin Mordmüller. 2014. Antimalarial Activity of the Myxobacterial Macrolide Chlorotonil A. *Antimicrob. Agents Chemother*. 2014, 58(11):6378-84.

Additional publications not included in this dissertation:

1. Sulyok M, Rückle T, Roth A, Mürbeth RE, Chalon S, Kerr N, Samec SS, Gobeau N, Calle CL, Ibáñez J, Sulyok Z, Held J, **Gebru T**, Granados P, Brückner S, Nguetse C, Mengue J, Lalremruata A, Sim KL, Hoffman SL, Möhrle JJ, Kremsner PG, Mordmüller B. DSM265 for *Plasmodium falciparum* chemoprophylaxis: a randomised, double blinded, phase 1 trial

with controlled human malaria infection. *Lancet Infect Dis.* 2017 Mar 28. pii: S1473-3099(17)30139-1.

2. Mordmüller B, Surat G, Lagler H, Chakravarty S, Ishizuka AS, Lalremruata A, Gmeiner M, Campo JJ, Esen M, Ruben AJ, Held J, Calle CL, Mengue JB, **Gebru T**, Ibáñez J, Sulyok M, James ER, Billingsley PF, Natasha KC, Manoj A, Murshedkar T, Gunasekera A, Eappen AG, Li T, Stafford RE, Li M, Felgner PL, Seder RA, Richie TL, Sim BK, Hoffman SL, Kremsner PG. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature.* 2017 Feb 23;542(7642):445-449.
3. Manego RZ, Mombo-Ngoma G, Witte M, Held J, Gmeiner M, **Gebru T**, Tazemda B, Mischlinger J, Groger M, Lell B, Adegnika AA, Agnandji ST, Kremsner PG, Mordmüller B, Ramharter M, Matsiegui PB. Demography, maternal health and the epidemiology of malaria and other major infectious diseases in the rural department Tsamba-Magotsi, Ngounie Province, in central African Gabon. *BMC Public Health.* 2017 Jan 28;17(1):130.
4. Mekonnen SK**,** Aseffa A**,** Berhe N**,** Teklehaymanot T**,** Clouse RM**, Gebru T,** Medhin G and Velavan TP**.** Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia. *Malaria Journal.* 2014, 13:244.
5. **Tamirat Gebru Woldearegai**, Kremsner PG, Kun JFJ, Mordmüller B. Plasmodium vivax malaria in Duffy-negative individuals from Ethiopia. Trans R Soc Trop Med Hyg, 2013, 107(5):328-31.

1 General introduction and background

1.1 Malaria: global distribution and clinical burden

Malaria is a preventable and treatable disease that is transmitted by female *Anopheles* mosquitoes as vector. The disease is of global health importance especially in sub-Saharan Africa (Figure 1). According to current estimates, approximately 3.2 billion people are globally at risk of being infected with *Plasmodium,* the causative agent of malaria[1]. In 2015, 212 million cases and 429,000 deaths occurred globally, with 92% of these malaria deaths occurring in sub-Saharan Africa[2]

The nature of malaria morbidity and mortality can be affected by the level of disease transmission. In stable transmission areas, fatality rates may reach 2-3%[3]; while in low seasonal transmission settings, where the population fails to sustain disease-modulating immunity, it is even 10 times higher[4]. In areas with unstable malaria transmission, high risk for severe disease is expected in younger children[5] while cerebral manifestations is likely to happen in older children and adults[6] leading to different socioeconomic consequences.

With the expansion of malaria interventions through an improved access to accurate diagnosis, introduction of effective antimalarial medicines and scale up of vector control coverage, the burden of the disease significantly declined globally between 2000 and 2015 with a reduction of malaria incidence and mortality by 41% and 62%, respectively[2]. In Africa, the mortality rate has reduced by 66% in the same period[1], even though the disease is still a major cause of morbidity and mortality on the continent (Figure 1).

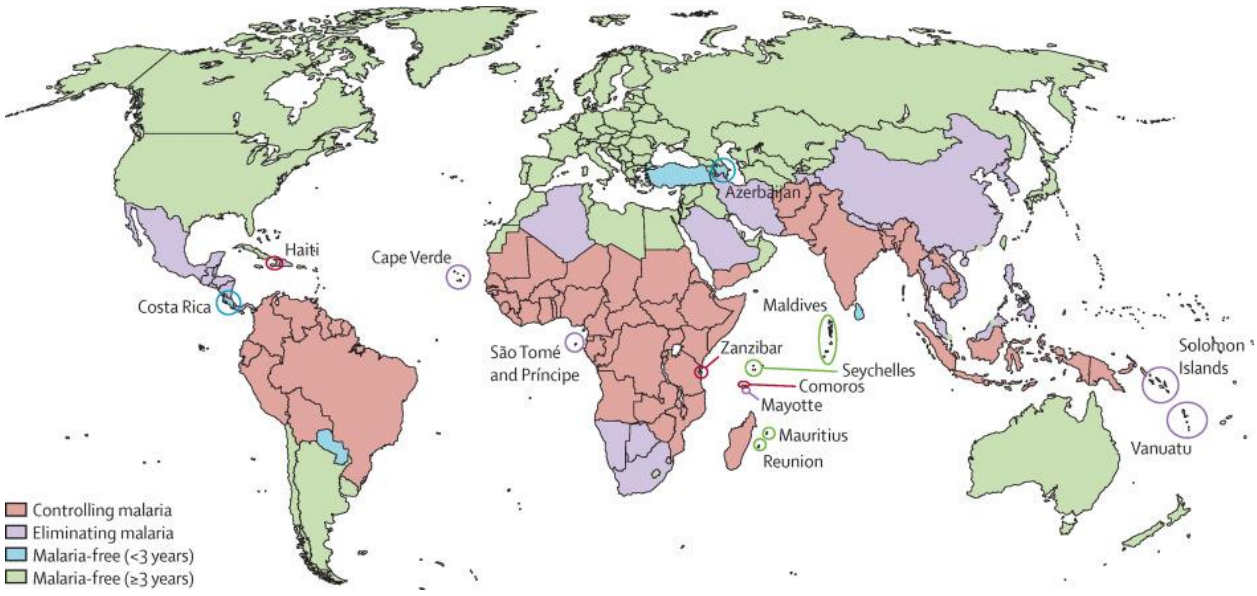

Figure 1. Categorization of countries as malaria-free, eliminating malaria, or controlling malaria, 2015

Source: *Newby et al 2016. The Lancet; v*olume 387, Issue 10029, Pages 1776.

DOI: 10.1016/S0140-6736(16)00230-0

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However, the effect of these interventions on the reduction of malaria transmission in much of sub-Saharan Africa have been only modest[7]. An increasing trend in *Plasmodium falciparum* burden has been recently reported from Mozambique after a successful decline in the prevalence, emphasizing the need for sustained malaria control with proven interventions[8]. Additionally, the declining trends of malaria prevalence achieved during the past decade are threatened by emerging resistance to the pyrethroid group of insecticides[9] and by the emergence of artemisinin-resistant malaria parasites[10].

1.2 Life cycle and causative agents of malaria

Malaria is caused by a protozoan parasite of the genus *Plasmodium* that infects humans and female *Anopheles* mosquitoes. There are five species of *Plasmodium*

that infect humans, namely *P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi* (a simian malaria parasite that can be transmitted to humans and causes disease[11,12]). *P. falciparum* is the most virulent species, responsible for almost all mortality due to malaria in Africa. Malaria parasites have complicated and fascinating life cycles with the development of the parasite both in humans and in the female *Anopheles* mosquito (Figure 2). When an infected mosquito bites a human for a blood meal, the parasites are injected in the form of sporozoites into the skin and capillaries with the mosquito's saliva. The sporozoites travel to the liver and multiply asexually in the liver cells to produce tens of thousands of merozoites from each liver cell within 5-16 days depending on the species. Some *Plasmodium* species (*P. vivax* and *P. ovale)* can remain dormant for extended periods in the liver, resulting in relapses weeks up to years later[13–15]. After the exo-erythrocytic schizogony, the merozoites leave liver cells and enter the bloodstream and infect red blood cells where they initiate asexual multiplication cycles (blood schizogony) that produce 8 to 24 new infective merozoites when the cell ruptures within 1-3 days depending on the species. The newly formed merozoites continue to repeatedly invade more erythrocytes and result in millions of parasite-infected cells in the host bloodstream, causing fever commonly when parasites egress from erythrocytes and invade new red cells. Some individuals control parasitemia and remain afebrile in malaria-endemic areas and an age-dependent fever threshold effect is apparent[16].

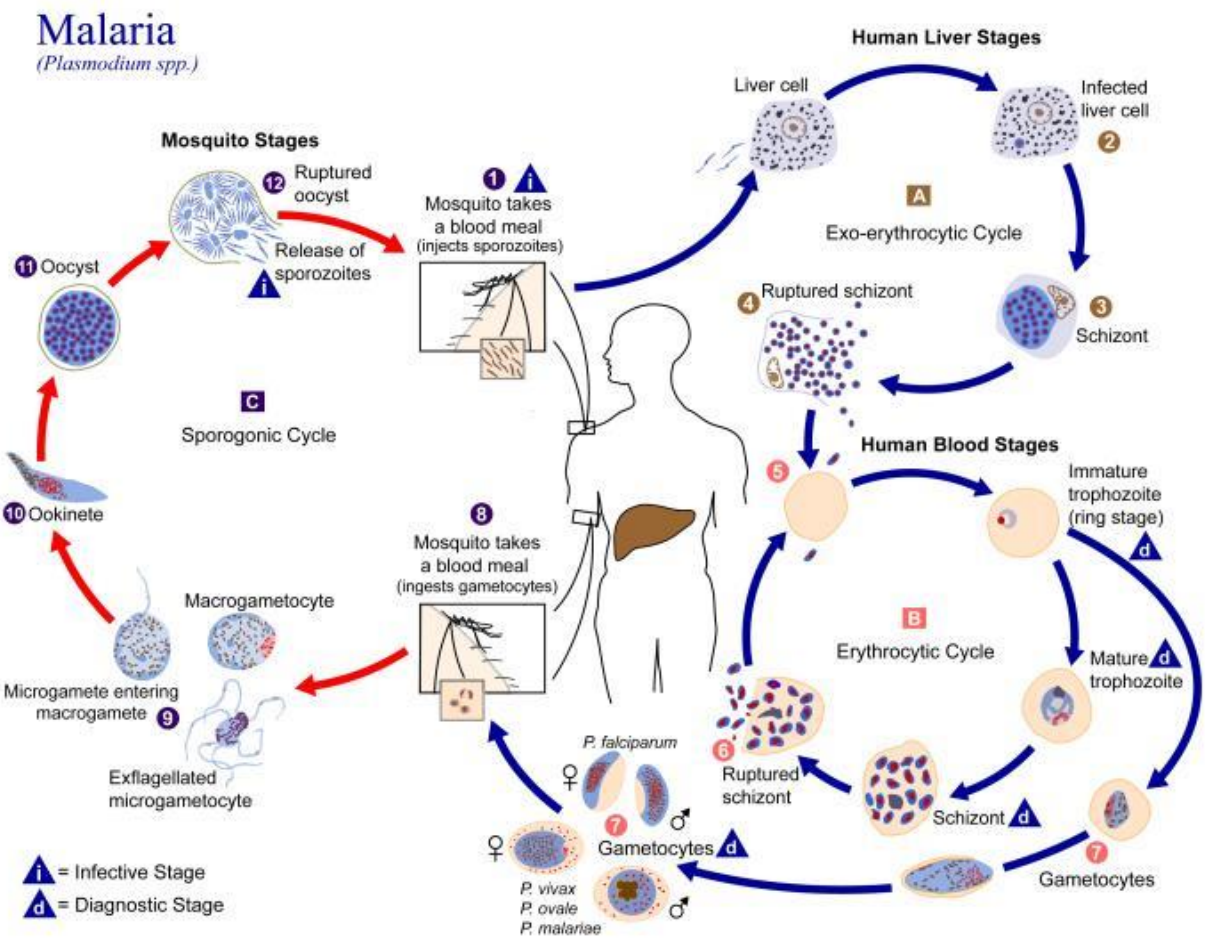

Figure 2. *Plasmodium* life cycle.

Source: https://www.cdc.gov/dpdx/malaria/index.html ("DPDx - Malaria Image Library")

Reprinted with permission from Centers for Disease Control and Prevention (CDC).

Some merozoites leave the cycle of blood-stage asexual multiplication and differentiate into sexual erythrocytic stages forming male and female gamete precursors called gametocytes. Gametocytes are highly specialized form of the parasite responsible for its transmission. The gametocytes are ingested when a female *Anopheles* mosquito bites an infected human and develop into mature cells called gametes and complete the sexual cycle in the mosquito that takes 10-18 days depending on species and temperature. In the midgut, the male and female gametes

fuse and form diploid zygotes that develop into actively moving ookinetes. The ookinetes move into the mosquito midgut wall and form oocysts. Inside the oocyst, thousands of sporozoites are produced that travel to and invade the mosquito salivary glands. When the infected mosquito bites another human, the malaria life cycle perpetuates through the inoculation of the sporozoites from its salivary glands. The mosquito is acting as a transmission vector, commonly without significant impairment by the presence of the parasites[17,18].

1.3 Plasmodium falciparum infection and gametocyte carriage

In the natural *P. falciparum* infection cycle, a continuous production of gametocytes results from untreated asexual infections[19]. After commitment to gametocytogenesis immature forms (stage I-IV) sequester in the body (mainly in bone marrow) for a maximum of 12 days until they become mature (stage V)[20] gametocytes and appear in the circulation (Figure 3). During gametocyte development, stage-specific mRNA is transcribed even though no genomic replication is present. Gametocyte carriage refers to the presence of sexual forms of the parasite in the body of infected person being sequestered in the tissues of internal organs and later circulating in peripheral blood till they decay spontaneously, by immune attack or drugs.

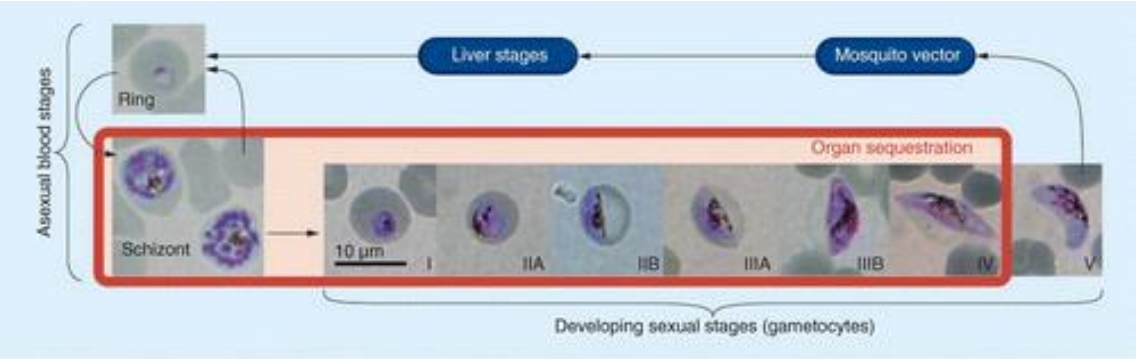

Figure 3. Simplified *Plasmodium falciparum* life cycle, focusing on gametocytogenesis.

Source: Lucantoni and Avery 2012[21], Future Medicinal Chemistry, Vol. 4, No. 18, Pages 2338.

DOI 10.4155/fmc.12.188

1.3.1 *P. falciparum* gametocyte commitment and longevity

Gametocyte commitment and carriage have been associated with the occurrence of a variety of conditions unfavorable for asexual growth. The commitment rate commonly depends on the type of treatment taken[22], density of asexual infection[23], period of infection[24], acquired immunity, presence of anemia[24,25], and other hematological and parasite genetic factors. After clearance of the asexual infections by drug or immune response, gametocytes can still be detected in peripheral blood of the infected person for prolonged time till they decay or are killed by the effect of immune response or treatment against these transmission stages[26]. The mean and maximum circulation time of mature gametocytes (after period of sequestration and being released to the circulation) are estimated *in vivo* by microscopy as 6.4 and 22 days, respectively[20]. This was further confirmed by PCR with a mean circulation time of 4.6-6.5 days[26]. The latter reported a much longer gametocyte carriage (sequestration plus circulation time) of low density gametocytes, as 13.4 and 55 days after ACT and non-ACT treatment, respectively[26]. These few gametocytes that remain in the blood for an extended period might be enough to be transmitted to mosquito as the transmission can occur even at a submicroscopic level[27]. A prolonged presence of mature gametocytes (their lifespan) in the blood has great epidemiologic importance as it increases the chances of infectivity and transmission to mosquito. However, except a handful of reports that rely on the estimation of the *in vivo* longevity (mostly using modelling), the gametocyte lifespan was not studied experimentally *in vitro*. Here in this thesis, additional data is presented that shows the *in vitro* lifespan of gametocytes.

1.4 Immune response to malaria and development of transmission blocking vaccine

Natural infection with malaria parasites leads to an immune response that is regulated by the innate (natural) and adaptive (acquired) immune system which is further classified into humoral and cell mediated. The interaction of malaria parasite proteins with the immune system of the host plays a role in the pathogenesis of the disease.

1.4.1 Innate immunity

Innate immunity is a fast-acting inherent refractoriness of the host against the parasite (sporozoites). It is not dependent on previous antigen encounter, but naturally present in the host and carried out mainly by the complement system (humoral) and effector immune cells (cell mediated). The humoral and cellular mechanisms of this 'nonspecific' defense are poorly defined, despite an emerging body of evidence emphasizing the importance of innate immune signaling in the activation of adaptive immune responses[28].

During the first encounter with the malaria parasite at the inoculation site, a generic immune response is initiated that induces the recruitment of immune cells (polymorphonuclear neutrophils, resident myeloid cells and inflammatory monocytes reviewed in[29]). During migration from the skin to the liver, sporozoites trigger signaling cascade in murine hepatocytes *in vitro*[30]. In the liver, the replication of sporozoites induces type I and II IFNs-dependent functional innate immune responses[31,32] in mice. The type I IFN signaling is needed for the recruitment of natural killer T cells that plays a role in reducing liver infection during secondary sporozoite infection[32], though it does not have impact on the growth of exoerythroctic forms released from the hepatocytes after the primary infection[31,32].

In humans the significance of innate immune response is not clear. Generally, the response to sporozoite inoculation is not measurable, presumably due to the low number of inoculated parasites, whereas systemic inflammatory response to asexual blood stage infection is strong and can lead to sepsis-like syndromes. Captured parasite antigens, after being ingested and processed by antigen presenting cells (mainly dendritic cells), are presented to T cells as antigen-MHC complexes. This leads to the expression of pro-inflammatory cytokines, and co-stimulatory molecules for subsequent initiation of the antigen-specific adaptive immunity mediated by B and T lymphocytes[33]. Several studies have shown that after activation by antigen presenting cells (APCs), CD4+ T cells initiate antibody-independent non-specific parasite killing by producing pro-inflammatory cytokines and stimulate macrophages to kill parasites intracellularly or via oxygen and nitric oxide radicals[34]. However, the CD4 cytotoxic effect is controversially discussed and the B-cell independent parasite killing is not achieved in some situations[35,36].

The mechanism of antigen capture and presentation for primary activation of $CD8^+$ T cell is not well known. However, it is assumed that sporozoite antigens are shed in the cytosol of various cell types during cell traversal during the migration of sporozoites from the skin to liver and these antigens are processed and presented by dendritic cells (reviewed in[29]). Additionally, antigens expressed in hepatocytes during the development of exoerythrocytic forms are shown to prime protective $CD8^+$ T cells[37], followed by the differentiation of these cells into liver-resident memory $CD8^+$ T cells, a key determinant of protective immunity against pre-erythrocytic stages[38].

1.4.2 The adaptive (acquired) immunity to malaria

Following the initial defense by the innate immune responses, naturally acquired adaptive mechanisms mediated by B and T lymphocytes are required for control of parasite-mediated pathology. Acquired immunity is provoked by the malaria parasite antigens which changes throughout the complex parasite life cycle. Acquired immunity is species-, stage-[39], and strain-specific[40]. Immune protection is usually

non-sterile and short-lived, requiring lifelong repeated parasite exposure and episodes of clinical disease[41]. Adaptive immunity recognizes pathogens by rearranged high affinity receptors and often involves cell proliferation, gene activation and protein synthesis. It is hence not rapid enough to act early in infection unlike the innate immune defense. Acquired immunity against *Plasmodia* have been defined as either anti-disease, anti-parasite, or sterilizing immunity[41,42]. Fully protective anti-parasite immunity can develop in areas with stable malaria transmission following frequent and multiple infection resulting in very low or undetectable parasitemia and an asymptomatic carrier status. Only partial immunity is acquired in people living in unstable endemic areas. Complete sterile immunity is usually not achieved naturally, except when administered experimentally as reported earlier in sporozoite-induced liver-stage immunity[43].

Although the pre-erythrocytic stage and gametocytes are also targeted, acquired immunity is directed predominantly against the asexual erythrocytic stages in circulation, mainly the extracellular merozoites, with little effect against developing or mature trophozoites[42]. Acquired immunity to malaria has two arms: antibody-mediated and cell-mediated immunity. Antibodies are particularly important for asexual and sexual blood stage immunity, whereas cell-mediated immune mechanisms are essential to suppress liver stage development.

1.4.3 Humoral (antibody-mediated) immunity

In areas with stable and intense malaria transmission children born to immune mothers are protected from malaria in the first months of life by means of passive immunity (by maternal antibodies) followed by few years of susceptibility before the acquisition of active immunity[44]. Antibodies are important mediators of immunity to malaria and their protective role has been shown earlier[45,46]: e.g. parasitemia and clinical disease were reduced in Thai *P. falciparum*-infected patients after passive transfer of IgG from immune donors[46]. A central role of humoral immunity has been additionally demonstrated in rodents: Mice lacking B cells were unable to clear blood-stage parasite infection[35].

Antibody production is induced during malaria infection with IgM and IgG being the predominant type. Antibodies protect against malaria by a variety of mechanisms including blocking the invasion of sporozoites into liver cells, inhibition of merozoite invasion of erythrocytes[47], prevention of intra-erythrocytic growth and release from schizonts[48]. Antibodies also prevent binding and sequestration of infected erythrocytes in small vessels to enhance their clearance from the circulation by the spleen[49,50]. They also disrupt resetting or spontaneous binding of uninfected RBCs to RBCs infected with late-stage asexual parasites[51]. Additionally, in collaboration with other immune cells, *Plasmodia* antigen-specific antibodies play a role in parasite clearance by opsonization of infected erythrocytes to increase their susceptibility to phagocytosis and cytotoxicity by various immune effector cells[52].

1.4.4 Cell-mediated immunity

Cell-mediated immunity in malaria may protect against both pre-erythrocytic and erythrocytic parasite stages. CD8 T cells have important effector functions in pre-erythrocytic immunity[53], however there is little evidence of their role against blood stage infection, possibly because CD8 T cells provide protection by lysing infected cells through major histocompatibility complex (MHC) antigen expression which is not present in erythrocytes[54]. In pre-erythrocytic stage, CD8 T cells attack parasite-infected liver cells by secreting lysis factors, like perforin and granzyme B[55].

In contrast, CD4 T cells are essential for immune protection against pre-erythrocytic and asexual blood stages via cytokine production and modification of B cell[56] and CD8+ T cell responses[57]. They play a major role in the acquisition and maintenance of protective immune responses to malaria infection[58]. It has been shown *in vitro* that CD4 T cells from malaria exposed people respond to *P. falciparum* blood stage antigens by proliferation and then production of IFN-γ and/or IL-4[56]. CD4+ T cells regulate humoral immune response by activating B cells to differentiate and produce *P. falciparum*-specific antibodies[59]. Some CD4 T cells (Tregs) play role in regulating

the pro-inflammatory immune response that may lead to severe illness and mortality[60,61].

1.4.5 Antibody response to *P. falciparum* gametocytes

Antibodies are the main mediators of sexual-stage immunity to malaria[62]. The presence of sexual-stage specific immune response to malaria is evidenced in several studies showing their partial transmission reducing effect. This immunity targets the formation and maturation of gametocytes in the circulation and also the fertilization and further sporogonic development in mosquito. A humoral immunity targeting epitopes expressed on the surface of gametocytes and/or gametocyte-infected erythrocytes plays a major role in limiting the transmission of the parasite, while the CD8+ T cells are not the main part of transmission reducing immunity as erythrocytes do not express major histocompatibility complex (MHC) molecules and lack processing organelle for the complex of MHC-I and antigen, as reviewed by Stone et al[63].

During natural infection, following exposure to the sexual stage parasites, individuals from malaria endemic areas develop transmission reducing antibody responses[64] against proteins expressed on the surface of gametocytes[65]. These sexual-stage specific antibodies are commonly detected during *P. falciparum* infection and their transmission blocking effect are shown by standard membrane feeding assays[66–68]. Sexual stage-specific antibodies affect gametocyte maturation[69] and density[70,71] in infected individuals. More importantly, these antibodies are taken up by mosquito during blood meal together with other components of the immune system, including cytokines and complement, and during the sporogonic phase of the *Plasmodium* life cycle, these molecules mediate the lysis of gametocytes, prevent fertilization and interfere with further parasite development in the mosquito[67,72,73].

Antibody response to gametocyte-infected erythrocyte surface antigens are not well investigated compared to the commonly evaluated gametocyte surface proteins,

pfs230 and pfs48/45. A strategy that aims to identify gametocyte-specific epitopes on the surface of infected erythrocyte might be a new approach in finding a novel gametocyte vaccine target. It was shown in a Thai cohort that naturally acquired antibodies bound to gametocyte-infected erythrocytes, distort the morphology and maturation of immature gametocytes and limit parasite transmission to mosquitos[69]. Sexual-stage antibody immunity was also observed in sera of Gambian children against surface antigens of mature gametocyte-infected erythrocytes that increased with age and associated with decreased gametocyte density, indicating that humoral immunity may interfere with gametocyte production and viability[71]. In line with this, in this PhD work, the natural immune response to mature gametocyte-infected erythrocytes was assessed in clinical samples to confirm previous findings and evaluate the influence of vaccination and co-infection on antibody response towards gametocytes.

1.4.6 Transmission blocking candidate vaccines

Several malaria candidate vaccines targeting the different stages of the parasite are under development as new and cost effective interventions for the control of malaria[74]. In particular with the aim to inhibit parasite development within the mosquito and interrupt the transmission of the parasite to the vector, transmission blocking vaccines (TBVs) that target surface protein on the sexual/sporogonic stages of the parasite or mosquito midgut antigens are in development[75] though at a much earlier stage, with the leading candidate of the TBVs (Pfs25) at phase I clinical trial[76].

Even though the potential of this approach has been appreciated, the development and licensure pathways for TBVs are not well defined. A number of *P. falciparum* antigens have been developed in pre-clinical studies as transmission blocking vaccine candidates. The ability of leading TBV candidate antigens Pfs230, Pfs25 and Pfs48/45 to induce a transmission-blocking response has been examined in different laboratories. Transmission blocking antibodies targeting sexual-stage specific antigens were evaluated in several clinical samples using a standard membrane feeding (functional) assays. Antibodies to the above-mentioned antigens have shown

in vitro transmission blocking effect in mosquito feeding experiments supporting their candidacy as transmission blocking vaccine. However, efficacy of such a vaccine can only be measured on population level. Therefore they are often combined as part of multistage vaccines.

Among the different lead candidate TBVs that target parasite surface antigens expressed either in the pre-fertilization stages within the human (for example Pfs48/45[77] and Pfs230[78]) or post-fertilization stage within the mosquito (for example Pfs25[77] and Pfs28[79]), only Pfs25 has been tested in phase I human clinical trials and induced transmission blocking antibodies even though the study was terminated due to the associated systemic adverse events[76], possibly related to the specific antigen/adjuvant combination used in the preparation. Adjuvant related limitations and immunogenicity of Pfs25 TBVs has been improved by using purified Pfs25 protein expressed in *E. coli*[80] and other organisms and through conjugation of the antigen to carrier proteins with known high immunogenicity[81]. Currently, Pfs25 antigen chemically conjugated to *E. coli*-expressed ExoProtein A (EPA) is advancing to a phase 1 human trial in endemic settings (reviewed in[82]).

The post-fertilization proteins, Pfs25 and Pfs28, are not expressed in the vertebrate host, and hence not exposed to the host immune selection pressure and appear to have limited antigenic variations[83,84], which qualify them to be broad-acting vaccine candidates. A very different transmission blocking approach targets the mosquito midgut ligands required for ookinete interaction. Antibodies elicited against mosquito midgut proteins (such as *A. gambiae* midgut glycoprotein, Anopheline Alanyl aminopeptidaseN [AnAPN1]) have demonstrated transmission blocking activities[85].

Despite very slow progress in the last decades, there is currently a promising improvement in the development of transmission blocking and other malaria vaccines. However, still more work has to be done to explore different targets of parasite or mosquito stage proteins so as to fill the gap in finding a good

transmission blocking vaccine. As sexual stage antigens are under less immune selection pressure, they are likely to have minimal antigenic variation compared to vaccine antigens to other stages of the malaria lifecycle. The development and inclusion of TBVs as malaria control strategy would therefore make a significant change in malaria epidemiology. TBVs could prevent the spread of the disease in the community including in countries with high malaria transmission. The vaccine could help to accelerate elimination of the disease from areas with low and seasonal malaria transmission and eventual eradication.

1.5 Treatment and control of malaria

Malaria is a curable disease if promptly diagnosed and treated. Treatment should be initiated as soon as possible as the disease can be deadly if there is a delay in diagnosis especially when caused by *P. falciparum*. The primary aim of malaria treatment is a rapid and complete elimination of the parasite. Antimalarial drugs or combinations with an additional transmission blocking effect would be particularly beneficial on the population level, because they would reduce spread of parasites; in particular, strains that developed resistance.

A good antimalarial treatment for uncomplicated cases needs to be available as oral formulation, administered with limited number of doses (preferably only one), with very good therapeutic effect and safety profile, with long term protection and delay for emergence of resistance, whereas treatment for severe cases needs a parenteral formulation with fast-acting drugs that are fully active against all strains. In the past, *P. falciparum* has shown a widespread resistance to various antimalarial chemotherapies. There are several antimalarial drugs that are administered depending on the species of *Plasmodium* and the sensitivity of the parasite species. Artemisinin-based combination therapies (ACTs) are the most effective antimalarial drugs available today for uncomplicated *P. falciparum* malaria[86]. Five different ACTs have been recommended by the World Health Organization (WHO)[87] and among

these combinations artemether-lumefantrine (AL) has been the most widely prescribed ACT in sub-Saharan Africa.

Different malaria control strategies work together to interrupt the transmission of parasites (vector control), the establishment of blood-stage infection in humans (chemoprevention) and the development of illness and severe disease (management of cases)[1]. It is estimated that the reduction of malaria cases in sub-Saharan Africa between the year 2000 and 2015 was achieved mainly (70%) by malaria control interventions. Among the averted cases, 79% were estimated to be averted through the use of vector control measures and 21% by artemisinin based combination therapy[1].

However, the emergence of parasite resistance to the currently used combination drugs (ACT) in five countries of the so called 'Greater Mekong sub region (GMS)' and resistance of mosquitoes to various insecticides in almost all African countries are challenges of malaria control strategies that may reverse the declining trend of malaria incidence if unaddressed. A recent report has shown the development of *P. falciparum* resistance to both fast-acting (artemisinin derivative) and long-acting (partner) combination drugs in a multisite prospective study in Cambodia[10]. The distribution of insecticide treated mosquito nets is still inadequate in malaria transmission areas of sub-Saharan Africa. The coverage of indoor residual spraying is decreased these days and mosquito resistance to at least one insecticide occurred in 60 countries since 2010[1]. Despite the improved intervention, millions of people still lack access to accurate diagnosis and treatment. The progress towards the improvement of preventive therapies for pregnant women and adoption of preventive therapies for children under five is slow.

1.5.1 Effect of antimalarial drugs on *P. falciparum* gametocytes

Gametocidal activity of antimalarials is highly desirable for malaria control and prevention of spread of resistant parasites and for the final elimination and

eradication agenda[88]. Although most currently used antimalarial drugs, though they are active against the asexual blood-stage parasites[89–91], they have no effect against mature *P. falciparum* gametocytes[92], aside from a modest effect on the early stages of the sexual forms of the parasite. Some antimalarials may even increase the gametocyte carriage rate and transmission of malaria[93], which would be devastating if it results in the selection of drug resistant parasites. A recent report has shown that patients carrying artemisinin resistant parasites more often develop gametocytemia before or after treatment with artemisinin combination therapies than those having artemisinin sensitive parasites indicating an increased transmission potential of artemisinin resistant parasites[94]. This remains a big challenge for the control of malaria and resistance containment unless effective and safe gametocidal drugs are accompanied with the drugs targeting the asexual stages.

Based on the effect on the asexual[95] and early gametocyte stages[96] artemisinin combination therapies have been shown to have an important public health benefit in reducing gametocyte carriage[97] and thereby reducing patient infectivity and malaria transmission[98]. However, ACTs are not active against the matured sexual forms responsible for the transmission of the parasite from human to the vector.

Even though primaquine, an 8-aminoquinoline, has shown proven gametocytocidal activity against matured gametocytes *in vivo*[99,100], its use is limited due to its serious safety problems (risk of hemolysis) in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals. 8-aminoquinoline drugs are also reported to have low efficacy in poor metabolizers of CYP2D6 in humans[101,102]. The same safety and efficacy issue is the concern of other related 8-aminoquinolines; bulaquine with a potential gametocidal activity[103] and tafenoquine[104], which is at the late-phase of clinical development. A single low dose of 0.25 mg/kg primaquine as a *P. falciparum* gametocytocide was recently recommended by World Health Organization (WHO) replacing the previously recommended dose of 0.75 mg/kg for a use together with artimisinin combination therapy[105]. As reviewed by Uthman et. al.[106], some studies tested low-dose primaquine (single-dose of 0.1 to 0.25 mg/kg) in G6PD-deficient individuals and have reported less marked falls in average haemoglobin compared to

the higher dose (0.75 mg/kg). In line with this, a recent meta-analysis study reviewed that primaquine was not significantly different from other drugs in the incidence rate ratios for adverse effects[107]. However, another recent study showed that even the lower (0.25 mg/kg) single-dose of primaquine resulted in greater fall in haemoglobin concentration in G6PD-deficient patients who received primaquine than in those who did not[108]. Currently, there is no alternative safe and effective gametocidal drug licensed for the control of *P. falciparum* transmission.

1.5.2 Novel compounds with gametocidal activities

Chemotherapy plays a great role in the treatment and control of malaria, however, emergence of drug-resistance is a major threat of chemotherapies. The parasite has developed resistance in the past to several registered antimalarials[109], including growing reports recently of delayed response to artemisinin derivatives in Southeast Asia[110]. Therefore, new drugs need to be developed to back up the currently available drugs before they fully lose their activity. Much work has to be done especially in exploring novel compounds against the sexual stages of *P. falciparum* where currently there is no effective and safe drugs proven for public use. Such drugs would be central to fight malaria and the spread of resistance on the population level and shall be included in the pipeline for discovery and development. In this PhD work, the gametocidal effects of several fluorescent dyes and a macrolide compound chlorotonil A are evaluated.

1.5.2.1 Gametocidal activities of fluorescent dyes

Since the late 19th century, several synthetic dyes have been tested for their antimalarial activity[111,112], starting with the pioneering work of Guttmann and Ehrlich who discovered the antimalarial activity of the thiazine dye methylene blue[113]. Methylene blue has been shown to have strong gametocytocidal effect and potent transmission-blocking activity *in vivo*[114,115] and *in vitro*[116,117].

A recent *in vitro* study identified several synthetic dyes with high levels of antimalarial activity against the asexual blood stages of *P. falciparum* parasites[118]. To further explore the activity of fluorescent dyes against the sexual stages of *P. falciparum* responsible for the transmission of malaria, the effect of a panel of fluorescent dyes against gametocytes was assessed in this PhD work using an ATP-based bioluminescence assay. In this work, fluorescent dyes were found with high levels of inhibitory activity against mature *P. falciparum* gametocytes of the 3D7 laboratory strain and clinical isolates from Gabon[119].

1.5.2.2 Gametocidal activities of chlorotonil A

Plants, fungi and bacteria are a rich source of biologically active natural compounds and their derivatives to develop effective chemotherapeutics against infectious diseases including malaria. Among these, the soil-dwelling myxobacteria are known producers of novel biologically active natural products[120–122]. In primary screening of a library of secondary substances from myxobacteria, particularly *Sorangium cellulosum* strains, at the Swiss Tropical and Public Health Institute, the antiplasmodial activity of a chlorine-containing metabolite, chlorotonil A was identified. Gerth *et. al.* have first isolated and described the structure of chlorotonil A[123] and Rahn and Kalesse later reported the total synthesis and the dehalogenated form of the substance[124]. The compound is complex and poorly soluble in water and organic solvents[123]. In this PhD work, the gametocidal activity of chlorotonil A was investigated *in vitro* against late-stage *P. falciparum* gametocytes with an established bioluminescence assay. Here it is shown that chlorotonil A had an inhibitory effect at concentrations well below any of the available antimalarials.

2 Aim of the PhD work

The institute and the study group where there PhD project was completed is involved in several studies on the investigation of malaria transmission, immunity and drugs targeting the *P. falciparum*, the most important causative agent of malaria. There is little information about the biology of the transmissible stage of the parasite and no

safe and effective drug and vaccine is on the market to block the transmission of the parasite from human to mosquito.

In order to contribute to the knowledge of *P. falciparum* gametocyte biology, a gametocyte *in vitro* culture was established and several experiments were performed to assess the development and longevity of mature gametocytes, to explore acquisition of anti-gametocyte immunity and evaluate susceptibility of mature gametocytes to drugs. The assessment of gametocyte lifespan is done here experimentally for the first time *in vitro* to confirm the previous *in vivo* modelling estimate of gametocyte longevity. The survival of *in vitro* differentiated gametocytes was evaluated by microscopy, flow cytometry, and real time quantitative polymerase chain reaction (RTqPCR). Moreover, natural antibody response to the surface and intracellular antigens of mature gametocyte-infected erythrocytes was explored using previously collected samples from two separate studies (two phase I clinical trials of GMZ2, a malaria candidate vaccine) from Gabon. For this purpose, a flow cytometry based immunoassay was used to quantify anti-gametocyte IgG antibody response to *P. falciparum* sexual stage antigens. Additionally, new compounds (several fluorescent dyes and chlorotonil A) were tested for their activity against the transmission stages of *P. falciparum* clinical isolates and laboratory strains. To achieve this aim, an ATP based bioluminescence assay was used to measure the viability of the parasites.

The specific objectives of this PhD work are presented as follows.

1. To investigate the life span of *in vitro* cultivated *P. falciparum* gametocytes.
2. To explore natural antibody response to *P. falciparum* gametocyte-infected erythrocytes and evaluate the effect of vaccination with GMZ2 (candidate malaria vaccine) and intestinal parasite infection on the development of antibody response to gametocytes.
3. To evaluate the activity of fluorescent dyes against mature (stage V) gametocytes of the laboratory strain 3D7 and clinical isolates in comparison to previously described antimalarials.
4. To assess activity of chlorotonil A against mature sexual stages of the *P. falciparum* 3D7 laboratory strain.

3 Results

The summary of the results from different studies making up this PhD work is presented here. Figures and tables from each of the papers are referred to in the text.

3.1 Manuscript I (published)

Life-span of *in vitro* differentiated *P. falciparum* gametocytes

Tamirat Gebru, Albert Lalremruata, Peter Kremsner, Benjamin Mordmüller, Jana Held

Malar Journal. 2017;16 (1): 330

The sexual stages of *Plasmodium* parasite (gametocytes) are responsible for the transmission of the parasite from humans to mosquitoes. It has been reported that mature gametocytes stay in infected individuals for several weeks being infective to the mosquito for a number of days. Previous assessments were made *in vivo* by microscopy[20,125] and PCR[126] following clearance of the asexual stages, and the longevity was estimated by mathematical modelling[20,126] assuming a constant sequestration period and an exponential decrease of circulating gametocytes with their age. To confirm the previously reported modelling estimate and present a better representation of the natural survival time, the gametocyte lifespan was assessed here using three viability methods (microscopy, flow cytometry, and RT-qPCR) for the first time *in vitro.* Gametocytes of one clinical isolate and one laboratory strain were followed in culture until they decayed naturally.

The gametocytes appeared healthy for several days by microscopy (Figure 1 - Manuscript I) and were detected for 39-46 days after initiation of the gametocyte culture, with a half-life of 2.6 days on average. The highest gametocytemia was observed between 20 to 22 days (Figure 2 – Manuscript I). *In vitro* longevity after reaching maturity reflects the *in vivo* circulation time of the mature forms. Assuming gametocyte maturation at the peak of gametocytemia (as some gametocytes did not

mature before peak), the mature gametocyte longevity was calculated by subtracting the number of days needed to reach peak gametocytemia from the recorded longevity. Therefore, mature gametocyte longevity, mean 50% and 10% survival rate were 18-26 days, 2.6 days, and 10 days, respectively (Table 1 – Manuscript I). NF54 had longer longevity than the tested clinical isolate (JH013). The ex-flagellation assay also demonstrated that mature male gametocytes can ex-flagellate for two weeks (Figure 2 – Manuscript I).

Gametocyte longevity was also evaluated by flow cytometry using a previously published assay of hydroethidine staining. Hydroethidine needs to be metabolized by live parasites to be converted to ethidium which can emit a red fluorescence when excited after interaction with the parasite nucleic acids. Gametocytes were detected by flow cytometry for 45 – 49 days after initiation of culture (Figure 3 – Manuscript I) and peak gametocytemia was recorded on day 16. Considering the day of peak gametocytemia as the day of maturation, mature gametocytes had longevity of 16-24 days. Mature gametocytes had on average 50% and 10% survival rate of 6.5 and 12 days, respectively (Table 1 – Manuscript I). Gametocyte longevity was higher in NF54 laboratory strain, followed by *P. falciparum* clinical isolate (JH013, collected from plasmodium infected people in Gabon) and 3D7 laboratory strain.

When assessed by molecular technique using RNA based quantitative real-time polymerase chain reaction (RTqPCR), the gametocytes were detected for longer than 50 days of the gametocyte culture. Two previously published, PF14_0367 (middle and late stage) and Pfs25 (late stage), gametocyte markers were used (Figure 4 – Manuscript I). Peak gametocytemia was recorded on 18 and 20 days after initiation of culture for NF54 and JH013, respectively. NF54 had longer lifespan than the clinical isolate (JH013). Estimated gametocyte circulation time (mature gametocyte longevity) was 31 and 26-32 days for NF54 and JH013, respectively. The mean 50% and 10% survival rate was 3.5 days and 12 days, respectively (Table 1 – Manuscript I).

Additionally, rate of gametocytogenesis was assessed *in vitro* in different strains of *P. falciparum* in the presence or absence of serum supplementation. Highest gametocytemia was observed in NF54 followed by JH013 and 3D7 (Figure 5 – Manuscript I) and serum supplementation enhanced the gametocyte yield. There was no statistically significant difference among the strains and between the serum supplemented and non-supplemented cultures. All the strains had similar patterns of parasitemia.

Overall, the longest gametocyte life span was measured by RTqPCR followed by flow cytometry and microscopy. The NF54 strain survived longer than the clinical isolates in all the three viability assays and has also shown the highest gametocyte yield. This finding is consistent with infectivity and cycle propagation capability of strains used in controlled human malaria infection.

3.2 Manuscript II (published)

Recognition of *Plasmodium falciparum* mature gametocyte-infected erythrocytes by antibodies of semi-immune adults and malaria exposed children from Gabon

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Malar Journal. 2017; 16 (1): 176

With the aim of assessing the natural immune response to gametocytes and evaluating the effect of the candidate malaria vaccine (GMZ2) and intestinal helminths on the development of natural antibody (Ab) response to gametocytes, Ab recognition of *P. falciparum* mature gametocyte-infected erythrocytes was evaluated

in vitro by microscopy- and flow cytometry-based immunofluorescence assay in samples collected from 30 children and 32 adults from two GMZ2 trials in Gabon (Table 1-Manuscript II). *In vitro* antigen recognition by Abs considerably increased after fixation and permeabilization of *P. falciparum* gametocyte infected erythrocytes (Figure 1-Manuscript II). However, to measure the gametocyte-specific response, non-permeabilized cells were used (Figure 2A-manuscript II) for all subsequent measurements with the intention to capture erythrocyte surface-expressed antigens. The antibody recognition of permeabilized erythrocytes (Figure 2b-Manuscript II) will not be fully gametocyte-specific as the detected internal antigens could be shared by the asexual stages.

The level of Ab recognition of NF54 laboratory strain showed was similar to the clinical isolate (JH013) that was additionally tested. Therefore, the NF54 strain which is currently being used in clinical trials was chosen to evaluate the level of anti-gametocyte Ab response in adults and children. Adults showed higher level of Ab response to mature gametocytes compared to children (Figure 3-Manuscript II) with the sero-prevalence also being higher in adults (77%) than in children (57%).

Antibody response to gametocytes may be modulated due to GMZ2 vaccination. This immune modulation may occur through the diversion of the immune response towards the vaccine or by reducing gametocyte density (via limiting the blood stage asexual infection) despite the low efficacy of the vaccine in younger children between one and five years[127]. To investigate this, the effect of GMZ2 vaccination on the development of anti-gametocyte immune response was assessed in adults and children. In adults, significantly lower levels of Abs were observed in GMZ2 vaccinated individuals on Day 84 (Figure 4A-Manuscript II) compared to baseline (Day 0). This difference was not seen in rabies vaccinated adult control group or in children vaccinated with either GMZ2 or rabies vaccine (Figure 4B-Manuscript II).

Even though some overlap is expected between antigens expressed at the sexual and asexual stages, sexual stage immunity may be developed independently from

the asexual-stage immunity. Therefore, possible associations were tested between the level of anti-gametocyte Abs and the previously published data of Abs to asexual blood stages antigens or GMZ2 vaccine antigen. A positive correlation was shown between Ab response to the sexual and asexual antigens in the semi-immune adult population but not in children (Table 2-Manuscript II). No association was observed between sexual Ab response and Ab to GMZ2 antigen in adults and children.

Prompted by earlier reports on the effect of intestinal helminths on anti-GMZ2 Ab responses, the effect of intestinal helminths infection on the anti-gametocyte immune response was evaluated in children using the previously published data of intestinal parasite burden of all 30 children included in the GMZ2 phase Ib trial. *Trichuris trichiura* and *Ascaris lumbricoides* were the dominant parasites followed by others with very low (< 7%) prevalence including *Strongyloides*, *Schistosoma*, and *Ancylostoma*. It was observed that children infected with *Ascaris* had significantly higher anti-gametocyte Ab response compared to uninfected children (Figure 5A-Manuscript II). When gametocyte-infected erythrocytes were permeabilized and used in the assay (which is not sexual stage specific), it was found that naturally acquired Ab response was significantly lower in children infected with *T. trichiura* compared to uninfected children (Figure 5B-Manuscript II).

3.3 Manuscript III (published)

Effect of fluorescent dyes on *in vitro*-differentiated, late-Stage *Plasmodium falciparum* gametocytes

Tamirat Gebru, Benjamin Mordmüller, Jana Held

Antimicrobial Agents and Chemotherapy. 2014; 58 (12): 7398-7404

Drugs that target the transmission of the parasite from an infected human host to mosquito vector are highly demanded to block the transmission as currently there is no safe and proven anti-gametocyte drug approved for use. Primaquine acts on

gametocytes and is licensed but cannot be safely used in G6PD-deficient individuals, who represent a significant population of most malaria-endemic areas. In this study, based on a previous report on the activity of fluorescent dyes against the asexual blood stages of *P. falciparum,* several potential synthetic compounds were selected and investigated for their activity against mature sexual stages using a bioluminescence cell viability assay. Viability of gametocytes was assessed by microscopy after purification and the gametocytes appeared non-compromised with an intact cytoplasm (Figure 1-Manuscript III), male gametocytes showed exflagellation, and the mature gametocytes consistently remained viable for an average of three weeks. The ATP readout was validated before testing the activity of the compounds and showed a close correlation of the ATP level (relative luminescence unit) with the number of gametocytes (R^2 = 0.99).

Among the nine fluorescent dyes tested (Figure 2-Manuscript III) five showed IC_{50}s below 1 µM (Table 1-Manuscript III) and among these, three mitochondrial dyes, MitoRed, DiOC6, and rhodamine B, had IC_{50}s below 200 nM, which is even lower than the IC_{50} of the control dye, methylene blue (770 nM). In particular, MitoRed showed high activity against laboratory strain 3D7 as well as against clinical isolates. The laboratory strain 3D7 was consistently more sensitive than the clinical isolates (2- to 10-fold) for the different dyes as well as the control drugs, particularly artesunate and DHA. Activity of primaquine (a prodrug) was low, which is in contrast to its strong effect against gametocytes *in vivo*. This is expected in *in vitro* assays as primaquine is a prodrug that requires activation by cytochrome P450 2D6 (CYP2D6)[128]. Epoxomicin, which was used as internal positive control was highly active against both; the 3D7 laboratory strain and clinical isolates. With the exception of epoxomicin, gametocytes were inhibited at higher drug concentration by all compounds compared to asexual blood stages (Table 1-Manuscript III).

Methods used in different gametocyte assays are poorly standardized and different approaches are used to assess viability and time of incubation to test gametocidal effects of compounds. In this study, the effect of incubation time on the activity of compounds was evaluated by incubating fluorescent dyes and standard antimalarial drugs with mature gametocytes of 3D7 parasites and clinical isolates for either 24 h

or 48 h. The incubation time had no significant effect for most compounds, however, MitoRed, the most active fluorescent dye in this assay, was 3-fold more active when incubated over 48 h than when used over 24 h (Table 2-Manuscript III). There was an even more pronounced (10-fold) increase in the activity of epoxomicin and artesunate when parasites were exposed for 48 h.

3.4 Manuscript IV (published)

Antimalarial Activity of the Myxobacterial Macrolide Chlorotonil A

Jana Held, **Tamirat Gebru**, Markus Kalesse, Rolf Jansen, Klaus Gerth, Rolf Müller, Benjamin Mordmüller

Antimicrobial Agents and Chemotherapy. 2014; 58 (11): 6378–6384

Similar to the investigation of fluorescent dyes, the activity of Chlorotonil A was evaluated *in vitro* and *in vivo* against asexual and sexual forms of *P. falciparum* laboratory strains and clinical isolates. Chlorotonil A (Figure 1-manuscript IV) was active against 3D7 and Dd2 laboratory strains of *P. falciparum* (Table 1-Manscript II) and did not show a delayed-death effect in a six-day assay with 10.6 nM (3D7) and 23.5nM (Dd2) IC_{50}s. Chlorotonil A has also shown activity against clinical isolates of *P. falciparum* isolated from patients with uncomplicated malaria in Lambaréné, Gabon with a narrow range of IC_{50}s close to those obtained against the laboratory strains (Table 2-Manuscript IV).

At least 25 clinical isolates were successfully grown in culture and tested *in vitro* for their sensitivity to chlorotonil A and the control drugs chloroquine and artesunate. Chlorotonil A arrested parasite development in different asexual blood stages of the parasite. Activity was highest in the ring and trophozoite stages (Figure 2A and 2B-Manuscript IV), and was still measurable in schizont stages (Figure 2C-Manuscript

IV), where artesunate at the same concentration (500nM) could not prevent the egress of merozoites from schizonts. Chlorotonil A acts quickly showing activity against the ring stage of the parasite, even when incubated only for 1 h with IC_{50} close to the one obtained in the standard *in vitro* susceptibility assay. *In vivo*, chlorotonil A was active against *P. berghei* in BALB/c mice (97%) administered orally at 36 mg/kg, 68 mg/kg, and 110 mg/kg and in Swiss CD1 mice (98%) at 100 mg/kg compared to the control mice in a 4-day suppression test with no obvious signs of toxicity. However, none of the mice were cleared of their parasitemia completely (Figure 3-Manuscript IV)

Chlorotonil A was evaluated *in vitro* if it has any effect on the viability of mature sexual stages of the laboratory strain 3D7 *P. falciparum* parasites by an *in vitro* bioluminescence assay. Chlorotonil A was active against stage IV to V gametocytes at concentrations close to that obtained against asexual blood stages. However, comparator drugs, artesunate and dihydroartemisinin act only when used in very high concentrations (Table 3-Manuscript IV). Epoxomicin, the internal control for the assay, was highly active against late-stage gametocytes.

4 General discussion

Malaria remains one of the biggest global health threats, even though increased funding and implementation of improved tools and strategies of malaria control have reduced the global malaria burden since 2000 and revived interest in the elimination and eradication of malaria[129,130]. Development of effective transmission blocking drugs and vaccines are considered as an essential components of the Malaria Eradication Research Agenda (malERA)[88], which complements the primary aim of antimalarial treatment that are directed towards removal of the asexual blood-stage parasites to cure the illness and prevent or treat severe forms of the disease.

To interrupt malaria transmission, intervention strategies and tools should focus on the reduction of the reservoir of infection, the period that a person or a vector is infectious, and the speed at which infections are spread. Drugs are essential tools for malaria elimination. If they effectively attack all stages of the parasite, they have the potential to eliminate last residual foci of infection and interrupt transmission.

Neglected in the past, currently there is an encouraging revival of interest on transmission stages of the parasite. However, it is still a long way ahead to understand fully the biology, commitment, development, sequestration, longevity, infectivity, immune response and drug susceptibility towards *P. falciparum* gametocytes. The four studies presented in this dissertation address the knowledge gap in this area.

4.1 In vitro life-span of *P. falciparum* gametocytes

Gametocyte maturity, density, sex ratio, and longevity are the main features of gametocytes that influence their infectivity to the mosquito. More specifically, the circulation time of mature gametocytes in the blood of an infected person will have an influence on the transmission of the parasite to the vector. To determine the *in vivo* circulation time of mature gametocytes, the first investigation of this PhD study

explores, for the first time, the period that the sexual stages of *P. falciparum* can stay viable in culture[131]. It was observed here using three viability assays that gametocytes were detected in the range of 39-52 days after initiation of the gametocyte culture. This period is assumed to reflect the *in vivo* sequestration and circulation time of the parasite. The average half-life ranged from 2.6 to 6.5 days.

The early forms of gametocytes get sequestered in the body for a maximum of 12 days[20] and appear in the peripheral blood and become infective after further maturation in the circulation in 2-3 days[125,132]. By our protocol, day of maturity was not the same for the whole gametocyte population of one culture. We observed that all gametocytes got matured on peak gametocytemia after day 12. Assuming peak gametocytemia as day of maturation, the mature gametocytes in our assay had 16-32 days survival time with parasitemia gradually declining. As some gametocytes got matured in our culture before the day of the peak gametocytemia, the exact gametocyte longevity after maturation (circulation time) could be a bit longer than the result we provided. A relatively shorter longevity of mature gametocytes was estimated earlier *in vivo* by microscopy, average 4.6 days (with maximum 24 days)[125] and 6.4 days (with maximum 22.2 days)[20]. The *in vivo* evaluation of gametocyte longevity might be affected by the host factors (which vary in different individuals) and the difficulties of the commonly used mathematical modelling method as it is not possible to follow the exact lifespan of parasites in a way similar to the *in vitro* experiment shown here. Other reason for the aforementioned shorter longevity period in the *in vivo* report could be the limit of microscopic identification of lower parasitemia of gametocytes in the blood, especially in the later days of follow up.

In a previous study, after evaluating gametocytemia by PCR, Bousema *et al.* reported longer gametocyte longevity (55 days) estimating parasitemia using mathematical modelling after clearance of asexual parasites[126]. This is in line with the *in vitro* data presented here observing gametocyte longevity for more than 50 days when using RTqPCR. For the clinical isolates, the gametocytes were detected for 6 more days when Pfs25 is used compared to PF14_0367. This could be due to

the relatively higher expression rate of Pfs25 at the sexual stage[133]. Even though the gametocytes persist for longer periods, it is not possible to answer the infectivity of old gametocytes from this approach unless a standard membrane feeding assay is done. However, it was observed that the mature male gametocytes were able to exflagellate for at least two weeks. Earlier, Smalley and Sinden have shown that ability of exflagellation by gametocytes persisted for three weeks[134].

Additionally, in this investigation, *in vitro* gametocytogenesis of different *P. falciparum* strains were evaluated. It was found that NF54 parasites are more prone to commit into gametocytes than JH013 clinical isolate and 3D7 laboratory strain. This confirms an earlier report that showed gametocyte yield depends on the parasite line[135], as the commitment rely on genetic and environmental factors[136]. The gametocyte production was improved in all *P. falciparum* parasite strains used when serum (from malaria-naïve individuals) was supplemented in the gametocyte culture medium. Similar finding was shown earlier with serum from semi-immune individuals[137]. This could be due to an increased production of the asexual parasites (a precursor for gametocytes) in the presence of serum from naïve or semi-immune people.

Despite their long longevity, the gametocytes in this study have short half-life or 50% survival rate (2.5-6.5 days) similar to a previous report (2.4 days)[134]. The 10% survival rate was 10-12 days and there was a steady decline of gametocytemia at the later days. Based on previous reports of mosquito infectivity studies, sub-microscopic gametocytemia could be enough to infect mosquitoes[138,139]. Therefore, the few gametocytes remaining at late time points of incubation in culture might be potentially capable of infecting mosquitoes. This should be further investigated with membrane feeding experiments. The extended *in vitro* longevity of gametocytes recorded in this study needs to be taken in to account in malaria control programs especially for the aim to eliminate the parasite.

4.2 Antibody recognition of *Plasmodium falciparum* mature gametocyte-infected erythrocytes

In the second study, antibody recognition of *in vitro* differentiated *P. falciparum* gametocyte-infected erythrocytes[140] was explored. Antibody response generated to sexual stage *P. falciparum* parasites has been reported to not only suppress their development[69], but also influence their survival in the circulation[70,71] and also interrupt the fertilization of gametes in the vector[77]. Natural antibody response to gametocytes could be initiated towards the parasite internal and membrane bound antigens or to the infected erythrocyte membrane proteins. Specific antibody response to few gametocyte-specific membrane proteins (mainly Pfs230 and Pfs48/45) have been studied earlier and their correlation with transmission blocking activities were shown. However, antibody response to gametocyte-infected erythrocyte surface antigens is rarely investigated. As this information is vital for the design and development of transmission blocking vaccines, here an exploratory study was done to investigate the antibody response to *P. falciparum* mature gametocyte-infected erythrocyte surface *in vitro* in sera from malaria-exposed children and semi-immune adults from Gabon. Additionally, the effect of the malaria vaccine candidate GMZ2 and worm infection on the development of anti-gametocyte antibody response have been evaluated.

In this study, adults had high antibody response compared to children. There are contradictory reports on the relation of age with anti-gametocyte antibody response. The result here is similar to some of the previous reports[141,142] but are not in line with others[143] that assume antibody response to gametocytes to be not long-lasting. Difference between the studies on the age-dependency of anti-gametocyte response might be due to different methodologies, varying age-ranges and epidemiological settings. Similarly, anti-gametocyte sero-prevalence was higher in adults (77%) than children (57%). According to earlier reports, sera from children in Ghana[144] and Gambia[71,141] also have shown similar prevalence of antibody response to the surface of gametocyte infected erythrocytes.

In addition, the association between antibody response to the asexual antigens (whole parasite antigen and GMZ2 [a fusion of MSP3 and GLURP protein]) and the development of anti-gametocyte antibody response was evaluated. In adults, there was a significant but weak positive correlation between sexual antibody and whole asexual antibody response, though not with the GMZ2 antigens. The result shows that parasite exposure is associated to antibody response to gametocytes. When the effect of GMZ2 vaccination was additionally assessed on the level of antibody response, the vaccination led to a reduced sexual antibody response in adults. This could be due the influence of the candidate vaccine to divert the immune response towards the vaccine or reduce the asexual parasite population leading to a lower gametocyte density and sexual antibody response, though the recent report shows only 10-14% efficacy of the vaccine in children[127]. To our knowledge, there is no evidence for the expression of GMZ2 component proteins on the surfaces of gametocyte-infected erythrocytes that could be the reason why boosting of anti-gametocyte antibody response was not seen in the vaccinated individuals. In children, no correlation was observed between asexual and sexual antibody response and the antibody level did not change after vaccination. This might be due to the low level of gametocyte-antibody response observed in children that decreases the ability to detect differences between the two variables. Additionally, the statistical power is limited due to the small sample size. It would be interesting to investigate the larger cohort of the recently completed phase II trial.

Previously it was shown that antibody response to the gametocyte-infected erythrocytes get enhanced after permeabilization[141]. Interestingly, the same effect of increased antibody recognition with permeabilization and also fixation of the cell was seen, as some fixatives might also simultaneously permeabilize the cell[145]. This could be due to the presence of intracellular targets of transmission blocking antibodies[146]. However, the intracellular antigens are largely shared by the asexual stages[147] and antibody response measured after permeabilization might not be gametocyte specific. Therefore, to explore factors associated to the sexual antibody response, non-fixed and non-permeabilized gametocyte-infected erythrocytes were

used for the analysis of the effect of age, asexual antibody response, GMZ2 vaccination, parasite strain and worm-infection.

No difference in antibody recognition was detected between the laboratory strain (NF54) and a clinical isolate (JH013) from Gabon, though a higher recognition was expected from JH013 as it was collected from the area where the study participants were recruited from. In agreement to this finding, Dinko et. al. reported earlier that plasma antibodies from Ghanian children recognize both 3D7 lab strain and a clinical isolate from Kenya with no significant variation in the detection level[148]. Therefore, the antibody response generated towards gametocyte-infected erythrocyte surfaces might not be strain specific.

Co-infection with other parasites have been shown to affect antibody response towards *Plasmodium*[149] or its candidate vaccine[150]. In this study, using permeabilized and non-permeabilized gametocyte-infected erythrocytes, effect of intestinal helminth infection on the anti-gametocyte antibody response was evaluated in children. When permeabilized parasites were used, low antibody response towards mature gametocytes in *Trichuris trichiura* infected children was observed. Nevertheless, this might not represent suppression of gametocyte specific antibody response by the co-infected parasite, even though it confirms the previous finding shown earlier for the malaria blood-stage asexual candidate vaccine (GMZ2) in the same population where the antibody response to the vaccine was 3.4-fold reduced in *T. trichiura*-infected subjects[150]. Similarly, in a cross-sectional study it was shown that another helminth (*Schistosoma haematobium*) infection resulted in reduced gametocyte specific (Pfs48/45) antibody titers.

The effect of *Trichuris* was not seen in the gametocyte-specific assay using non-permeabilized cells. This might be due to the low level of antibody response to non-permeabilized gametocyte-infected erythrocytes. This could be due to the presence of only small fraction of gametocyte transport proteins that are expressed on the surface of infected erythrocytes. To confirm this, the assay was also run using the

clinical isolate (JH013) and found similar level of low detection. Surprisingly it was observed that *Ascaris*-infected children had an increased anti-gametocyte immune response compared to uninfected subjects. Confirmatory studies with a bigger cohort are sought to corporate these findings performed in small sample cohort. Antibody response to gametocyte-infected erythrocytes and immune modulatory effects of helminths need to be studied further in a big sample size and populations with a different level of malaria transmission and co-endemicity.

4.3 Effect of fluorescent dyes on *in vitro*-differentiated, late-stage *P. falciparum* gametocytes

In addition to the search for transmission blocking antibodies and vaccines, new compounds (drugs) with activity against the transmission stages of *P. falciparum* are needed to sustain malaria control as this aspect is missing in many of the antimalarials. In the third investigation of this PhD study, synthetic dyes that are normally used for parasite/microbial staining are tested for their activity against late-stages of *P. falciparum* gametocytes using ATP-based bioluminescence assay[119]. In this study, the activity of several fluorescent dyes was assessed against this stage. Five active dyes were identified with a submicromolar IC_{50} value against mature gametocytes. Three of the dyes (mitochondrial) were more active than the control drug (methylene blue) which is known to have a gametocytocidal effect[114,115]. These mitochondrial dyes were not toxic when evaluated against HeLa cells[118]. Among all dyes, MitoRed (that belongs to the rhodamine dyes) was the most active and it was also previously recommended for further development based on its high activity against asexual stages and least toxicity against HeLa cells[118], Further toxicity profiles of rhodamine derivatives has been reported in relation to their use for cosmetic industry[151,152], however, additional safety information on MitoRed is missing. Further toxicity studies need to be done for the promising active compounds.

The enhanced gametocytocidal activity of mitochondrial dyes draws attention to consider this target organelle for new antimalarial drug development. Dyes select a specific organelle in an organism for targeted inhibitory activity. Similar to mitochondrial dyes, the antimalarial drug atovaquone targets the mitochondrion of the parasite for its inhibitory activity[153], acting as a potent inhibitor of the cytochrome bc1 complex of the organelle[154]. Despite having similar targets to the gametocytocidal mitochondrial dyes, atovaquone is not active against the late-stage gametocytes[89]. Therefore, mitochondrial dyes might use a different mechanism of action.

In this study, gametocytes from 3D7 lab strain and three clinical isolates from Lambaréné, Gabon were evaluated for their sensitivity to the fluorescent dyes and control drugs. The clinical isolates were less sensitive compared to 3D7 to all fluorescent dyes and comparator drugs except primaquine. This shows the importance of drug screening in different strains including clinical isolates collected from the field where the drugs will be used. Clinical isolates might be exposed to various drugs pressure and it is expected that they might show a different level of sensitivity compared to the lab strains. The difference of drug-sensitivity between the clinical isolates and the laboratory strain is observed more pronounced in case of the artemisinin derivative drugs. One can hypothesize that the exposure is the reason for this, but it is not possible to conclude from this study as the drug exposure profile of participants was not assessed.

The compounds were more active when tested with a prolonged incubation time (48 h) than a short exposure (24 h). This could be due to the fact that after application of the drugs, the metabolic changes are better detected by the reader after extended incubation. A difference in the *in vitro* anti-gametocyte activity of compounds was also observed with a change in the content of culture medium; e.g. artemisinin derivatives (artesunate and DHA) showed 10-fold higher sensitivity in this study when medium is supplemented with serum[119] compared to albumax used in our previous assay[155]. However, artemisinin was not effective[156] or had only modest effect[116,157] in membrane feeding assays, a different and functional way of anti-

gametocyte drug evaluation. Different assay protocols might result in different outcomes. Among the commonly used gametocyte drug-assay protocols, variation is commonly observed in number of gametocytes used, incubation period, parasite strains, content of culture medium and type of readouts that might lead to up to 1000-fold difference in the results of the same compound[89,157–160].

In general, gametocyte drug assays rely on the evaluation of the viability of the parasite. Assays that reflect the parasite biomass are not helpful here since the sexual forms are not multiplying unlike the asexual stages. Though new protocols for gametocyte production[161,162] and drug assay[89,116,159,163–166] are being developed, better standardization and validation of assay procedures and agreement between labs are required to identify compounds with promising activities.

In conclusion, fluorescent dyes, in particular MitoRed, show very high activity against mature gametocytes and should be considered for further evaluation to develop a new gametocytocidal or transmission blocking drugs. Clinical isolates need to be included in the screening of compounds as they can show a different drug sensitivity profile.

4.4 Antimalarial activity of chlorotonil A

In the fourth study[155], after exploring susceptibility of asexual parasites, the activity of a novel compound, chlorotonil A (produced by a Myxobacterium: *Sorangium cellulosum*) was evaluated against mature gametocytes of *P. falciparum*. Myxobacteria are a valuable source of new chemotherapeutic agents. Among these, the genus *Sorangium* is commonly used to produce new compounds of which some are in advanced pre-clinical and clinical development[167]. *S. cellulosum* was the main producer of chlorotonil A, the compound explored here for its activity against *P. falciparum*. Chlorotonil A has many features that entitles it to be a likely lead compound. Multiple antimalarial antibiotics have delayed-effect against *P. falciparum*[168], but chlorotonil A is very fast acting, even after short, transient

exposure. It is effective against all blood stages of the asexual forms at low nanomolar concentrations. As it acts immediately after contact at the early stages of the asexual blood stages (ring and trophozoites), it is quite effective in reducing parasite biomass and may suppress the release of toxins. Unlike the comparator drugs used in this study, chlorotonil A has very rapid onset of action with full activity shown in 1 h of contact with the parasite. This rapid onset is quite helpful for quick reduction of parasitemia in case of severe malaria and for the immediate action of drugs having a short half-life[169]. The compound is active against chloroquine-resistant strains and also clinical isolates isolated from individuals living in an area with high rates of chloroquine[170] and sulfadoxine-pyrimethamine[171] resistance.

The mode of action of chlorotonil A is not known, but it is active only in the chlorine containing pharmacophore. The dehalogenated form is inactive. Activity of chlorotonil A is independent compared to control drugs. This means that it shall have a different mechanism of action than the comparator drugs are performing. Chlorotonil A was also active *in vivo* in the murine *P. bergei* model with low toxicity and with possibility for oral administration though it had poor solubility. The solvent (tetrahydrofuran) used here to dissolve the substance would be toxic to the mice[172], and therefore, the compound was administered to the mice as a powder in peanut butter.

Chlorotonil A has good activity against the late stage gametocytes, which is usually not seen in most commonly used antimalarials. Late-stage gametocytes appear in the circulation after a period of sequestration in the tissues of internal organs and stay in the blood for an extended period even after treatment with the clinically used antimalarials[126,173]. This stage is not easy to target by drugs. Except the 8-amioquinolines (which require pre-testing for G6PD deficiency)[174], all currently available antimalarial drugs including ACT are not effective against the transmission stage of *P. falciparum*, though they are active against the asexual blood stages[89–91], and have a limited activity against the preerythrocytic stages[157,175] and early stages of the gametocytes[114,176,177]. Treatment with ACT has a profound effect in gametocyte carriages compared to other non-ACT antimalarials[126], possibly due to

its effect in reducing asexual biomass and activity against early gametocytes. Chlorotonil A was very active against mature gametocytes in the current assay at low nanomolar level as measured by an ATP-based bioluminescence assay, whereas the activity of artimisinin derivatives to this stage was low as it was also documented elsewhere[89,92,116,157]. A new safe and effective gametocytocidal drug that block the infectivity of *P. falciparum* gametocytes is needed to have an impact on transmission of the parasite and for the elimination of the parasite especially in areas with low endemicity. In the emergence of antimalarial resistance to the currently used first line treatment (artemisinin combination therapy), gametocytocidal drugs would be a valuable tool to contain and eliminate resistant parasites.

Based on its unique characteristics of low toxicity, oral availability, rapid onset of action, activity against all blood stages including the transmission stages of the parasite, chlorotonil A fulfills the criteria to be considered for further assessment from a lead compound toward a drug development candidate as antimalarial and transmission blocking drug. Recently, improved derivatives of this compound have shown enhanced activities against both asexual and mature sexual stages of the parasite (unpublished data).

4.5 Conclusions

After the global decline of malaria prevalence, malaria elimination is on the current agenda of malaria control, and now considered as attainable goal for a number of malaria-endemic regions, as reviewed by Newby et al[178]. As the presence of gametocytes are responsible for the transmission of the parasite from human host to mosquito, these stages of the parasite need to be targeted if elimination or eradication of the disease is the final goal. Gametocytes appear in humans within 7-12 days after initial parasitemia. Human infectious reservoirs, who carry the transmissible gametocyte stages are important targets of successful malaria control strategies. Transmission blocking drugs and vaccines are considered as important tools in limiting the parasite transmission combined with other strategies which are already in use in malaria control programs.

Despite a key role in the transmission of the parasite from human to mosquito, the transmission stages of *P. falciparum* were neglected in the past and little is known about their biology. Here, in this PhD work, lifespan of mature gametocytes, natural antibody recognition and susceptibility to novel compounds were investigated using established standard procedures. Gametocytes survive *in vitro* 16-32 days after reaching maturity, representing the *in vivo* transmission period once appearing in circulation. Erythrocytes infected with *in vitro* differentiated laboratory strain and clinical isolate were observed to be recognized by serum antibodies from malaria exposed children and semi-immune individuals with the antibody response influenced by vaccination and co-infecting helminths. Mitochondrial fluorescent dyes and chlorotonil A were found to be very active against the mature sexual stages of the parasite and are recommended to be considered as lead compounds for further development and validation.

5 Personal contributions

My contribution to the four papers included in this thesis is as follows:

Paper I (published in Malaria Journal): Lifespan of *in vitro* differentiated *Plasmodium falciparum* gametocytes.

- Contributed to the study design,
- Established and performed laboratory experiments,
- Analyzed and interpreted the data set,
- Drafted, revised, and approved the manuscript for publication.

Paper II (published in Malaria Journal): Recognition of *Plasmodium falciparum* mature gametocyte-infected erythrocytes by serum-antibodies of semi-immune adults and malaria exposed children from Gabon.

- Contributed to the study design,

- Established and performed laboratory experiments,
- Analyzed and interpreted the data set,
- Drafted, revised, and approved the manuscript for publication.

Paper III (published in the journal of Antimicrobial Agents and Chemotherapy): Effect of Fluorescent Dyes on *In Vitro*-Differentiated, Late-Stage *Plasmodium falciparum* Gametocytes.

- Contributed to the study design,
- Established gametocyte culture and adapted ATP-based bio-luminescence drug assay,
- Performed laboratory experiments,
- Analyzed and interpreted the data set,
- Drafted, revised, and approved the manuscript for publication.

Paper IV (published in the journal of Antimicrobial Agents and Chemotherapy): Antimalarial Activity of the Myxobacterial Macrolide Chlorotonil A.

- Established gametocyte culture and adapted ATP-based bio-luminescence drug assay,
- Performed laboratory experiments,
- Analyzed and interpreted the data set from gametocyte drug assay,
- Contributed in the draft and reviewed the manuscript for publication.

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8 Curriculum Vitae

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9 Publication list

1. **Tamirat Gebru**, Albert Lalremruata, Peter G. Kremsner, Benjamin Mordmüller, Jana Held. Life-span of *in vitro* differentiated *Plasmodium falciparum* gametocytes. Malar Journal 2017 Aug 11;16(1):330.
2. **Tamirat Gebru**, Anthony Ajua, Michael Theisen, Meral Esen, Ulysse Ateba Ngoa, Saadou Issifou, Ayola A. Adegnika, Peter G. Kremsner, Benjamin Mordmüller, and Jana Held. Recognition of Plasmodium falciparum mature gametocyte-infected erythrocytes by antibodies of semi-immune adults and malaria-exposed children from Gabon. *Malar Journal*. 2017 Apr 26;16(1):176.
3. **Tamirat Gebru**, Benjamin Mordmüller, Jana Held. Effect of fluorescent dyes on *in-vitro* differentiated, late-stage *Plasmodium falciparum* gametocytes. *Antimicrob. Agents Chemother*, 2014 58(12):7398-404.
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9. **Tamirat Gebru Woldearegai**, Kremsner PG, Kun JFJ, Mordmüller B. Plasmodium vivax malaria in Duffy-negative individuals from Ethiopia. Trans R Soc Trop Med Hyg, 2013, 107(5):328-31.
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10 Annexes: Publications I - IV

Annex I: Life-span of *in vitro* differentiated *Plasmodium falciparum* gametocytes.

Annex II: Recognition of *Plasmodium falciparum* mature gametocyte-infected erythrocytes by serum-antibodies of semi-immune adults and malaria exposed children from Gabon.

Annex III: Effect of fluorescent dyes on *in vitro*-differentiated, late-stage *P. falciparum* gametocytes.

Annex IV: Antimalarial activity of the Myxobacterial Macrolide chlorotonil A.

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Malaria Journal

RESEARCH Open Access

Life-span of in vitro differentiated *Plasmodium falciparum* gametocytes

Tamirat Gebru[1,2,3], Albert Lalremruata[1,2], Peter G. Kremsner[1,2], Benjamin Mordmüller[1,2] and Jana Held[1,2*]

Abstract

Background: The sexual stages (gametocytes) of *Plasmodium falciparum* do not directly contribute to the pathology of malaria but are essential for transmission of the parasite from the human host to the mosquito. Mature gametocytes circulate in infected human blood for several days and their circulation time has been modelled mathematically from data of previous in vivo studies. This is the first time that longevity of gametocytes is studied experimentally in vitro.

Methods: The in vitro longevity of *P. falciparum* gametocytes of 1 clinical isolate and 2 laboratory strains was assessed by three different methods: microscopy, flow cytometry and reverse transcription quantitative real-time PCR (RT-qPCR). Additionally, the rate of gametocytogenesis of the used *P. falciparum* strains was compared.

Results: The maximum in vitro lifespan *of P. falciparum* gametocytes reached almost 2 months (49 days by flow cytometry, 46 days by microscopy, and at least 52 days by RT-qPCR) from the starting day of gametocyte culture to death of last parasite in the tested strains with an average 50% survival rate of 6.5, 2.6 and 3.5 days, respectively. Peak gametocytaemia was observed on average 19 days after initiation of gametocyte culture followed by a steady decline due to natural decay of the parasites. The rate of gametocytogenesis was highest in the NF54 strain.

Conclusions: *Plasmodium falciparum* mature gametocytes can survive up to 16–32 days (at least 14 days for mature male gametocytes) in vitro in absence of the influence of host factors. This confirms experimentally a previous modelling estimate that used molecular tools for gametocyte detection in treated patients. The survival time might reflect the time the parasite can be transmitted to the mosquito after clearance of asexual parasites. These results underline the importance of efficient transmission blocking agents in the fight against malaria.

Keywords: Malaria, Clinical isolate, Gametocytogenesis, Gametocyte viability, Gametocyte circulation time, Gametocyte longevity, Exflagellation

Background

Malaria is one of the most important infectious diseases, leading to approximately half a million annual deaths globally [1]. Among the different *Plasmodium* species that cause disease in humans, *Plasmodium falciparum* is the most virulent form and is responsible for most of the malaria morbidity and almost all malaria mortality in sub-Saharan Africa. The parasite is transmitted from the human host to the mosquito through mature sexual stages of the parasite (gametocytes) that circulate in peripheral blood. In the life cycle of *P. falciparum*, a certain percentage of asexual blood-stage parasites develop stochastically to gametocytes [2]. The immature forms (stage I–IV) sequester in internal organs [3, 4] for a maximum of 12 days [2], before developing to mature gametocytes (stage V) that appear in the peripheral circulation and can be taken up by a blood-sucking female *Anopheles* mosquito. Once in the mosquito's midgut the sexual cycle continues leading to infective sporozoites in the salivary glands that render the mosquito infective to the next person it will bite.

As gametocytes are responsible for bridging the human/vector transmission of the parasite, studying their biology, development and viability or life span is of

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paramount importance for designing an effective transmission-blocking strategy. Neglected in the past, this field is now getting more attention with a revival of interest for the regional elimination and worldwide eradication of malaria [5, 6].

It has been shown earlier that even sub-microscopic densities of gametocytes in humans can establish mosquito infection [7, 8]. The chance of transmission of the parasite to the mosquito increases with the circulation time of the parasite [9, 10]. Most anti-malarials are not effective against the transmission stages and only act indirectly by reduction of asexual stages or immature gametocytes [11, 12]. After an effective clearance of asexual parasites either by drugs or the immune response, the duration of gametocyte carriage depends on the sequestration and subsequent circulation time of gametocytes in the blood stream before the natural decay of the parasite happens. Once in the circulation, they remain infective for several days and may be even weeks, able to continue the transmission [9], but the exact time span is not known. Mathematical modelling in vivo after clearance of asexual stages by anti-malarial treatment in patients and subsequent detection by microscopy or molecular methods was so far the main method by which longevity of gametocytes was estimated. In these modelling approaches, assumptions were made considering gametocyte conversion probability, sequestration periods and decay of gametocytes [2, 10, 13].

Even though transmission of the parasite to the mosquito is associated with circulation time [14, 15] and density of mature gametocytes [16, 17] in the infected person, this parameter is not well studied as it is technically difficult to measure gametocyte longevity in vivo. The density of gametocytes in the circulation is often below the detection limit of commonly used methods and to follow in vivo a single mature gametocyte when released from the site of sequestration to the peripheral circulation has never been achieved. However, in vitro there is the possibility to follow gametocytes for their life span and evaluate their natural decay. In this study, the longevity of gametocytes was assessed in vitro by three different methods: microscopy, flow cytometry and reverse transcription quantitative real-time polymerase chain reaction (RT qPCR). In addition, the rate of gametocytogenesis was evaluated in the different *P. falciparum* strains by microscopy, to characterize variation in gametocyte development.

Methods

Gametocyte culture

Gametocyte culture was initiated from an asexual culture of 1 clinical isolate (JH013) and 2 laboratory strains (3D7 and NF54) of *P. falciparum.* The clinical isolate (JH013) was selected from a previous study conducted in Lambaréné, Gabon [18]. The isolate was collected from a patient presenting with *P. falciparum* mono-infection and preserved in glycerolyte at −196 °C until culturing. JH013 and the *P. falciparum* laboratory strains 3D7 (Malaria Research and Reference Reagent Resource (ATCC, Virginia, USA) and NF54, isolated from a volunteer inoculated with PfSPZ Challenge for controlled human malaria infection (Sanaria Inc, Rockville, MD, USA) [19], were kept in asexual culture at 5% haematocrit in RPMI 1640 supplemented with 25 mM HEPES, 28 mM NaHCO3, 50 µg/mL gentamicin, 0.5% w/v Albumax II, 2.4 mM L-glutamine, and 0.14 mM hypoxanthine at 5% CO_2 and 5% O_2. Naïve serum (5%) was added for the cultivation of the asexual parasites of the clinical isolate. The medium was replaced every 24 h.

Gametocyte culture was performed as described before [11] with modifications: the culture was started from asexual parasites that were kept in a continuous culture with sorbitol synchronization twice weekly. The same culture medium was used for the gametocyte development as for the asexual culture except the addition of 5% naïve serum for both clinical and laboratory strains. The culture was initiated with 9% haematocrit and 0.5% parasitaemia in 20 mL total volume and was kept in a 5% O_2/CO_2 atmosphere at 37 °C with daily change of medium without parasite dilution. In the second week, the volume of medium was doubled and thereby the haematocrit reduced to 4.5%. On days 11–15 and from day 19 on every 5 days for 2 consecutive days, cultures were treated with 50 mM *N*-acetyl-D-glucosamine (MP Biomedicals GmbH, Santa Ana, CA, USA) to remove or suppress asexual parasites to avoid emergence of new gametocytes. The culture was followed until all gametocytes were judged as dead with daily change of complete medium without any purification or enrichment step.

Assessment of gametocyte longevity

Microscopy, flow cytometry and RNA-based RT-qPCR were used to assess the longevity of gametocytes of different strains of *P. falciparum*. Day 0 is the starting day of gametocyte culture and the culture was followed until the last positive signal representing a gametocyte detected by the respective technique. Longevity after reaching maturity represents the estimated in vivo circulation time as only mature gametocytes circulate freely in the blood. This was calculated by subtracting the number of days needed to reach peak gametocytaemia (see day of peak gametocytaemia in Table 1). On the day of peak gametocytaemia all gametocytes in the respective culture reached maturity (stage V), as checked by microscopy. The 50 and 10% survival rate of mature gametocytes was also calculated starting with the day of peak parasitaemia.

Table 1 Summary of in vitro gametocyte longevity data evaluated by three viability assays from cultures of one *Plasmodium falciparum* clinical isolate and two laboratory strains

Plasmodium falciparum	Gametocyte longevity in days: mean (range)			
	Microscopy	Flow cytometry	RT-qPCR	
			Pfs25	PF14_0367[a]
3D7	–	46 (43–49)	–	–
NF54	46	49 (48–50)	>52	>52
JH013	39 (38–40)	45 (43–49)	>52	46
Average number of days of gametocyte culture to reach peak gametocytaemia (maturation)[c]	20.5	16	19	19
Mature gametocytes 50% survival rate (days)	2.6	6.5	4.5	2.5
Mature gametocytes 10% survival rate (days)	10	12	13.3	10.4
Mature gametocyte circulation time (days)[b]	18–26	16–24	31–32	26–31

[a] Mid and mature gametocyte marker [23]

[b] Circulation time is calculated by subtracting the number of days needed to reach peak gametocytaemia

[c] On the day of peak gametocytaemia all gametocytes in the respective culture have reached maturity (stage V)

After initiation of gametocytogenesis a sample was taken on days 3, 5, 8, 10, 12, 18, 20, 22, 27, 33, 35, 38, 40, 43, 46, 49, and 52 and analysed by microscopy, flow cytometry and RTqPCR.

Assessment of gametocyte longevity by microscopy

Viability of gametocytes was assessed microscopically in two parasite strains, the NF54 laboratory strain and JH013 clinical isolate by evaluating the morphology of parasites in a Giemsa-stained thin blood smear. Gametocytes were classified either as viable (normal intact morphology of mature gametocytes) or dead (deformed cells with a decrease in width, a thin needle-like appearance or degraded cytoplasmic content). Parasitaemia of mature gametocytes ((number of mature gametocyte infected erythrocytes/number of erythrocytes) × 100) was determined by two trained and qualified readers blinded to each other and the slide identification (ID). Samples were declared negative if no intact and mature gametocytes were seen in 20,000 erythrocytes. In case of discordant results a third reading was performed.

To verify viability, an exflagellation assay (incubation for 20 min in exflagellation medium supplemented with xanthurenic acid as described earlier [20]) was additionally performed on days 17, 18, 19, 20, 22, 27, 33, 35, and 38 to assess exflagellation of male gametocytes in NF54 and JH013.

Assessment of gametocyte longevity by flow cytometry

Viability and life span of gametocytes of *P. falciparum* isolates (3D7, NF54, JH013) was assessed also by hydroethidine (HE) staining evaluated by flow cytometry to measure metabolic activity. HE staining has been used earlier in a flow cytometric gametocyte drug assay [21]. HE staining (Polysciences Europe GmbH, Eppelheim, Germany) was performed at a final concentration of 50 µg/mL in medium for 20 min at 37 °C in the dark before analysis by flow cytometry (BD FACS Canto II) with the FACSDiva software version 6.1.2 (BD Biosciences, San Jose, USA). Metabolically active parasites convert HE to ethidium that interacts with the parasites' nucleic acids showing red fluorescence emission when excited (see panel B of Additional file 1: Figure S1). The result is expressed as the percentage of positive fluorescent cells (PPFC) in 50,000 erythrocytes. As a negative control, uninfected erythrocytes (incubated with medium in the same way as the culture) were used for gating (see panel A of Additional file 1: Figure S1). The gametocyte culture was followed until no positive signal above the negative control could be seen after HE staining. After day 15, a control slide was additionally done and read by microscopy to exclude presence of asexual parasites.

Assessment of gametocyte longevity by reverse transcription quantitative PCR

RNA extraction

On the sampling days, 40 µL of erythrocytes was taken out of the culture for molecular detection of gametocytes and was added to 100 µL Trizol reagent (Life Technologies GmbH, Darmstadt, Germany) for RNA stabilization and stored at −80 °C until RNA extraction. RNA purification was done automated on the QIAsymphony SP (QIAGEN GmbH, Hilden, Germany) using the QIAsymphony RNA Kit (QIAGEN GmbH, Hilden, Germany) and RNA CT 400 protocol provided with the instrument. Before purification, frozen samples were thawed and vortexed followed by an incubation at room temperature

(RT) for 5 min. Then 20 µL chloroform (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added to the homogenized mixture and shaken vigorously for 15 s and incubated for 3 min at RT before centrifugation at 4 °C for 15 min at 11,500 rpm. The upper aqueous phase was transferred to 2-mL sample tubes (Sarstedt, Numbrecht, Germany) and the volume adjusted to 400 µL with buffer RTL Plus (Qiagen) and loaded onto the QIAsymphony SP. All samples were eluted in 100 µL elution buffer in 96-well plates (Qiagen). Gametocyte density calibration standards were generated from tenfold serial dilution of gametocytes (10^7–10) in 40 µL of erythrocytes.

Molecular detection of *Plasmodium falciparum* gametocytes by RT-qPCR

Plasmodium falciparum gametocytes (NF54 and JH013) were quantified by RT-qPCR analysed by the LightCycler® 480 Instrument II (Roche Diagnostics, Mannheim, Germany). Gametocyte-specific targets published earlier were used to measure transcript levels of *Pfs25* mRNA [22] and Pf14_0367 [23] with modifications. Custom one-step RT-qPCR assay was performed using Taqman RNA-to-C_T™ 1-Step kit (Thermo Fisher Scientific). The reaction consisted of 1 × Taqman RT-qPCR mix, 1 × Taqman enzyme mix, 150 nM probe, and 400 nM of each primer and 2.5 µL of RNA extract. The thermal conditions consisted of reverse transcription (48 °C for 20 min), enzyme activation (96 °C for 10 min), and two-temperature cycling step (95 °C for 15 s, 62 °C for 1 min, for 45 cycles). The amplification curves were assessed for true amplification and the quantification cycle value (C_q) was automatically calculated by the second derivative maximum method integrated in the LightCycler 480 software (version 1.5.1.62). The positivity of the amplification was defined by C_q value less than 40 in at least two technical replicates. The gametocyte density was derived from extrapolation of the C_q value obtained from the standard curve (Additional file 2: Figure S2). Primer and probe sequences are listed in Additional file 3: Table S1 and PCR conditions in Additional file 4: Table S2. All samples including the gametocyte standards were tested in triplicate in the presence of positive and negative (non-template and non-RT) controls.

In vitro gametocytogenesis of different strains of *Plasmodium falciparum*

In order to compare the rate of gametocytogenesis of different strains of *P. falciparum*, the same culture conditions were used for gametocytes as described above. After removal of asexual parasites by the application of *N*-acetyl-D-glucosamine between days 11 and 15 of the gametocyte development, the culture was purified first by NicoPrep 1.077 cushions (AXIS-SHIELD PoC AS, Oslo, Norway) and later by LD-MACS magnetic columns (Miltenyi Biotec, Gladbach, Germany) resulting in highly purified (above 95% stage V) gametocytes (Additional file 5: Figure S3). This purification procedure was not applied to the cultures used for the longevity assay. Parasites were kept at 37 °C during the whole procedure to avoid exflagellation, as described earlier [24]. The efficiency of gametocytogenesis of three different strains of *P. falciparum* was assessed: the laboratory strains 3D7 and NF54 and 1 clinical isolate (JH013) from Lambaréné, Gabon in complete culture medium in the presence or absence of 5% serum. The parasites of all the three strains were cultured in 75-cm^2 flasks with a starting volume of 20 mL gametocyte culture that contained 2 mL erythrocyte pellet. Gametocyte yield was determined after 15 days of culture by counting the purified stage V gametocytes using a Neubauer improved cell counting chamber. The tests were done three times in duplicate for each strain and the mean was determined.

Statistical analysis

Descriptive statistics were calculated with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) for determining mean, percentages, standard deviation and plotting of graphs. Excel 2013 and GraphPad Prism 6 was used for the calculation of gametocytaemia of microscopic and flow cytometric data. A sample was declared positive by flow cytometry if the percentage gated cells positive for HE was above the negative control (erythrocytes cultured in complete culture medium in the incubator). PCR data were analysed by Excel and gametocytaemia was extrapolated with the help of a gametocyte standard curve with known parasitaemia. To estimate the 50 and 10% survival rate of gametocytes, Excel was used to develop a linear regression equation using the log-transformed data of the different viability assays starting on the day of highest parasitaemia. Mann–Whitney test and Wilcoxon matched-pairs signed rank test were used in GraphPad Prism 6, for non-parametric analysis of the difference in the gametocytogenesis rate among the different isolates in the presence or absence of serum.

Results

Longevity of gametocytes was assessed by three different viability methods.

Microscopic reading

The gametocytes appeared viable with intact cytoplasm for several weeks after reaching stage V (Fig. 1). Live gametocytes were detected on average for a maximum of 39 and 46 days after initiation of gametocytogenesis in JH013 and NF54, respectively. The longevity after reaching maturity was 18–26 days. The mean 50

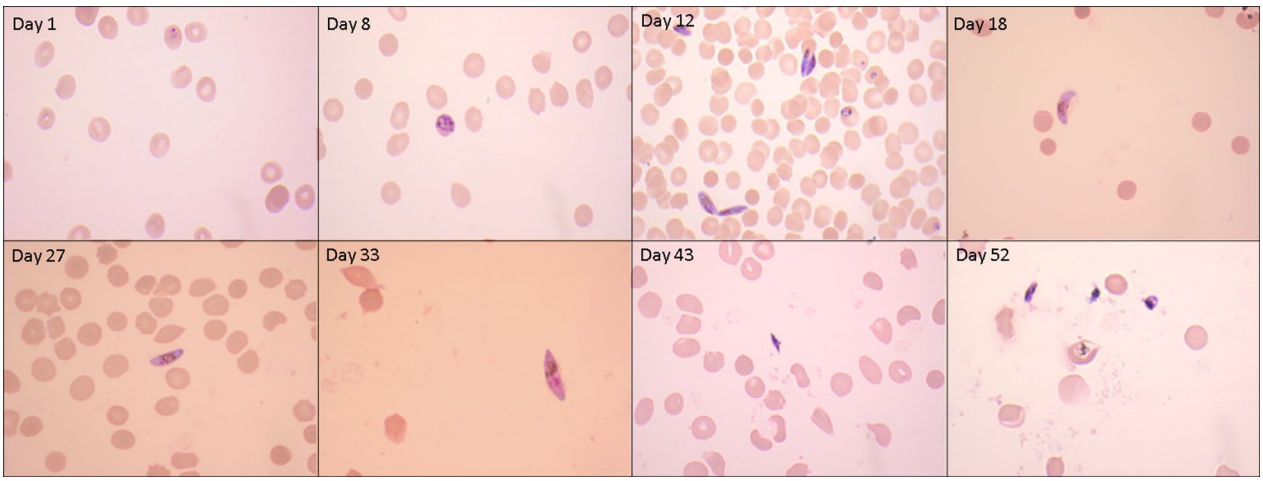


Fig. 1 Microphotograph of Giemsa-stained thin smears of *Plasmodium falciparum* gametocyte (NF54) culture on different days of gametocyte development (magnification ×1000). *N*-Acetyl-glucosamine has been added on days 11–15 and from day 19 on every 5 days for 2 consecutive days

and 10% survival rate were 2.6 and 10 days, respectively. The highest number of gametocytes can be seen on days 20–22. The percentage of live, mature, gametocyte-infected erythrocytes for the different days of the gametocyte culture evaluated by the blinded readers is shown in Fig. 2. The last viable gametocyte was seen on days 40 and 46 for JH013 and NF54 *P. falciparum* strain, respectively.

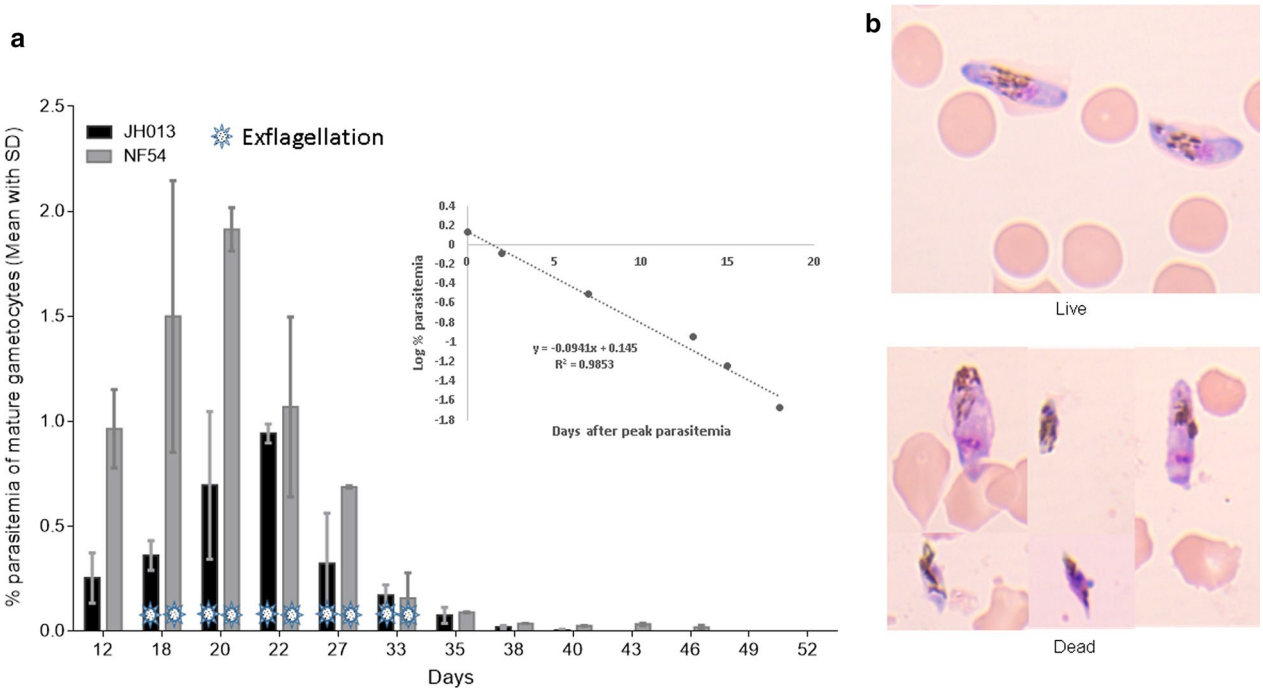


Fig. 2 Longevity of gametocytes evaluated microscopically by morphological assessment. Viability of live gametocytes (1 clinical isolate and 1 NF54 laboratory strain) was assessed by microscopy. **a** The percentage of mature gametocytes in erythrocytes (parasitaemia) from days 12 to 52 of gametocyte culture. *Values* indicate the mean percentage (and SD) of live gametocytes counted by two qualified microscopists for the separate experiments. The days on which exflagellation was detected is indicated by a *white/blue star* at the *bottom of the bar graph*. **a** Also shows a scatter chart with a trend line and equation made using log.transformed gametocytaemia data from microscopy displayed on the y-axis of the graph. **b** Giemsa-stained thin smears of gametocyte culture with live and dead gametocytes. *Log.* logarithmic, *SD* standard deviation

Exflagellation assay

Exflagellation of male gametocytes was observed reproducibly until day 33, whereas from day 35 on no exflagellated males could be detected (Fig. 2). The experiment was repeated three times.

Flow cytometry

Analysis of flow cytometry data measuring HE staining of *P. falciparum* gametocytes showed a longevity of 43–49 days in the tested clinical isolate and laboratory strains with an average 50% survival rate of 6.5 days, 10% survival rate of 12 days and peak gametocytaemia on day 16, following the removal of asexual parasites (Fig. 3). On day 12 there were still asexual parasites left in the culture that were also stained by HE. Therefore, these data are excluded from Fig. 3 to avoid confusion. The estimated circulation time (longevity after reaching stage V) was 16–24 days.

Molecular detection of *Plasmodium falciparum* gametocytes by RT-qPCR

RT-qPCR was used to assess a marker for mature (Pfs25 [22]) and a marker for both middle and mature stage (PF14_0367) [23]) gametocytes. The peak gametocytaemia was higher in NF54 gametocytes compared to JH013 (Fig. 4). Gametocyte longevity was longer when evaluated by PCR compared to microscopy and flow cytometry. For JH013, the PF14_0367 signal was detected until day 46 and the Pfs25 signal until day 52. Both markers were detectable until day 52 in NF54. The mature gametocyte 50 and 10% survival rate were on average 3.5 and 12 days, respectively. Peak parasitaemia was recorded on days 18 and 20 and the estimated circulation time was 31 and 26–32 days for NF54 and JH013, respectively.

In vitro gametocytogenesis

Rate of gametocytogenesis was assessed in three different parasite lines (3D7, NF54 and JH013) of *P. falciparum* in vitro after purifying mature gametocytes (Additional file 5: Figure S3). The highest yield of gametocytes was recorded in NF54 followed by the clinical isolate (JH013). The pattern of parasitaemia peak and decline was similar in all gametocyte cultures. Serum supplementation enhanced the gametocyte yield in all strains (Fig. 5). There was no statistically significant difference in gametocyte yield among the different strains and between the cultures with or without serum supplementation.

A summary of the longevity data evaluated by the different methods is given in Table 1, showing that analysis by RTqPCR was the most sensitive method, revealing the

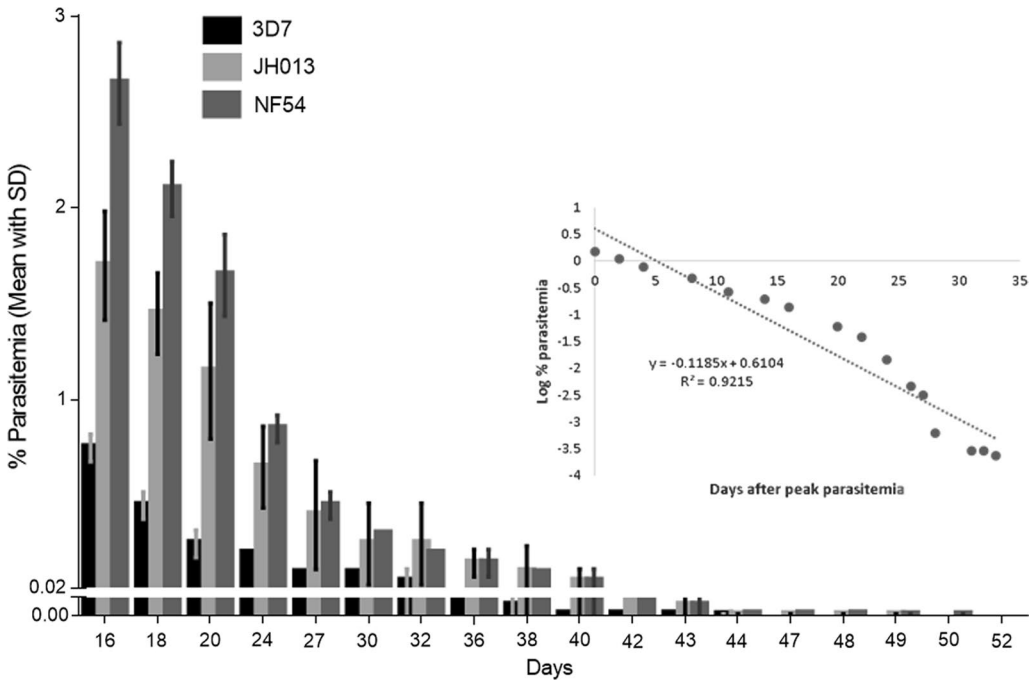


Fig. 3 In vitro longevity of *Plasmodium falciparum* gametocytes of 3D7, NF54 and one clinical isolate (JH013) of hydroethidine (HE) stained and analysed by flow cytometry. Mean (and SD) maximum longevity of gametocytes is shown in days. Viability of gametocytes is assessed by flow cytometry measuring the percentage of positive fluorescent cells (PPFC) of 50,000 erythrocytes gated against negative control (uninfected erythrocytes stained by HE). All strains were tested at least three times. The *figure* also shows a scatter chart with a trend line and equation made using log. transformed gametocytaemia data from flow cytometry displayed on the y-axis of the graph. *Log.* logarithmic, *SD* standard deviation

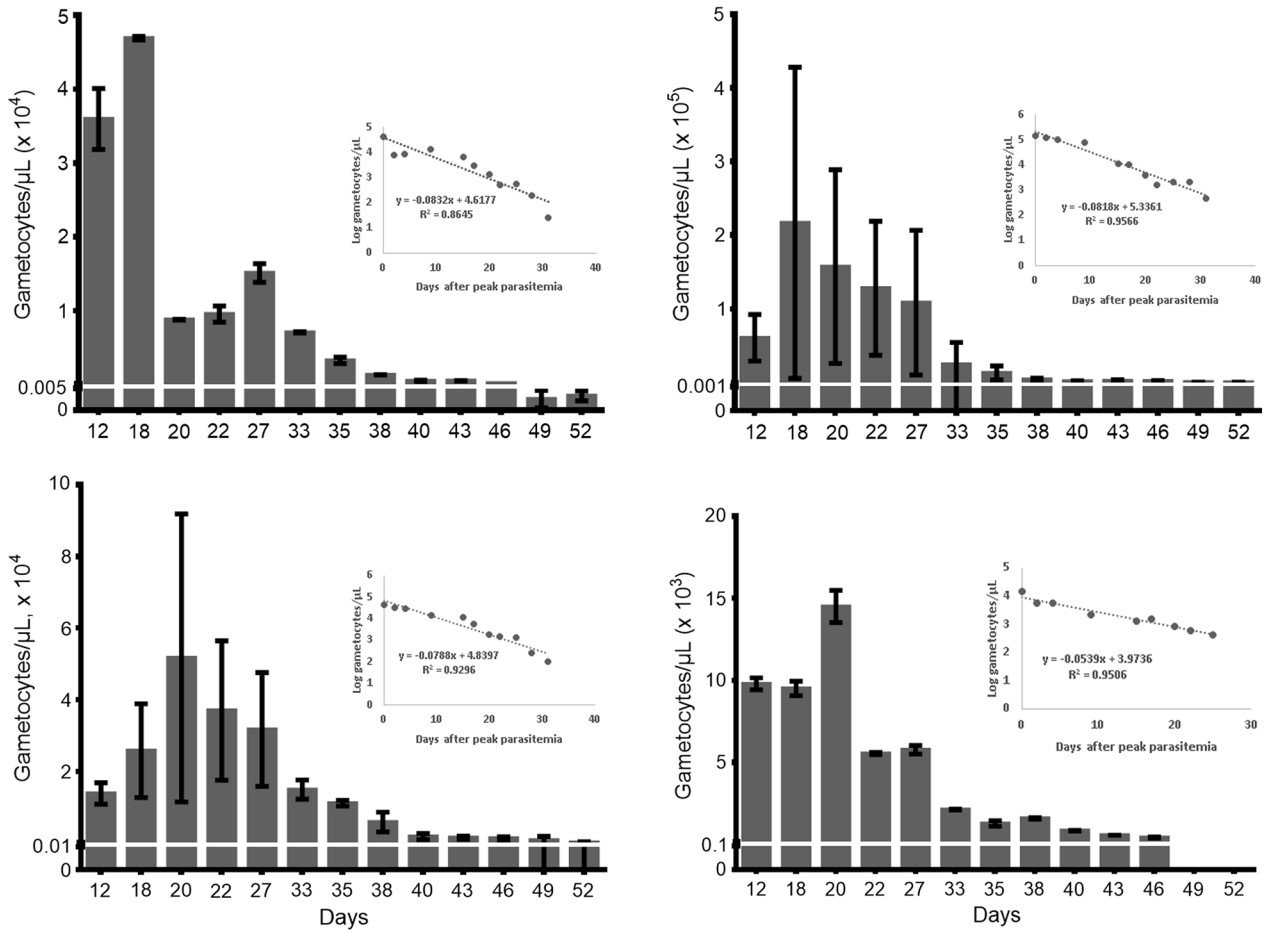
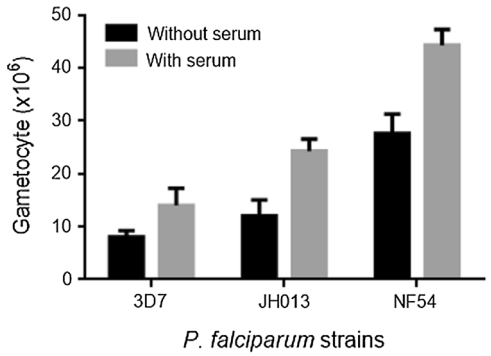


Fig. 4 Number of gametocytes/µL of JH013 and NF54 on the different days after culture initiation measured by RT-qPCR. Quantification of test sample was done with the help of a standard curve. The previously reported gametocyte markers (PF14_0367 [23] and Pfs25 [22]) were used. Y axis shows the mean and SD. *SD* standard deviation. The figure also shows a scatter chart with a trend line and equation made using log.-transformed gametocytaemia data displayed on the y-axis of the graph. *Log.* logarithmic

longest life span for gametocytes, followed by analysis by flow cytometry and microscopy. The strain NF54 had the longest life span by all methods and it was more prone for gametocyte commitment compared to the other two strains tested.

Discussion

Density, sex ratio and life span of stage-V gametocytes of *P. falciparum* are the most important factors determining the probability of transmission to the mosquito. The maximum life span of gametocytes was explored here for the first time in vitro. Viability of gametocytes ranged from 39 to 52 days (16–32 days after reaching maturity considering peak gametocytaemia as the day of maturation) depending on the different measurement methods used, with little variability between the different parasite strains (Table 1). This time-span corresponds to the in vivo gametocyte carriage or the sum of sequestration and circulation time of the parasite in an infected person. Mature gametocytes (stage V), that freely circulate in the peripheral blood, are developed latest after 12 days [2]. Based on the ex-flagellation assay, at least 14 days of longevity was recorded for mature male gametocytes. By the protocol used here, gametocytes are generated during the first few cycles of asexual culture and therefore age of gametocytes or the day of maturity is not the same for the whole gametocyte population of one culture. However, it was observed that all gametocytes matured at the day of peak gametocytaemia, and therefore gametocyte peak was used as the day of gametocyte maturity to calculate longevity of mature gametocytes (estimated in vivo circulation time). When subtracting this maturation time, the circulation time estimated here is 16–32 days. It was observed that some gametocytes were already mature



In vitro gametocytogenesis (Gametocytes x 10^6)		
P. falciparum	Without serum Mean (SD)	With serum Mean (SD)
3D7	8 (1.2)	14 (3.3)
Clinical isolate (JH013)	12 (3.0)	24 (2.3)
NF54	28 (3.7)	44 (3.0)

Fig. 5 In vitro gametocytogenesis of *Plasmodium falciparum* 3D7, NF54 and JH013 cultured in complete medium in presence and absence of serum. The results shown represent the mean of at least three experiments. Mean and standard deviations are presented. The gametocyte yield was measured on day 15 after start of gametocyte culture with a starting volume of 20 mL medium with 2 mL erythrocyte pellet in 75-cm^2 flasks. *SD* standard deviation

before peak gametocytaemia and the real life span or circulation time could be a bit longer than provided. In previous in vivo studies that assessed gametocytaemia by microscopy, the circulation time was estimated to be at maximum 24 [9] and 22.2 days (with mean circulation time 6.4 days) [2]. The differences in longevity to the present study likely result from the influence of host factors in in vivo studies but also from assumptions of mathematical modelling that may be incorrect, as well as the limit of detection of gametocytaemia by microscopy. It was observed in the here-presented in vitro assay that peak gametocytaemia is not yet reached on day 15, indicating the presence of some immature stages on this day. Commitment of gametocytes occurs not only on the first day of starting gametocyte culture but during the first 2–3 cycles of asexual multiplication. This might slightly affect the longevity estimate that is provided here, as some gametocytes might be some days younger than other gametocytes. To account for this the longevity of mature gametocytes (estimated circulation time) was calculated from day of peak gametocytaemia measured by the different approaches. The 50 and 10% survival rate is also calculated starting from day of peak-parasitaemia.

Another study estimated the maximum duration of gametocyte carriage (corresponding to the sum of in vivo sequestration and circulation time) to be 55 days based on molecular detection of Pfs25 and mathematical modelling [10]. Longevity of gametocytes reported in this study is similar with the current in vitro data presented here. Gametocytes were detected up to 52 days in culture by PCR when analysing the female gametocyte specific marker Pfs25. For PF14_0367 the signal was slightly weaker and in the tested clinical isolate (JH013) it was no longer detectable after day 46. The difference of the two markers is most probably due to the higher copy number and expression of Pfs25 at the gametocyte stage compared to PF14_0367 [23]. Slight differences in longevity of male and female gametocytes could also be the reason; however, no difference was seen in an earlier publication between male and female gametocyte longevity or mortality in vivo [9].

If these in vitro reared gametocytes are actually able to infect mosquitoes cannot be answered by this approach. However, in the exflagellation assay presented here, the male gametocytes were able to exflagellate for at least 2 weeks after reaching maturity. Similarly, Smalley and Sinden have reported that exflagellation of microgametes and infectivity to mosquitoes can take place for 3 weeks [9]. In line with this, a recent report showed in vivo the persistence of gametocytaemia following treatment with atovaquone–proguanil. This was later interrupted by primaquine treatment after 21 days of consistent gametocyte detection in a participant in a controlled human malaria infection trial [25].

One has to be careful to translate in vitro data to the in vivo situation as there are several contradictory factors influencing the longevity of gametocytes in vivo. On the one hand, the immune response of the host is negatively influencing longevity of the parasite, whereas on the other hand, the nutrition status might be more favourable for the parasite in the human host than in the culture flask. The in vitro culture shows longevity of gametocytes excluding the host factors.

Even though gametocytes have a long life-span in vitro, their half-life (50% survival rate) was short (2.6–6.5 days), similar to the result shown in a previous in vivo study (2.4 days) [9]. The peak gametocytaemia is followed by a steady decline in parasitaemia with few gametocytes remaining for several weeks, and around 10% surviving for 10–12 days after peak gametocytaemia. After day 40, on average less than 5% of the gametocytes remained viable. Studies have shown that if gametocytes remain viable, a small number of gametocytes (sub-microscopic level) is sufficient for transmission [7, 8]. These findings underline the importance of development of efficacious gametocidal drugs and its timely usage, especially if elimination and even eradication of malaria is the aim.

In this study, the NF54 strain of *P. falciparum* used for experimental human infection was more prone to develop into gametocytes followed by JH013 and 3D7. Similar to this report, it was shown earlier that the

gametocyte yield varies between different parasite lines [26]. Many factors are considered to influence sexual commitment of the parasite including genetic variation, environmental factors [27] and long-term in vitro culture [26, 28]. The same type of medium, erythrocytes and serum (in those cultures supplemented with serum) was used to avoid modulation of gametocytogenesis by culture conditions [28].

Serum supplementation in the gametocyte culture improved the gametocyte yield in all three strains of *P. falciparum* tested. It was previously reported that serum taken from infected individuals increased the sexual conversion rate of *P. falciparum* [29]. Here it is shown that supplementation of serum from non-infected person also improves the gametocyte yield in vitro. The enhanced yield of gametocytes could also be due to an increased multiplication of the asexual parasites of the gametocyte culture when serum is used as reported earlier [29].

Conclusions

Transmission of *P. falciparum* to mosquito depends on mature viable gametocytes in the peripheral blood of the host [30]. The in vitro life span (estimated in vivo circulation time) of mature gametocytes was 2–4 weeks in this study. These findings may help to design interventions to prevent transmission as part of elimination programs and containment of resistant *P. falciparum* isolates.

Additional files

Additional file 1: Figure S1. Flow cytometry analysis of *Plasmodium falciparum* (JH013) parasites. Samples were stained with hydroethidine (HE) and analysed by flow cytometry. Panel A shows the negative control. The erythrocytes are cultured in complete culture medium and stained with HE. Panel B shows the gating strategy and percent-infected erythrocytes of a sample taken from gametocyte infected erythrocytes (from gametocyte culture).

Additional file 2: Figure S2. Standard curves used for gametocyte quantification by RTqPCR. Dilution series of in vitro cultivated gametocytes were analysed by PF14_0367 and Pfs25. Standard curves were generated performing linear regression using the Log_{10} transformed number of gametocytes and the mean and SEM of the quantification cycles (C_qs) of triplicates. SEM: standard error of the mean.

Additional file 3: Table S1. Primer and probe sequences used in gametocyte-specific RTqPCR.

Additional file 4: Table S2. TaqMan One-Step Real-Time RT-PCR (RNA-to-Ct™ 1-Step Kit) PCR mix and cycling conditions.

Additional file 5: Figure S3. Microphotograph of Giemsa-stained thin smears showing gametocyte culture on day 15 before (A) and after (B) purification (magnification ×1000). Uninfected erythrocytes and remaining asexual parasites were removed by the two-step purifications for the in vitro measurement of gametocytogenesis of 3D7, NF54 and one clinical isolate (JH013).

Abbreviations

Cq: quantification cycle; HE: hydroethidine; ID: identification document; PCR: polymerase chain reaction; PPFC: percentage of positive fluorescent cells; RT-qPCR: reverse transcription quantitative real-time PCR; RT: room temperature.

Authors' contributions

The study was conceived and experiments designed by TG, AL, BM, and JH. TG performed the experiments and TG, BM and JH analysed the data. TG, AL, BM, and JH contributed reagents/materials/analysis tools and wrote the paper. All authors revised the first and the last draft of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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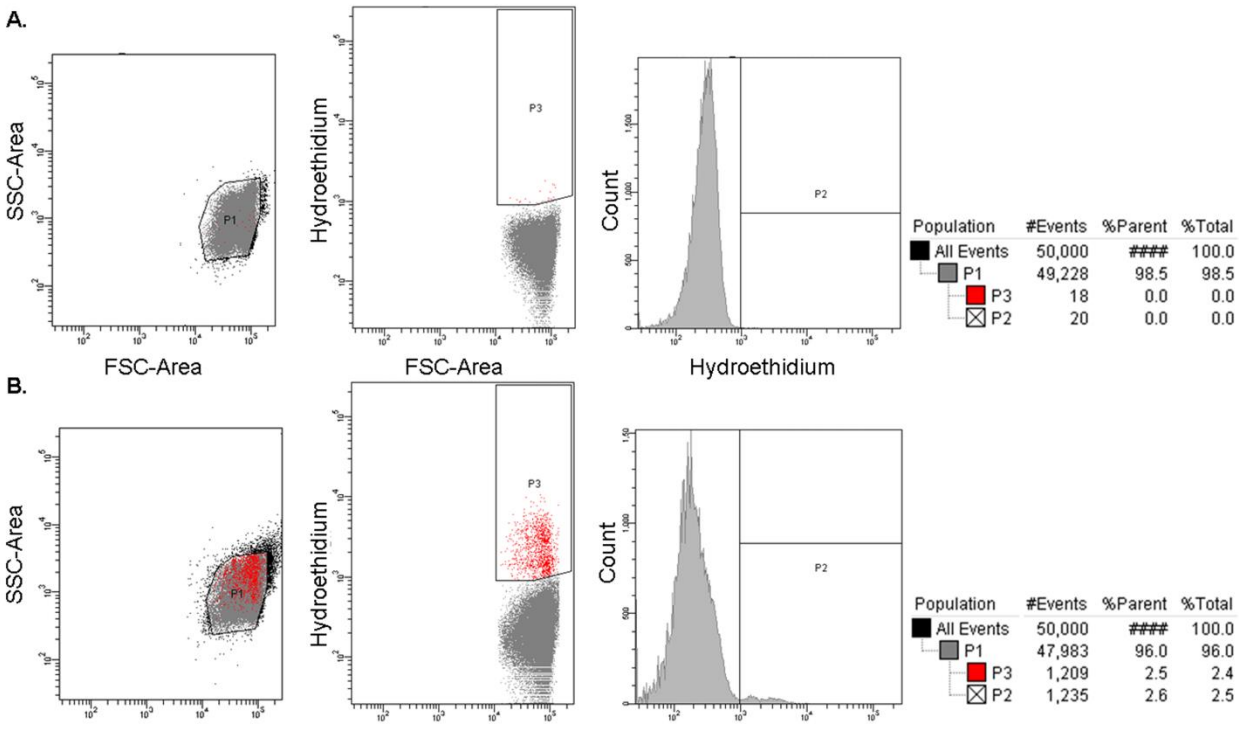
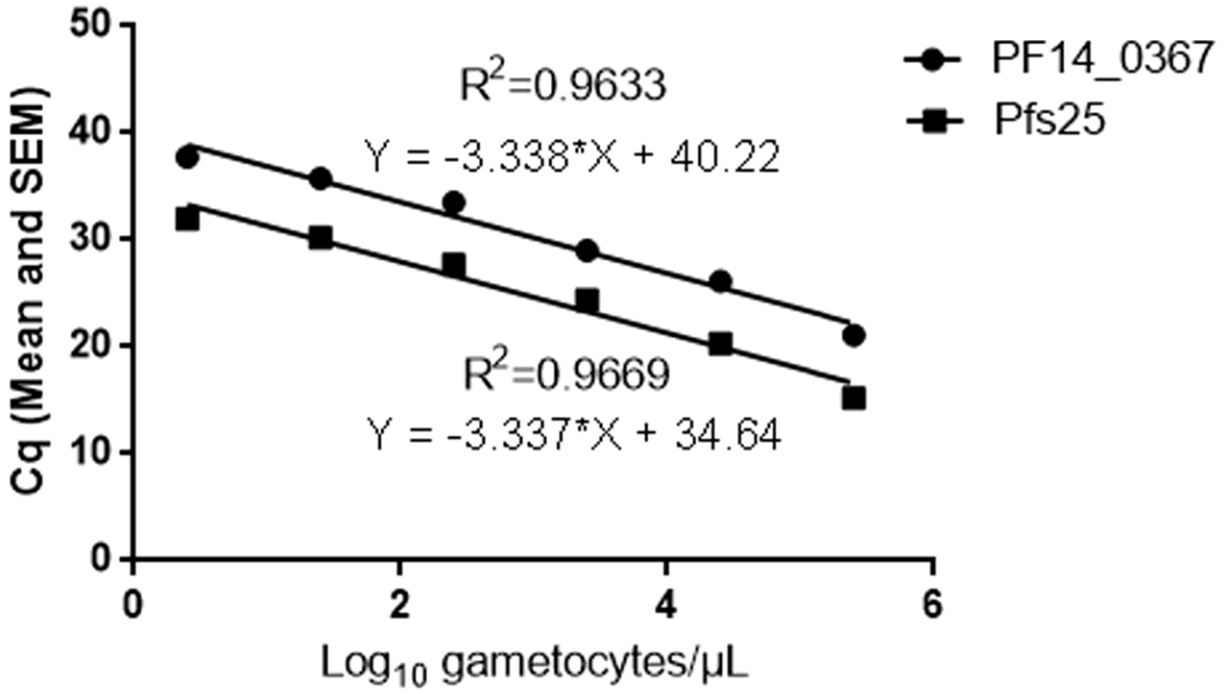
12936_2017_1986_MOESM1_ESM.tif **Additional file 1: Figure S1.** Flow cytometry analysis of *Plasmodium falciparum* (JH013) parasites. Samples were stained with hydroethidine (HE) and analysed by flow cytometry. Panel A shows the negative control. The erythrocytes are cultured in complete culture medium and stained with HE. Panel B shows the gating strategy and percent-infected erythrocytes of a sample taken from gametocyte infected erythrocytes (from gametocyte culture).



12936_2017_1986_MOESM2_ESM.tif **Additional file 2: Figure S2.** Standard curves used for gametocyte quantification by RTqPCR. Dilution series of in vitro cultivated gametocytes were analysed by PF14_0367 and Pfs25. Standard curves were generated performing linear regression using the Log_{10} transformed number of gametocytes and the mean and SEM of the quantification cycles (C_qs) of triplicates. SEM: standard error of the mean.



12936_2017_1986_MOESM3_ESM.docx **Additional file 3: Table S1.** Primer and probe sequences used in gametocyte-specific RTqPCR.

Table S1. Primer and probe sequences used in gametocyte-specific RTqPCR.

Species	Target gene	Primer name	Primer sequence (5'->3')
P. falciparum	**Pfs25** [1]	Pfs25_Fwd	GAC TGT AAA TAA ACC ATG TGG AGA
		Pfs25_Rev	CAT TTA CCG TTA CCA CAA GTT A
		Pfs25_Probe	LC640 - AGA TGG AAA TCC CGT TTC ATA CGC TTG T
	PF14_0367 [1] (mid-late gametocytes)	MG_Fwd	GTTACATTTCGACCCAGCATAAATT
		MG_Rev	GTTACATTTCGACCCAGCATAAATT
		MG_Probe	VIC - CAG TGC ATA TTG TTG CCT GT - MGBNFQ

[1] The primer and probe sequences of mid-late stage gametocyte marker (PF14_0367) were previously published (Joice *et al* 2013), whereas we have designed a new sequences for the Pfs25 marker.

12936_2017_1986_MOESM4_ESM.doc **Additional file 4: Table S2.** TaqMan One-Step Real-Time RT-PCR (RNA-to-Ct™ 1-Step Kit) PCR mix and cycling conditions.

Table S2. TaqMan One-Step Real-Time RT-PCR (RNA-to-Ct™ 1-Step Kit) PCR mix and cycling conditions.

A. RT-qPCR Reaction Mix [1]

PCR mix	**Volume (µL)**
TaqMan® RT-PCR Mix (2×)	5
Forward primer	0.4
Reverse primer	0.4
TaqMan probe	0.15
RNA template	2.5
RNase-free H2O	1.3
TaqMan® RT Enzyme Mix (40×)	0.25
Total	10

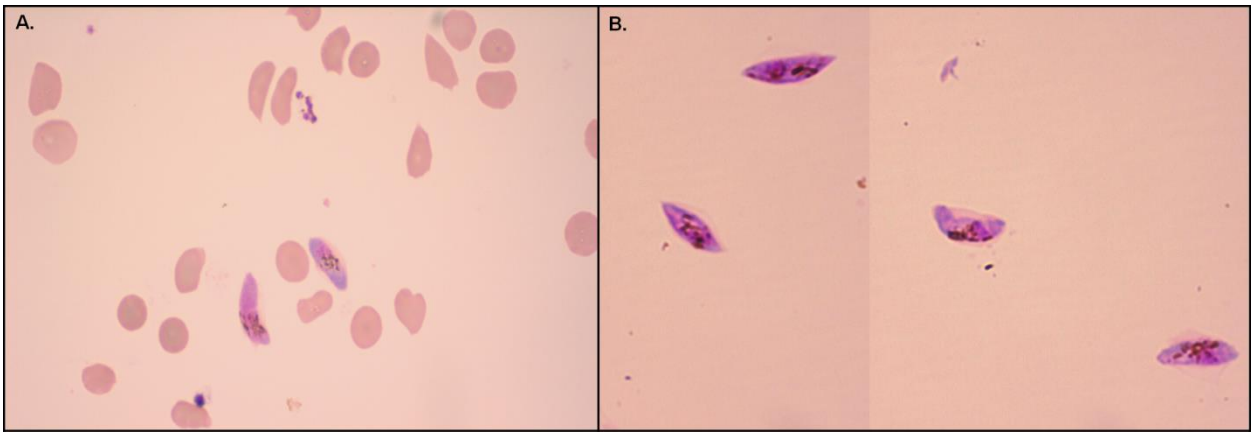
B. RT-qPCR Thermal cycling conditions [2]

Stage	**Step**	**Temperature**	**Time**
Holding	Reverse transcription	48°C	20 minutes
Holding	Polymerase activation	96°C	10 minutes
Cycling (45x)	Denaturation	95°C	15 seconds
	Annealing/Extension	62°C	1 minute
Holding	Cooling	48°C	10 seconds

[1] The reaction was prepared in a PCR cabinet and the mix was dispensed to the 384 wells using a Qiagen QIAgility High-Precision Automated PCR System.

[2] The PCR was run using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) and all samples with Ct value ≤40 were considered positive.

12936_2017_1986_MOESM5_ESM.tif **Additional file 5: Figure S3.** Microphotograph of Giemsa-stained thin smears showing gametocyte culture on day 15 before (A) and after (B) purification (magnification ×1000). Uninfected erythrocytes and remaining asexual parasites were removed by the two-step purifications for the in vitro measurement of gametocytogenesis of 3D7, NF54 and one clinical isolate (JH013).



Gebru *et al. Malar J (2017) 16:176*
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Malaria Journal

RESEARCH

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Recognition of *Plasmodium falciparum* mature gametocyte-infected erythrocytes by antibodies of semi-immune adults and malaria-exposed children from Gabon

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Abstract

Background: Transmission of malaria from man to mosquito depends on the presence of gametocytes, the sexual stage of *Plasmodium* parasites in the infected host. Naturally acquired antibodies against gametocytes exist and may play a role in controlling transmission by limiting the gametocyte development in the circulation or by interrupting gamete development and fertilization in the mosquito following ingestion. So far, most studies on antibody responses to sexual stage antigens have focused on a subset of gametocyte-surface antigens, even though inhibitory Ab responses to other gametocyte antigens might also play a role in controlling gametocyte density and fertility. Limited information is available on natural antibody response to the surfaces of gametocyte-infected erythrocytes.

Methods: Ab responses to surface antigens of erythrocytes infected by in vitro differentiated *Plasmodium falciparum* mature gametocytes were investigated in sera of semi-immune adults and malaria-exposed children. In addition, the effect of immunization with GMZ2, a blood stage malaria vaccine candidate, and the effect of intestinal helminth infection on the development of immunity to gametocytes of P. falciparum was evaluated in malaria-exposed children and adults from Gabon. Serum samples from two Phase I clinical trials conducted in Gabon were analysed by microscopic and flow-cytometric immunofluorescence assay.

Results: Adults had a higher Ab response compared to children. Ab reactivity was significantly higher after fixation and permeabilization of parasitized erythrocytes. Following vaccination with the malaria vaccine candidate GMZ2, anti-gametocyte Ab concentration decreased in adults compared to baseline. Ab response to whole asexual stage antigens had a significant but weak positive correlation to anti-gametocyte Ab responses in adults, but not in children. Children infected with *Ascaris lumbricoides* had a significantly higher anti-gametocyte Ab response compared to non-infected children.

Conclusion: The current data suggest that antigens exposed on the gametocyte-infected red blood cells are recognized by serum antibodies from malaria-exposed children and semi-immune adults. This anti-gametocyte immune response may be influenced by natural exposure and vaccination. Modulation of the natural immune response to gametocytes by co-infecting parasites should be investigated further and may have an important impact on malaria control strategies.

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Keywords: Malaria, Transmission blocking, *Plasmodium falciparum*, Clinical isolates, Helminths, *Ascaris lumbricoides*, *Trichuris trichiura*, GMZ2, Immune modulation

Background

Malaria remains a major global public health problem affecting hundreds of millions of people annually, mainly in sub-Saharan Africa. Each year approximately half a million people die, mostly children younger than 5 years [1]. Gametocytes, the sexual stage of the parasites, are essential for transmission of the parasite from man to mosquito. Malaria transmission can be interrupted by drug treatment affecting gametocytes [2, 3], causal chemoprophylaxis, vector control as well as the acquisition of immunity to sexual stage parasites by the human host [4, 5].

Transmission blocking interventions that target gametocyte development and gamete fertilization are considered an essential part of malaria control, especially if containment or eradication of the disease is the aim. Transmission blocking vaccines (TBVs) would have a great public benefit in malaria-endemic countries by breaking the life cycle and decreasing the number of new infections. In addition, it is assumed that TBVs could help in containing the spread of parasites resistant to drugs or malaria vaccine components directed against asexual blood stage or pre-erythrocytic stages [6, 7]. To better understand immunity against the sexual stage of the plasmodial life cycle and for the design and development of TBVs, profiling the response to mature gametocytes is of relevance.

Antibodies (Abs) are important mediators of sexual stage immunity against *Plasmodium* and other apicomplexan parasites [8–12]. Such Abs can affect malaria transmission either by inhibiting gametocyte development [5] or by directly affecting viability of mature sexual stages [13–15]. The latter might happen within the body or once they are ingested by mosquitoes [5, 16–18], e.g. through opsonization of gametes followed by phagocytosis [12]. In malaria-endemic areas, the age-dependent decline of the duration of gametocyte carriage [19, 20] is most likely due to an increase in gametocyte exposure and development of sexual stage specific immune responses, in parallel to the asexual immunity acquired with age [21].

Indirectly, immune responses to asexual stage antigens may decrease transmission by limiting the number of asexual parasites that develop to gametocytes [21], similar to the decrease of gametocytogenesis that results from the elimination of asexual infections by drugs [22]. However, development of sexual-stage immunity is different from the immune response directed to asexual stage antigens [13, 15]. Gametocytes have distinct gene expression patterns [23] and proteomic profiles [24] compared to asexual stages. Similarly early and late stage gametocytes differ; for example, the latter have a comparatively low representation of active export machinery proteins. However, some overlaps are expected in the proteomic profiles and exported proteins between the different stages of the parasite's life cycle [24].

Naturally acquired sexual-stage antibodies are produced against gametocyte-infected erythrocyte surface antigens or gamete-specific antigens in the circulation and also against mosquito-stage parasites that act following ingestion of the parasite [25]. There are only few studies on natural immune responses to gametocyte-infected erythrocyte surface antigens. Saeed et al. [15] showed that 34% of Gambian children had plasma antibodies recognizing stage V gametocyte-infected erythrocytes in vitro, with no recognition of stages I–IV. In the same study Abs to gametocyte surface antigens were associated with lower gametocyte densities indicating the importance of Abs in reducing gametocyte carriage. Most other studies on immune responses to sexual stage antigens have focused on few specific antigens, mainly the TBV candidates Pfs230 [18, 26–31] and Pfs48/45 [18, 27–32]. The association of Ab response to these single antigens and transmission reducing activity is not consistent. After testing antibody response to both antigens, some authors reported a correlation of transmission reduction with both antigens [31], while others found associations only with Pfs230 [18, 28] or only with Pfs48/45 [29, 30]. Even though correlation might be confounded by exposure history to earlier malaria infections, these results suggest that Ab responses to other gametocyte-specific antigens may play an additional role in controlling transmission [5]. Here, Ab responses to gametocyte-infected erythrocyte surface antigens were measured in individuals from a malaria-endemic country (Gabon).

In the present study, the concentration of anti-gametocyte Abs against in vitro differentiated mature gametocytes of one *Plasmodium falciparum* clinical isolate and one laboratory strain (NF54) was measured by flow cytometric immunofluorescence assay (IFA) in sera from malaria-exposed children and semi-immune adults. Since exposure to asexual blood stage antigens and co-infection with other highly prevalent parasites may modulate immune responses [33, 34], here the anti-gametocyte responses were related to infection status with intestinal helminths. Assuming a reduced anti-gametocyte antibody response after vaccination with a malarial vaccine,

additionally the anti-gametocyte antibody response to antibodies induced by vaccination was related with the asexual blood stage vaccine candidate GMZ2, a recombinant fusion protein of *P. falciparum* glutamate-rich protein (GLURP) and merozoite surface protein 3 (MSP3) [35]. Therefore, the collected serum samples during the GMZ2 trials were used to investigate this in depth.

Methods

Study samples and sources

Gametocyte recognition of antibodies were investigated in adults and children who had a different history of exposures to malaria and infection status with intestinal helminths. The serum samples analysed in the current investigation are from two studies [36, 37] conducted between July 2007 and October 2008 at the Centre de Recherches Médicales de Lambaréné (CERMEL), Lambaréné in Gabon. The two sets of serum samples were from Phase I clinical trials designed to assess safety and immunogenicity of GMZ2, an experimental blood-stage malaria vaccine candidate, in malaria-exposed adults and children, respectively [36, 37], which shows partial efficacy [38].

As part of the GMZ2 trials, samples were collected from 30 children aged 1–5 years [36] and 40 semi-immune adults (18–45 years) [37]. At the time of this investigation samples were only available for 36 adults. Samples collected before (Day 0) and on Day 84 after vaccination (4 weeks after the last immunization) were analysed to assess if vaccination has an effect on the development of antibody response to the sexual stages of *P. falciparum*. During the GMZ2 trials, participants received three doses of either 30 or 100 µg GMZ2, adjuvanted with aluminum hydroxide or a control vaccine (Verorab) and were followed for 1 year. Further details of the studies are published elsewhere [36, 37]. Serum samples were stored at −80 °C until analysed.

The studies were conducted according to the principles of the Declaration of Helsinki and good clinical practice guidelines. The respective competent ethics committees: the Comité d'Ethique Régional Indépendant de Lambaréné, CERIL and the Gabonese Ministry of Health reviewed and approved the studies. A signed informed consent was obtained from each participant or parent/guardian of participant aged less than 18 years. Analysis of Ab levels against *P. falciparum* was part of the respective study protocols.

Gametocyte culture and purification

Gametocyte culture was initiated from a continuous culture of asexual *P. falciparum* parasites as described earlier [39]. Briefly, the asexual cultures were kept at 5% haematocrit and less than 2% parasitaemia at 37 °C, 5% CO_2 and 5% O_2 with weekly sorbitol synchronization and daily change of medium (RPMI 1640 supplemented with 25 mM HEPES, 28 mM $NaHCO_3$, 50 µg/mL gentamycin, 0.5% w/v Albumax II, 2.4 mM L-glutamine, and 0.14 mM hypoxanthine); medium of the clinical isolate contained in addition 5% human serum. The *P. falciparum* laboratory strain NF54 (Sanaria Inc., Rockville, MD, USA) and a laboratory adapted clinical isolate from Lambaréné, Gabon where the samples had been collected were used for the experiments. The clinical isolate (JH013) was cultivated from a blood sample collected in 2009 from an individual with *P. falciparum* mono-infection and cryopreserved at −150 °C in glycerolyte as reported previously [40].

Gametocyte culture was performed as described earlier [41, 42] with some modifications. Human serum (5%) was added to the gametocyte growth media and the culture was initiated with a parasitaemia of 0.5 and 9% haematocrit and kept at 5% O_2/CO_2 at 37 °C with daily change of medium without parasite dilution. On Day 7 the haematocrit was lowered to 4.5% by doubling the volume of medium. Beginning with Day 11 the parasites were treated with 50 mM *N*-acetyl-D-glucosamine (MP Biomedicals GmbH, Santa Ana, CA, USA) for 4 days to eliminate asexual stage parasites.

To enrich stage V gametocytes from approximately 2% to more than 90% on Day 15 a density gradient centrifugation (800*g* for 20 min) on 33% NycoPrep 1.077 cushions (AXIS-SHIELD PoC AS, Oslo, Norway) followed by magnetic separation with LD-MACS magnetic columns (Miltenyi Biotec, Gladbach, Germany) was performed.

Fluorescence microscopy and flow cytometry-based IFA

For IFA, gametocyte-infected erythrocytes were analyzed in three different ways: live [15], fixed, or fixed and permeabilized [43]. For assessment of Ab response to plasmodial antigens, a *P. falciparum* culture was used, that was enriched for late developmental stages of asexual [44] and stage V gametocytes. In brief, for fixation, parasites were incubated for 30 min in a mixture of 4% Electron Microscopy (EM) grade paraformaldehyde (Merck, Germany) and 0.0075% EM grade glutaraldehyde (Sigma-Aldrich, Germany), then washed once with PBS and stored at 4 °C until the IFA was performed. Prior to Ab staining, a fraction of fixed cells was permeabilized by PBS/0.1% Triton-X-100 for 10 min and washed once with PBS.

All live, fixed or fixed and permeabilized parasites were blocked using PBS/3%BSA for 1 h before the addition of test or negative control sera. Following the blocking step, evaluation of anti-gametocyte Ab response was done using cytometry-based IFA as described earlier [44]. Serum samples (in PBS/3% BSA diluted 1/4000 and 1/50,

respectively) from adults and children were added and incubated for 1 h. Serum dilutions that gave the best discrimination between negative and positive were used. In the cohort children are less reactive compared to adults, hence lower dilutions were used [45, 46]. After three washes with PBS, secondary Ab staining was carried out for 1 h with Alexa Fluor 488-labeled rabbit anti-human IgG (Life Technologies GmbH, Darmstadt, Germany) at a 1:3000 dilution and washed three times before the quality of the staining was assessed by fluorescence microscopy.

Subsequent acquisition of flow cytometry data was done using a FACSCanto II cytometer equipped with the FACSDiva software version 6.1.2 (BD Biosciences, San Jose, USA). Fluorescence of each event was analysed and the result expressed as percentage of positive fluorescent cells (PPFC) based on 20,000 erythrocytes (events) acquired.

ELISA for measurement of Abs to GMZ2 antigens

For comparison, a subset of previously reported GMZ2-specific IgG ELISA values (Days 0 and 84) of clinical trial participants were used for the current analyses [36, 37]. ELISA data were generated using a standardized ELISA protocol as described previously [36, 37].

Stool examination for worm infection

During follow up of study participants enrolled in the Phase I paediatric clinical trial of GMZ2 [36], stool samples were collected. They were freshly examined to evaluate the prevalence of soil-transmitted helminths at enrolment and on Day 84 as reported earlier [34].

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software, San Diego California) and SPSS version 16 were used for statistical analysis. Nonparametric tests were used for statistical testing. The significance level was set to a two-sided alpha of 0.05 and corrected using the Bonferroni method where applicable. Paired samples were tested with the Wilcoxon signed-rank test whereas the Mann–Whitney test was applied in case of unpaired data. Spearman correlation between Ab responses to whole sexual and asexual stage (including recombinant GMZ2) antigens were calculated and presented with 95% confidence intervals [47]. A sample was declared sero-negative by cytometric IFA if the percentage gated cells positive for a given marker was within three standard deviations above the mean of the negative controls (serum samples from malaria naïve individuals).

Results

Recognition of gametocyte antigens by Abs is enhanced by fixation and permeabilization

The level and prevalence of Ab response to *P. falciparum* mature gametocytes was evaluated in sera from adults (n = 36) and children (n = 30) from the two GMZ2 trials (Table 1) using live, fixed or fixed and permeabilized NF54 *P. falciparum* mature gametocyte infected erythrocytes. As expected, the level of antigen recognition by Abs was significantly higher after fixation and permeabilization of NF54 *P. falciparum* gametocyte-infected erythrocytes, two-fold and four-fold change, respectively (Fig. 1). Apparently, immune recognition of the parasite includes antigens not expressed on the surface of erythrocytes (Fig. 2a, b). However, these intracellular antigens could also be shared by the asexual and sexual antigens of the parasite. Therefore, for all subsequent measurements,

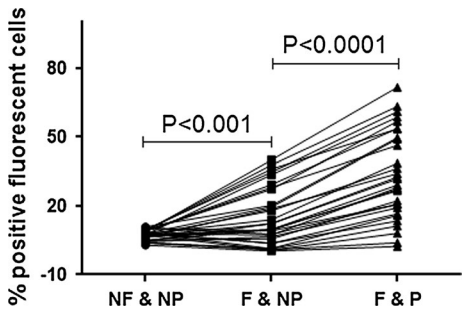

Fig. 1 Antibody response to in vitro differentiated live, fixed or permeabilized *Plasmodium falciparum* gametocytes. The figure shows the flow cytometric data obtained using live, fixed or permeabilized gametocyte-infected erythrocytes. *Dots* represent percentage positive cells (erythrocytes positive for AlexaFluor-488), one dot per tested sample. Gates were set against controls, which are serum from malaria naïve individuals. *NF* non fixed, *NP* non permeabilized, *F* fixed, *P* permeabilized. The *Y-axis* shows percentage of erythrocytes positive for the used marker (Alexa.Fluor-488)

Table 1 Study name, background characteristics, grouping, hematological profiles and time of data collection of samples used within the study

Study identifier (population)	Sampling period (month/year)	No. of participants	Median age in years (range)	Mean hemoglobin (g/dL)	Vaccine allocation	Samples time points
GMZ2_3_08 *(Children)* [36]	09/2008–10/2009	30	3.5 (1–5)	10.5 10.5 10.3	GMZ2 100 µg GMZ2 30 µg Rabies	Days 0 and 84
GMZ2_2_07 *(Adults)* [37]	07/2007–08/2008	32	26.3 (18–45)	13.8 14.1	GMZ2 100 µg Rabies	Days 0 and 84

non-fixed and non-permeabilized gametocyte-infected erythrocytes were used.

Anti-gametocyte Ab responses of malaria-exposed individuals in different *P. falciparum* parasite strains

The anti-gametocyte Ab-binding patterns of one clinical isolate (JH013) and NF54 were compared to identify the strain that is best recognized by participants' Abs [37]. There was no significant difference between the two strains in the recognition of gametocytes by serum antibodies from adults and children of the studies and, therefore, further experiments were done only with the *P. falciparum* laboratory strain NF54. The Ab response to mature gametocytes was higher in adults compared to children (Fig. 3), as well as the seroprevalence of anti-gametocyte Ab response (77% in adults and 57% in children).

Asexual and sexual stage anti-malarial immune response in GMZ2 vaccinated adults and children

The effect of GMZ2 vaccination on the development of anti-gametocyte immune response in adults and children was assessed. It was hypothesized that immune responses generated by the GMZ2 vaccine may divert the immune response towards the vaccine or lead to a lower gametocyte density, despite its relatively low efficacy (11–14%)

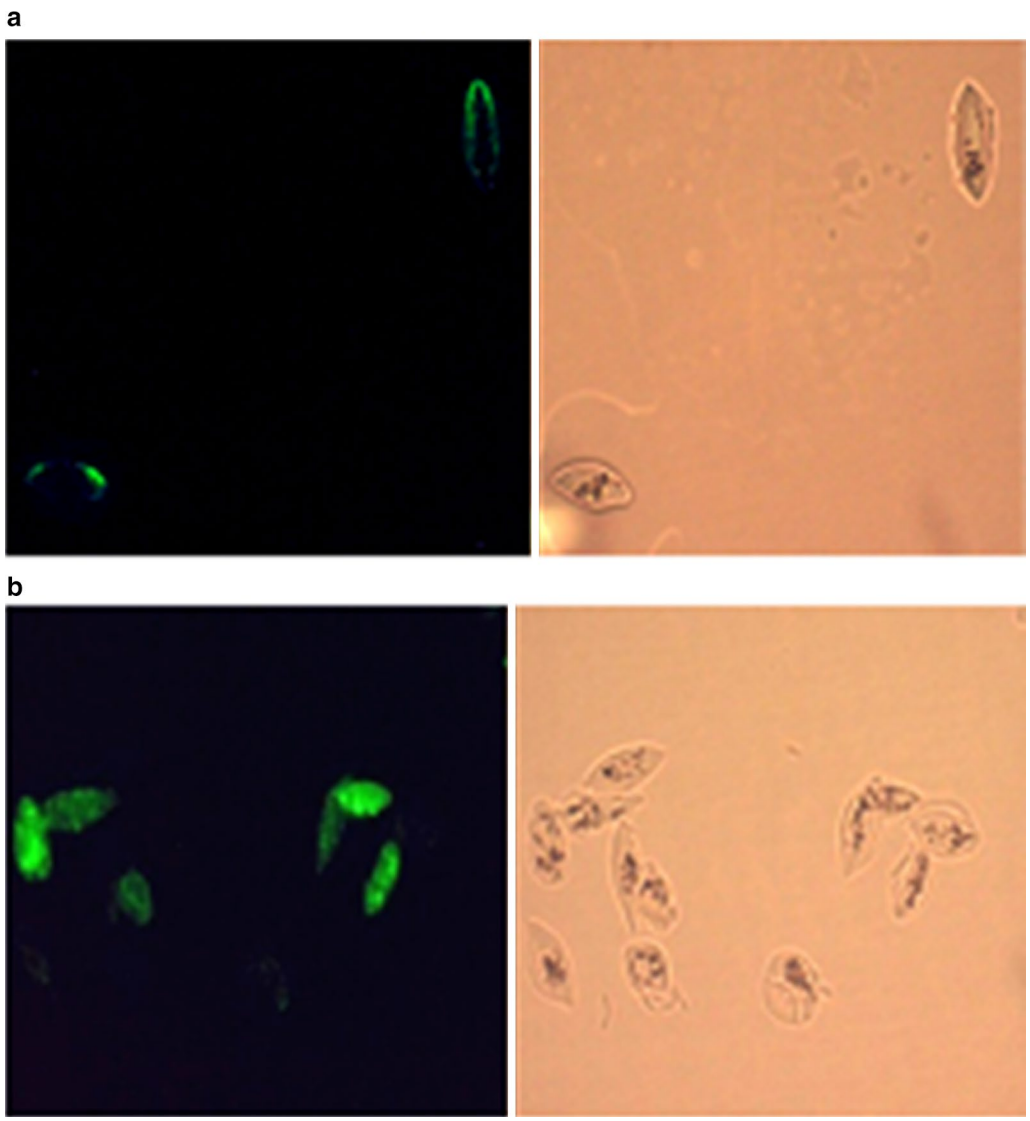

Fig. 2 Microscopic IFA of antibody response to *P. falciparum* gametocytes. The figures show an example picture of AlexaFluor-488 stained parasites by fluorescence microscopy before (**a**) and after (**b**) permeabilization

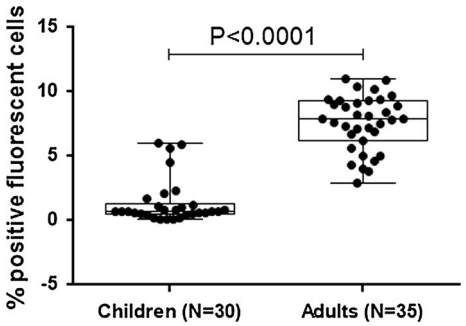

Fig. 3 Baseline levels of antibody response to gametocytes of *P. falciparum* in semi-immune adults and malaria exposed children. Antibody response (percentage of positive cells) against in vitro developed matured gametocytes (*P. falciparum* NF54 strain) of the serum samples of the different trials conducted in Gabon (GMZ2 children on Day 0 [36] and GMZ2 adults on Day 0 [37]) at baseline. Median, 25th and 75th percentiles, and minimum and maximum ranges are indicated

in children between 1 and 5 years [38]. Interestingly, the anti-gametocyte Ab response in GMZ2 vaccinated adult individuals was lower on Day 84 after vaccination compared to baseline (Day 0) (Fig. 4a). In children, the anti-gametocyte Ab response was not different after vaccination in both, the GMZ2 and rabies vaccinated control group (Fig. 4b).

Since sexual stage immunity may be developed independently from asexual-stage immunity [13, 15], possible associations were tested between sexual stage immune response (anti-gametocyte Abs) to the asexual stage immunity (Abs against asexual blood stages) and Ab responses to the GMZ2 vaccine antigen in the two vaccinated groups. For this analysis, our previously published data on anti-plasmodial (against whole asexual stage antigens) [44] and anti-GMZ2 [36, 37] Abs were used to assess correlations with anti-gametocyte Abs. There was a positive correlation on Day 0 before vaccination between the sexual and asexual Ab responses in the semi-immune population (Table 2). No correlation was found in data from children. The Ab response to GMZ2 was not associated with sexual Ab response neither in adults nor in children (Table 2).

Effect of helminth infection on development of anti-gametocyte immunity

The anti-plasmodial immune response is modulated by co-infection of *P. falciparum* with other infectious agents including helminths [48–50]. Previously an effect of intestinal helminths on anti-GMZ2 responses in 20 GMZ2 vaccinated children was found [34] and, therefore, also in this study the effect of intestinal parasites co-infection on the modulation of anti-gametocyte immune responses in children was assessed. Parasitological data of all 30 children included in the GMZ2 Phase Ib trial [36] were used to explore the modulation of anti-gametocyte immune response by helminths. Overall, five different helminths were recorded. *Trichuris trichiura* and *Ascaris lumbricoides* were present in a relative high proportion on enrolment (14/30; 47% and 8/30; 27%) and on Day 84 (15/30; 50% and 11/30; 37%), respectively. There was also a low rate of infections (<7%) by *Strongyloides*,

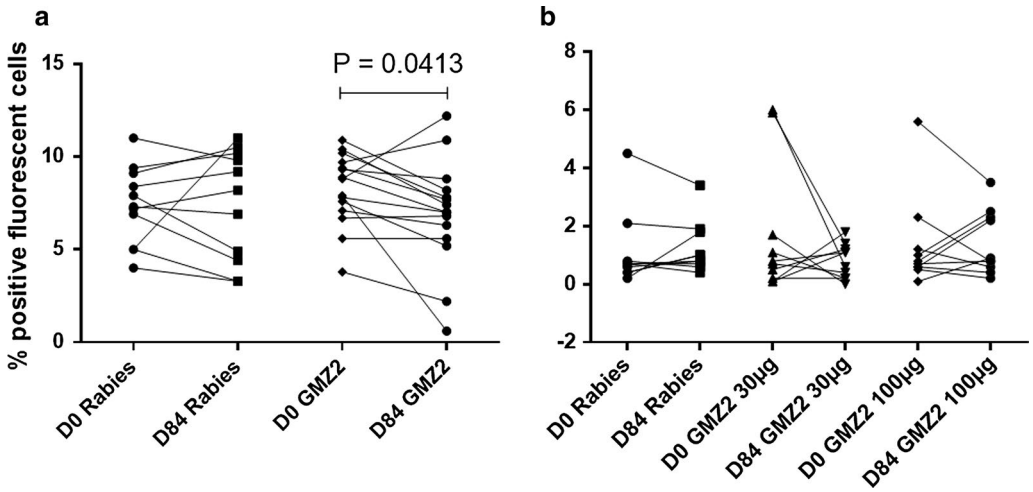

Fig. 4 Effect of GMZ2 vaccination on the development of antibody response to mature *P. falciparum* gametocytes. **a**, **b** Show the antibody response in semi-immune adults and malaria-exposed children, respectively. In adults, 73.3% (11/15) show a decrease of Ab response after vaccination while 20% (3/15) show an increase and one person has shown no change (6.7%). *D0* Day 0, *D84* Day 84, GMZ2 100 µg or GMZ2 30 µg: participants who received 100 or 30 µg of GMZ2 vaccine, respectively. Rabies: participants who received the control rabies vaccine

Table 2 Spearman correlation of the antibody response to sexual stage antigens with asexual stage antigens and the GMZ2 vaccine antigen of *P. falciparum* in adults/children from Gabon

Study participants and vaccination	Antibody against whole asexual stage antigens by C-IFA (Mean PPFC)	Antibody against GMZ2 by ELISA (Mean Ab titers)
	Rho (95% CI)	Rho (95% CI)
Antibody against stage V gametocytes by C-IFA (Mean PPFC)		
Adults		
D0[a]	0.39 (0.03, 0.66)*	0.17 (−0.18, 0.48)
D84 (GMZ2)	0.41 (−0.17, 0.78)	0.44 (−0.13, 0.80)
D84 (Rabies)	0.40 (−0.21, 0.79)	0.54 (−0.03, 0.85)
Children		
D0[a]	0.16 (−0.24, 0.51)	0.28 (−0.11, 0.60)
D84 (GMZ2, 30 µg dose)	0.02 (−0.62, 0.64)	−0.10 (−0.69, 0.57)
D84 (GMZ2, 100 µg dose)	0.06 (−0.59, 0.67)	0.22 (−0.48, 0.75)
D84 (Rabies)	−0.27 (−0.77, 0.44)	0.22 (−0.48, 0.75)

C-IFA Cytometric immunofluorescence assay, *ELISA* Enzyme-linked immunosorbent assay, *PPFC* percent positive fluorescent cells, *CI* Confidence interval

Significant p value is indicated as follows: * $P < 0.05$

[a] *D0* Values represent both GMZ2 and Rabies groups together. *D0* Day 0, *D84* Day 84

Schistosoma, and *Ancylostoma*. Interestingly, children infected with *Ascaris lumbricoides* had significantly higher anti-gametocyte Ab response compared to uninfected children when using live non-permeabilized gametocytes (Fig. 5a). When the assay was done using fixed and permeabilized parasites, a significantly lower Ab response was observed in *Trichuris trichiura*-infected children (Fig. 5b).

Discussion

Understanding the development of Ab responses to sexual stages of *P. falciparum* in malaria-exposed populations is important for understanding transmission patterns and the design and development of TBVs. In this study in a highly malaria endemic area, adults showed higher Ab responses to sexual stage antigens than children. The effect of age on anti-gametocyte Ab production and transmission reducing activity has been addressed in several studies and resulted in contradictory results. Some have shown an increase in antibody response to fixed whole parasites [51] and to Pfs48/45 and Pfs230 with age [46], others a decline in transmission reducing activity of sera with age [27]. The difference may be due to the varying age ranges, different epidemiological settings and inconsistent assays for anti-gametocyte Ab measurements between the studies. Since effective humoral

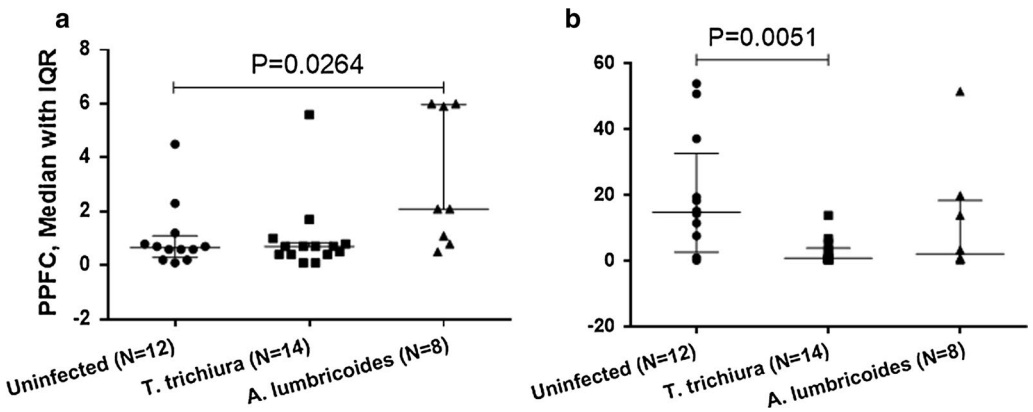

Fig. 5 Antibody response to *P. falciparum* mature gametocytes in children from Gabon infected with intestinal parasites. The *x-axis* shows helminth infection status on Day 0 before vaccination in children, N = 30. **a**, **b** show antibody recognition of live and permeabilized gametocyte infected erythrocytes, respectively. *Y-axis* shows percentage of erythrocytes positive for AlexaFluor-488. *PPFC* % positive fluorescent cells, *IQR* Interquartile range. Median and IQR are indicated

immunity to intraerythrocytic gametocytes requires prolonged exposure to the parasite [52], the epidemiological setting shall play an important role and the interpretation of divergent results needs to account for that.

The anti-gametocyte seropositivity rate was 77% in adults and 57% in children. Similar seropositivity rates have been recorded earlier, more than 50% in Ghanaian children with asymptomatic infection [53] and 34% in Gambian children with uncomplicated malaria and gametocytaemia [15]. In another cohort of Gambian children, who were gametocytaemic, an Ab response was detected in all participants [51]. This response might have been so high, as they fixed gametocytes with acetone that simultaneously permeabilizes the cell [54]. The same study reported a lower (42%) sero-prevalence when live gametes were used. Unfortunately, gametocyte carriage rates were not recorded in our studies. Due to different methodologies, direct comparison to the previously reported results is not possible. Of note, gametocyte prevalence is not always associated to antibody response to gametocytes [55] and transmission capacity [56, 57].

The effect of anti-GMZ2 Ab and Ab responses to whole asexual stage antigens and the effect of GMZ2 vaccination on the development of sexual-stage immunity was evaluated. The result showed a significant but weak positive correlation between Ab response to gametocyte antigens and whole asexual stage antigens, but not to GMZ2 antigen in adults, showing that exposure is correlated to Ab response to gametocytes in the investigated population. The level of sexual Ab response was significantly reduced after vaccination in GMZ2 vaccinated adults, but this difference was not seen in the rabies control group. An effective asexual malaria vaccine should lead to a reduced asexual parasite load and, therefore, reduced gametocyte production. The recently reported results of the GMZ2 vaccine shows 11–14% efficacy in children [38], therefore, the expected effect on gametocytes would be rather low. There is no evidence for the expression of either of the GMZ2 component proteins (MSP3 and GLURP) on the surface of gametocyte infected erythrocytes even though it was previously reported that GLURP is expressed in different stages of the parasite life cycle including the pre-erythrocytic stage [58]. The results show no boosting of the anti-gametocyte Ab response, supporting the data that none of the components is present at the sexual stage.

In children, there was no correlation between the Ab response to gametocyte antigens and GMZ2 (and other asexual antigens). The difference of the correlation results between adults and children might be due to the very low level of anti-gametocyte immune response in children complicating to see differences between groups. Additionally, Ab response to matured gametocytes did not change in children following vaccination with GMZ2 or the rabies control vaccine. Unfortunately our analysis has been underpowered to detect subtle differences in the study populations. Our result shall be confirmed with a bigger sample size.

As expected, antigen recognition by immune sera was significantly enhanced after fixation and permeabilization of gametocyte-infected erythrocytes as reported earlier [51]. This effect may be partly due to intracellular targets of transmission-blocking Abs [52] that are not exposed on the infected erythrocyte surface. However, the response measured after fixation and permeabilization of cells might not be gametocyte specific, but represent a response to the cocktail of internal asexual and sexual stage proteins [59]. Therefore, non-fixed and non-permeabilized gametocyte-infected erythrocytes were used to analyse the effect of age, vaccination, coinfection, and parasite strain variation on Ab responses to gametocytes. The natural Ab response to gametocyte antigens might inhibit gametocyte development and thereby interrupt the transmission of the parasite as shown in vitro by co-cultivation of early gametocytes with plasma from malaria patients [5]. In addition, Abs may also act following exflagellation in the mosquito midgut [60]. To get a deeper insight, characterization of naturally acquired transmission blocking Abs might improve the portfolio of TBVs.

Recognition of *P. falciparum* gametocytes of the laboratory strain NF54 and a clinical isolate by serum antibodies of semi-immune adults was assessed. Both lab strain and clinical isolate have been detected by the serum antibodies and no significant difference in the level of Ab recognition of the two strains could be seen. This was unexpected as the clinical isolate was collected from the area where the study participants were recruited. However, similar to the finding presented here, it has been shown earlier by Dinko et al. that the plasma antibodies from Ghanian participants recognizing the laboratory strain 3D7 and a clinical isolate from Kenia [55].

The immune response to malaria is modulated by co-infection with other infectious agents [33]. There were lower Ab responses in *Trichuris trichiura* infected children when the assay was done with permeabilized gametocytes. However this might not represent the suppression of the immune response to gametocytes but rather the response to the asexual or both stages of the parasite. This corroborates earlier findings in the same population, which showed that *Trichuris trichiura* infection is associated with 3.4-fold reduced Ab levels to the blood-stage asexual GMZ2 vaccine antigen while the response was increased in *Ascaris lumbricoides*-infected participants [34]. However, the effect

of *Trichuris trichiura* infection could not be seen in the assay using non-permeabilized gametocyte-infected erythrocytes. In line with this, a cross-sectional study has reported a significant reduction of gametocyte specific (Pfs48/45) antibody titers in individuals infected with *Schistosoma haematobium,* though this effect is not seen when comparing another gametocyte specific antigen (Pfs230) [61].

The response to any gamete-specific antigens was not measured and therefore activation of non-fixed gametocytes cannot be ruled out. Mature gametocytes should be handled with care and a drop in temperature could provoke exflagellation. Therefore, the small fraction of recognized erythrocytes with gametocytes could also be a small fraction that was accidentally activated. However, we tried to ensure that non-fixed and non-permeabilized erythrocytes in the assay were intact and controlled for exflagellation by microscopy. Therefore, the measured response most probably represents largely the antibody response to antigens displayed on the surface of gametocyte-infected erythrocytes. Another explanation for the low response could be that only a fraction of gametocytes transport proteins to the outside and express surface proteins on the surface of erythrocytes. A reduced transport of proteins to the erythrocyte surface is known for asexual parasites of laboratory strains in long-term culture. To check this, the prevalence of positive cells was tested also for one clinical isolate (JH013), but did not find a difference in the percentage of recognized cells when compared to NF54.

Infection with *Ascaris lumbricoides* resulted in an increased anti-gametocyte immune response compared to the uninfected participants. This was surprising but similar effects on the anti-malarial immune response have been observed earlier [34]. However, due to the relatively small sample size and exploratory nature of the experiment, confirmatory studies will be required. Therefore, monitoring of anti-gametocyte responses should be done in further larger studies. In addition, it would be interesting to validate the immuno-modulatory effect of co-infections on the development of asexual and sexual stage immune responses and the transmissibility of malaria in other co-endemic areas.

Conclusions

The level of Ab responses to mature gametocytes can be measured using flow cytometry. Adults show higher Ab responses when compared to children. Anti-gametocyte Ab responses are enhanced following permeabilization but may include responses to asexual antigens. Helminth infections and anti-malarial interventions modulate the humoral immune response to asexual and sexual blood stage *P. falciparum* parasitaemia. This may have a significant impact on malaria control strategies with the aim to reduce transmission of malaria.

Abbreviations

Abs: antibodies; *A. lumbricoides*: *Ascaris lumbricoides*; ELISA: enzyme linked immunosorbent assay; IFA: immunofluorescence assay; TBV: transmission blocking vaccines; *T. trichiura*: *Trichuris trichiura*.

Authors' contributions

The study was conceived and experiments designed by TG AA BM JH. TG performed the experiments and TG AA BM JH analysed the data. TG AA MT ME UAN SI AAA PGK BM JH contributed reagents/materials/analysis tools and wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The respective competent ethics committees, the Comité d'Ethique Régional Indépendant de Lambaréné, CERIL and the Gabonese Ministry of Health reviewed and approved the studies.

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Effect of Fluorescent Dyes on *In Vitro*-Differentiated, Late-Stage *Plasmodium falciparum* Gametocytes

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Plasmodium falciparum **gametocytes are not associated with clinical symptoms, but they are responsible for transmitting the pathogen to mosquitoes. Therefore, gametocytocidal interventions are important for malaria control and resistance containment. Currently available drugs and vaccines are not well suited for that purpose. Several dyes have potent antimicrobial activity, but their use against gametocytes has not been investigated systematically. The gametocytocidal activity of nine synthetic dyes and four control compounds was tested against stage V gametocytes of the laboratory strain 3D7 and three clinical isolates of *P. falciparum* with a bioluminescence assay. Five of the fluorescent dyes had submicromolar 50% inhibitory concentration (IC_{50}) values against mature gametocytes. Three mitochondrial dyes, MitoRed, dihexyloxacarbocyanine iodide (DiOC6), and rhodamine B, were highly active (IC_{50}s < 200 nM). MitoRed showed the highest activity against gametocytes, with IC_{50}s of 70 nM against 3D7 and 120 to 210 nM against clinical isolates. All compounds were more active against the laboratory strain 3D7 than against clinical isolates. In particular, the endoperoxides artesunate and dihydroartemisinin showed a 10-fold higher activity against 3D7 than against clinical isolates. In contrast to all clinically used antimalarials, several fluorescent dyes had surprisingly high *in vitro* activity against late-stage gametocytes. Since they also act against asexual blood stages, they shall be considered starting points for the development of new antimalarial lead compounds.**

Malaria leads to approximately 1 million deaths annually and remains a highly relevant public health problem in tropical and subtropical regions (1). *Plasmodium falciparum*, the most virulent species, is responsible for most of the morbidity from malaria and accounts for almost all mortality from malaria in sub-Saharan Africa. Gametocytes are the stage responsible for transmission of the parasite from the human host to the mosquito. During the asexual blood stage, a small fraction of parasites differentiates into gametocytes. In *P. falciparum*, differentiation from sequestered immature stages (stages I to IV) to the free circulating mature stage (stage V) occurs within 8 to 14 days in internal organs, mainly bone marrow (2–4). When a mosquito takes a blood meal, it ingests stage V male and female gametocytes, which can further develop in the mosquito. In the mosquito gut, gametocytes develop into gametes that join to become a zygote, which then matures into the ookinete, followed by the oocyst stage. This stage gives rise to sporozoites, which are released from the oocyst and migrate to the salivary glands of the mosquito, where they can be transmitted during a subsequent blood meal.

Even though gametocytes are essential for the spread of malaria, transmission-blocking agents have not been a priority in the search for antimalarial drugs for a long time. This has recently changed with the ambition to eradicate malaria (5). The development of a new generation of transmission-blocking tools and therapies is a top priority of the Malaria Eradication Research Agenda (malERA) since they are believed to be essential for the elimination and subsequent eradication of malaria (6, 7).

Most current antimalarial drugs show, at most, only modest activity against early gametocytes but have either negligible or no effect on stage V gametocytes, although all are active against the asexual blood-stage parasites (8–10) and some inhibit the growth of preerythrocytic stages. Primaquine, an 8-aminoquinoline, is the only licensed drug that has proven gametocytocidal activity *in vivo*. Its use is limited due to the risk of hemolysis in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a common trait in areas where malaria is endemic. In addition, primaquine is a prodrug that requires activation by cytochrome P450 2D6 (CYP2D6), which is polymorphic and can lead to treatment failures in poor and intermediate metabolizers (11). Other drugs with activity against gametocytes are bulaquine, a compound newly registered in India (12), and tafenoquine, which has been in late-phase clinical development for more than a decade (13). Both drugs are 8-aminoquinolines and are likely to share safety and efficacy problems with primaquine in G6PD-deficient individuals and poor metabolizers of CYP2D6, respectively (14). Dihydroartemisinin (DHA), the active metabolite of all artemisinin derivates, has been reported to be active against stage I to III gametocytes (15, 16), but it is uncertain whether DHA has clinically relevant activity against late-stage gametocytes (8). Evidence available from clinical studies shows that transmission is only partially blocked following treatment with artemisinin-based combination therapies (17, 18), the current mainstay of antimalarial therapy in areas where malaria is endemic.

In conclusion, no licensed drug with a satisfactory profile that can be used to control the transmission of *P. falciparum* is available. Some synthetic dyes have potent antimicrobial activity and have been successfully used as antimalarials since the late 19th

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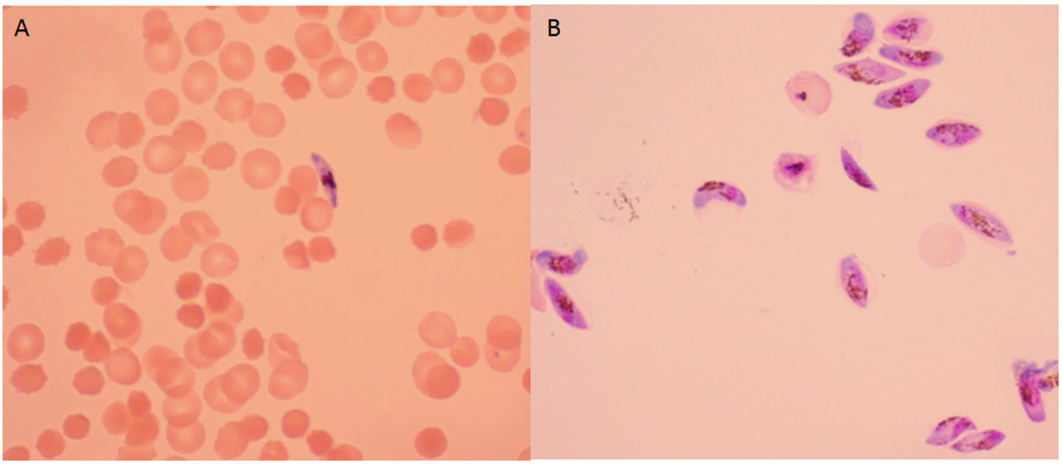

FIG 1 Giemsa-stained mature gametocyte culture on day 15 before (A) and after (B) purification.

century, when Guttmann and Ehrlich used the thiazine dye methylene blue for the treatment of malaria (19). Since then, several other dyes were tested for their antiplasmodial activity (20, 21). We revived this idea in a previous study by testing a panel of synthetic dyes for their *in vitro* activity against asexual stages and identified several compounds with high levels of *in vitro* activity (22). Methylene blue is known to show gametocytocidal activity (15, 23–25); therefore, we evaluated the activity of eight other antimalarial dyes against *P. falciparum* stage V gametocytes derived from the laboratory strain 3D7 and three clinical isolates from Lambaréné, Gabon, with an established bioluminescence assay (8).

MATERIALS AND METHODS

Cultivation of asexual parasites. *P. falciparum* strain 3D7 (Malaria Research and Reference Reagent Resource, ATCC, Manassas, VA, USA) and clinical isolates obtained from Lambaréné, Gabon, were kept in continuous culture as described earlier (26). Clinical isolates were from a study reported previously (27). Blood was sampled directly from patients with *P. falciparum* monoinfection and cryopreserved in glycerolyte at −150°C until culture for the experiment was initiated. The cultures of both 3D7 and clinical isolates were kept at 5% hematocrit in complete culture medium (RPMI 1640 supplemented with 25 mM HEPES, 28 mM $NaHCO_3$, 50 μg/ml gentamicin, 0.5% [wt/vol] lipid-rich bovine serum albumin [Albumax II], 2.4 mM L-glutamine, and 0.14 mM hypoxanthine at 5% CO_2 and 5% O_2). For clinical isolates, 5% human serum was added to the complete culture medium. Medium was replaced every 24 h.

Gametocyte culture and purification. Gametocyte culture was performed as described previously (8), with some modifications. Cultures were started from asexual parasites and kept in continuous culture with sorbitol synchronization twice weekly (28). Culture medium for gametocytes always contained 5% human serum. Initially, the culture was adjusted to 9% hematocrit and 0.5% parasitemia and kept at 5% O_2 and 5% CO_2 at 37°C with a daily change of medium without parasite dilution for 2 weeks. In the second week, the volume of medium was doubled and cultures were treated with 50 mM *N*-acetyl-D-glucosamine (MP Biomedicals GmbH, Santa Ana, CA, USA) for 4 days, beginning 11 days after initiation to remove asexual parasites. To enrich stage V gametocytes from approximately 2% to over 90%, a two-step purification was performed on day 15 using a NycoPrep 1.077 cushion density gradient (Axis-Shield PoC AS, Oslo, Norway), followed by magnetic separation with LD-MACS magnetic columns (Miltenyi Biotec, Gladbach, Germany) to remove the remaining erythrocytes. Purification was done at 37°C to keep the gametocytes viable and avoid exflagellation during the procedure.

Viability of mature gametocytes after separation. The viability of gametocytes after purification was assessed by microscopy (Fig. 1) and the capacity of male gametocytes to exflagellate in exflagellation medium (complete culture medium with 5% human serum and 100 μM xanthurenic acid) for 20 min at room temperature, as described earlier (25, 29).

Compounds. We selected eight fluorescent dyes (see the structures in Fig. 2) on the basis of their activity against asexual parasite stages evaluated previously (22), in addition to methylene blue and control compounds. The panel of fluorescent dyes consisted of three nucleic acid dyes, Hoechst 33342 (CAS number 23491-52-3), acridine orange (CAS number 65-61-2), and SYTO 9 (SYTO 9 green fluorescent nucleic acid stain; the structure is proprietary), and five mitochondrial dyes, MitoRed (MitoTracker Red CMXRos; CAS number 167095-09-2), MitoGreen (MitoTracker Green FM; CAS number 201860-17-5), dihexyloxacarbocyanine iodide (DiOC6; CAS number 53213-82-4), rhodamine 123 (molecular weight [MW], 380.83; CAS number 62669-70-9), and rhodamine B (hexyl ester perchlorate; MW, 627.17; CAS number 877933-92-1). We compared the activities of these dyes to those of methylene blue (MW, 319.86; CAS number 61-73-4), epoxomicin (MW, 554.7; CAS number 134381-21-8), DHA (MW, 284.35; CAS number 71939-50-9), artesunate (MW, 384.4; CAS number 88495-63-0), and primaquine (primaquine phosphate; MW, 455.33; CAS number 63-45-6). Methylene blue was obtained from AppliChem. All other dyes were from Invitrogen. Epoxomicin and DHA were obtained from Calbiochem and Shinpoong, respectively. Artesunate and primaquine were from Santa Cruz Biotechnology.

Methylene blue and acridine orange were dissolved in water, while all other tested dyes were dissolved in dimethyl sulfoxide (DMSO). Further dilutions were done with complete culture medium. Final DMSO concentrations were less than 0.5%, a concentration that does not interfere with gametocyte viability.

Epoxomicin was used as a positive control, as it is a potent inhibitor of all clinically relevant parasite stages, including gametocytes, *in vitro*.

ATP-based bioluminescence assay. We used a bioluminescence assay based on the luciferin-luciferase ATP-dependent reaction to assess the activity of the different compounds against mature gametocytes.

The assay was performed as previously described with some modifications (8, 30). In brief, compounds were 3-fold serially diluted in a 96-well plate. After purification, gametocytes were counted microscopically using an improved Neubauer cell-counting chamber, and 5×10^4 purified stage V gametocytes were added to each well in a final volume of 100 μl. In order to validate the test protocol and determine the linear relationship between the ATP content and the number of gametocytes in culture medium, we generated a standard curve using a 2-fold serial dilution starting

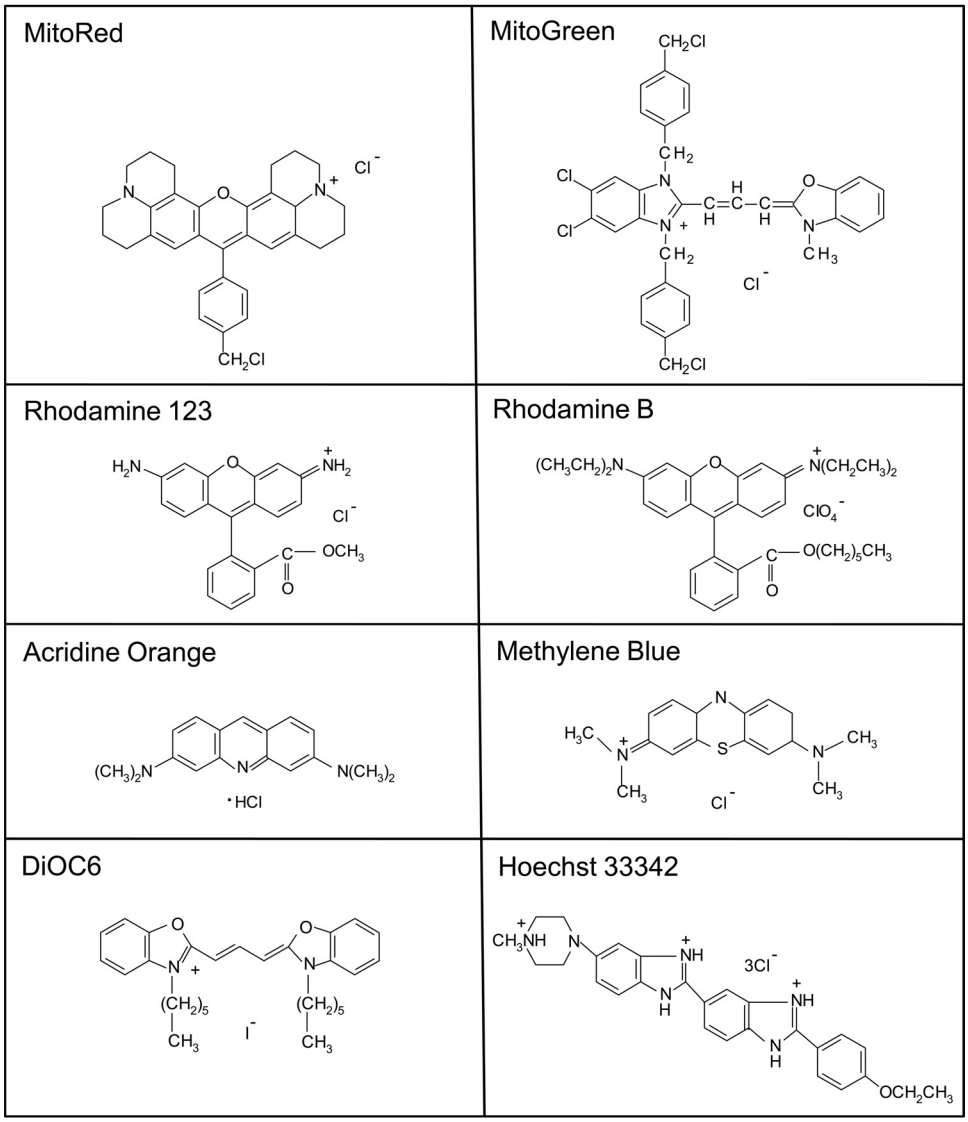

FIG 2 Chemical structures of the tested synthetic dyes. The structure of SYTO 9 is proprietary.

with 5×10^4 purified mature gametocytes. On every plate, a serial dilution of viable stage V gametocytes was added as a control.

ATP measurements were done using equal volumes (50 μl) of gametocyte culture and BacTiterGlo reagent (Promega, Mannheim, Germany) in white opaque-walled 96-well plates, which were incubated at room temperature in the dark for 5 min to allow cell lysis and stabilization of the luminescent signal. Subsequently, the luminescence signal was recorded with a luminometer (LUMO; Anthos Mikrosysteme, Krefeld, Germany).

Stage V gametocytes were exposed to serial dilutions of the test compounds for 24 h or 48 h to assess the effect of the incubation time on the activity. Experiments using 3D7 and a 48-h incubation time were performed at least three times independently.

Statistical analysis. We used the drc package (version 2.3.7) of R (version 2.15.1) to calculate the inhibitory concentrations of compounds by nonlinear regression analysis of log concentration-response curves. The 50% inhibitory concentrations (IC_{50}s) were determined for each compound. If more than two experiments were performed, the mean and standard deviation are given. The coefficient of determination (R^2) was calculated to assess correlations.

Ethical approval. We used three clinical isolates described in a previous study (27). The study was approved by the ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné and followed the principles of the Declaration of Helsinki (6th revision). The participating children and legal representatives gave assent and informed consent, respectively.

RESULTS

Viability of mature gametocytes and validation of luminescence. As a first step, we evaluated the viability of gametocytes after purification and validated the ATP readout. Gametocytes appeared noncompromised after purification with intact cytoplasm (Fig. 1), male gametocytes showed exflagellation, and stage V gametocytes consistently remained viable for approximately 3 weeks. ATP levels and the number of gametocytes in the assay correlated closely ($R^2 = 0.99$).

Activity of fluorescent dyes against mature stage V gametocytes. Five of the eight fluorescent dyes in our assay showed IC_{50}s below 1 μM (Table 1). Among the dyes tested, the three mitochondrial dyes, MitoRed, DiOC6, and rhodamine B, showed the best activity, with IC_{50}s being below 200 nM, which is even lower

TABLE 1 *In vitro* activities of standard antimalarial drugs and dyes against stage V gametocytes of the *P. falciparum* 3D7 laboratory strain and clinical isolates after 48 h of incubation[a]

Compound or dye	Mean IC_{50} ± SD (μM)				
	3D7 gametocytes	Clinical isolates (gametocytes)			3D7 asexual blood stages
		Isolate 1	Isolate 2	Isolate 3	
Standard compounds					
Epoxomicin	0.0016 ± 0.0007	0.0021	0.0024	0.0015	0.005
DHA	0.20 ± 0.07	2.81	2.66	1.80	0.002
Artesunate	0.38 ± 0.12	4.39	0.99	2.91	0.001
Primaquine	20.05 ± 5.67	4.88	7.11	10.55	
Methylene blue	0.77 ± 0.68	1.05	1.49	1.05	0.008[b]
Dyes with IC_{50}s of <300 nM for 3D7 gametocytes					
MitoRed	0.07 ± 0.04	0.21	0.18	0.12	0.008[b]
DiOC6	0.14 ± 0.03	0.11	0.72	0.58	0.011[b]
Rhodamine B	0.18 ± 0.08	0.23	0.99	0.34	0.026[b]
Hoechst 33342	0.23 ± 0.06	0.25	0.31	0.44	0.007[b]
SYTO 9	0.26 ± 0.07	1.34	1.13	1.24	0.021[b]
Dyes with IC_{50}s of >1 μM for 3D7 gametocytes					
MitoGreen	1.02 ± 0.23	1.66	1.24	1.45	0.116[b]
Acridine orange	3.04 ± 1.07	36.56		2.63	0.466[b]
Rhodamine 123	7.13 ± 2.97	13.83		10.39	0.388[b]

[a] The activities of the evaluated compounds against mature gametocytes of 3D7 and three different clinical isolates of *P. falciparum* were measured after 48 h of incubation in serum-supplemented medium. Results for 3D7 gametocytes are the means of three independent experiments.

[b] For comparison, the activity of dyes against asexual blood stages evaluated by a histidine-rich protein 2 enzyme-linked immunosorbent assay is given (results are from Joanny et al. [22]). The activities of epoxomicin, artesunate, and DHA were evaluated with the same method in our laboratory.

than the IC_{50} of the control dye, methylene blue (770 nM). Among all dyes, MitoRed was the most active compound against laboratory strain 3D7 as well as against clinical isolates. The activities of the different dyes as well as the control drugs were consistently higher against the laboratory strain 3D7 than against clinical isolates (2- to 10-fold). Particularly, artesunate and DHA had considerably higher IC_{50}s against clinical isolates than against 3D7. As expected for a prodrug, primaquine did not inhibit gametocytes at concentrations achievable *in vivo*. Epoxomicin served as our internal positive control and was highly active against the 3D7 laboratory strain and clinical isolates. The IC_{50}s of the single compounds against asexual blood stages are given for comparison (Table 1). All compounds except epoxomicin were less active against gametocytes than against asexual blood stages.

Activities of compounds after different incubation times (24 h and 48 h) with mature gametocytes. *In vitro* gametocyte assays are poorly standardized and use different incubation times to test gametocytocidal compounds (8, 30). To assess the influence of the incubation time on the antiparasitic activity, we incubated fluorescent dyes and standard antimalarial drugs for either 24 h or 48 h with stage V gametocytes of 3D7 parasites and clinical isolates. For most compounds the incubation time had no significant effect (Table 2). However, MitoRed was 3-fold more active when used over 48 h than when used over 24 h. This effect was even more pronounced for epoxomicin and artesunate incubations, with a 10-fold increase in activity when parasites were exposed for 48 h.

DISCUSSION

The primary aim of antimalarial treatment is to remove asexual blood-stage parasites in order to cure the disease and prevent or treat severe forms of malaria. In contrast, late-stage gametocytes do not cause significant harm to the infected individual and appear in the circulation after a lag period due to sequestration. Most of the current antimalarials act against asexual blood stages but have no significant effect on sexual stages, which remain in the circulation for extended periods after treatment and could therefore be transmitted (31, 32). To achieve sustained malaria control and contain resistance development, an efficient gametocytocidal drug is fundamental. We identified five synthetic dyes with good gametocytocidal activity. These five dyes, particularly the three mitochondrial dyes MitoRed, DiOC6, and rhodamine B, were more active than methylene blue, a drug with known gametocytocidal activity (15, 23–25). The cytotoxic concentration of the mitochondrial dyes was higher than 2.5 μM when evaluated against HeLa cells in our previous study (22). MitoRed was the most active compound against gametocytes. Interestingly, we had previously selected it as a promising candidate for development as an antimalarial due to its high activity against asexual blood-stage parasites and its favorable selectivity index when tested against HeLa cells. It belongs to the rhodamine dyes. Some of its derivatives are used in the cosmetic industry, where toxicity has been more extensively studied (33, 34). However, no further toxicity data on MitoRed are available. The results from the current gametocyte assay underline the interesting properties of mitochondrial dyes as starting points for new antimalarials, and further toxicity studies of the most promising compounds should be performed. Interestingly, all compounds except epoxomicin showed higher activity against asexual blood stages than against gametocytes. This is likely due to the less active metabolism during the late gametocyte stage. Dyes selectively target certain organelles in the

TABLE 2 *In vitro* activities of compounds against mature gametocytes of 3D7 and clinical isolates of *P. falciparum* after 24 h or 48 h of incubation[a]

Compound, *P. falciparum* strain	IC_{50} (μM) after incubation for:	
	24 h	48 h
Standard compounds		
Artesunate		
3D7	31.38	0.38[b]
Clinical isolate 1	24.04	4.39
Primaquine		
3D7	44.92	20.05[b]
Clinical isolate 1	4.64	4.88
Epoxomicin, clinical isolate 1	0.015	0.002
DHA, clinical isolate 1	2.86	2.81
Dyes		
MitoRed, 3D7	0.20	0.07[b]
Acridine orange		
3D7	3.19	3.04[b]
Clinical isolate 3	1.90	2.63
MitoGreen, 3D7	2.30	1.02[b]
DiOC6, clinical isolate 1	0.47	0.11
Rhodamine B		
Clinical isolate 1	0.18	0.23
Clinical isolate 3	1.41	0.34
Hoechst 33342, clinical isolate 1	0.25	0.24
Methylene blue, clinical isolate 1	2.04	1.05
Rhodamine 123		
Clinical isolate 1	14.71	13.83
Clinical isolate 3	14.85	10.39
SYTO 9		
Clinical isolate 1	0.48	1.34
Clinical isolate 3	1.03	1.13

[a] Individual values are presented unless indicated otherwise.
[b] Values are the means of three independent experiments (48-h assay of 3D7 laboratory strains).

organism, presumably inhibiting the targeted organelle. Interestingly, atovaquone, a potent inhibitor of the cytochrome bc1 complex of the mitochondrion, does not affect gametocyte development (8). Nevertheless, mitochondrial dyes are highly active, which is most likely because they do not share the same mechanism of action.

In addition to *P. falciparum* 3D7 gametocytes, we used three clinical isolates from Lambaréné, Gabon, an area of high drug pressure and widespread chloroquine and folate inhibitor resistance (35, 36). Gametocytes derived from the laboratory strain 3D7 seemed to be more sensitive to most compounds than the tested clinical isolates. This finding has important implications for larger screening programs, since hits achieved in laboratory isolates should be considered to be validated in clinical isolates. Primaquine presented one exception to this rule because it is more active against clinical isolates than against 3D7. However, the *in vitro* activity is likely not a good surrogate for the relevant activity of primaquine *in vivo*, as it has to be metabolized by CYP2D6 (37, 38) and monoamine oxidase A (38) to be active. Therefore, primaquine shows rather unspecific activity *in vitro* and can be used only as a comparator between assays, showing that our results are in line with results obtained by others (8, 30, 39).

The difference in activities between clinical isolates and 3D7 was particularly high for artemisinin derivatives. Whether or not this is due to drug pressure may only be hypothesized. Artemisinin combination therapy has been the recommended first-line therapy since 2003 in Gabon (40). Since then, local parasite populations may have adapted to the presence of artemisinin derivatives. It would be very interesting to test parasite isolates obtained before 2003. Unfortunately, such isolates were not available.

A prolonged incubation time influenced the activity of the drugs. In particular, the most active compounds and artemisinin derivatives were more active when incubated for 48 h than when incubated for 24 h. There are two possible explanations for this: (i) the drug needs a longer time to exert its effect, or (ii) the drug acts early but the readout detects only late changes in the metabolism of the gametocytes. Since artemisinin derivatives have a very short half-life (41), scenario (i) would result in very low *in vivo* activity, whereas scenario (ii) is more likely. When we compare the results to those of our previous assays in which we used medium supplemented with Albumax instead of human serum (42), we can also see an approximately 10-fold increase of the IC_{50} for artesunate and DHA for the Albumax-supplemented medium. The *in vitro* activity of artemisinin derivatives against late-stage gametocytes is highly variable and depends on the assay used. Commonly used methods differ in the numbers of gametocytes, parasite strains, incubation times, serum supplementation, and readouts used. These differences result in inhibitory concentrations between 3 nM and 11 μM for the same compound (8, 30, 43–45). These differences demonstrate that better standardization and validation of assays is required in order to compare results and identify compounds with promising activities. One method of independent validation would be the use of standard membrane feeding assays. Interestingly, most reports show modest effects (24, 44) or no effect (46) of artemisinins on oocyst counts following membrane feeding.

In conclusion, fluorescent dyes, especially MitoRed, show high *in vitro* gametocytocidal activities and should be considered starting points for the development of new antimalarial compounds with transmission-blocking potential. In addition to laboratory strains, gametocytes of clinical isolates should be considered for the screening of new compounds, as they have a different genetic background and might show a resistance profile different from that of laboratory strains.

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Antimalarial Activity of the Myxobacterial Macrolide Chlorotonil A

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Myxobacteria are Gram-negative soil-dwelling bacteria belonging to the phylum *Proteobacteria*. They are a rich source of promising compounds for clinical application, such as epothilones for cancer therapy and several new antibiotics. In the course of a bioactivity screening program of secondary metabolites produced by *Sorangium cellulosum* strains, the macrolide chlorotonil A was found to exhibit promising antimalarial activity. Subsequently, we evaluated chlorotonil A against *Plasmodium falciparum* laboratory strains and clinical isolates from Gabon. Chlorotonil A was highly active, with a 50% inhibitory concentration between 4 and 32 nM; additionally, no correlations between the activities of chlorotonil A and artesunate (rho, 0.208) or chloroquine (rho, −0.046) were observed. *Per os* treatment of *Plasmodium berghei*-infected mice with four doses of as little as 36 mg of chlorotonil A per kg of body weight led to the suppression of parasitemia with no obvious signs of toxicity. Chlorotonil A acts against all stages of intraerythrocytic parasite development, including ring-stage parasites and stage IV to V gametocytes, and it requires only a very short exposure to the parasite to exert its antimalarial action. Conclusively, chlorotonil A has an exceptional and unprecedented profile of action and represents an urgently required novel antimalarial chemical scaffold. Therefore, we propose it as a lead structure for further development as an antimalarial chemotherapeutic.

Malaria is the most important parasitic disease worldwide, with an estimated 207 million cases causing 627,000 deaths in 2012 (1). Among *Plasmodium* spp. causing malaria in humans, *Plasmodium falciparum* is responsible for almost all severe and fatal cases and is the predominant species in sub-Saharan Africa. Even though the scaling up of malaria control programs has led to a reduction in its incidence and mortality rates (1) and first-generation vaccines show some efficacy against it (2, 3), chemotherapy remains the mainstay of treatment for all forms of malaria. A major threat for chemotherapy is the development of resistance, since resistant phenotypes have been reported for most registered antimalarials (4). In sub-Saharan Africa, resistance against the former first-line drugs chloroquine and sulfadoxine-pyrimethamine is widespread (5), and decreased activity of artemisinin derivatives is well documented in Southeast Asia (6). The loss of artemisinin activity is of particular concern, since all current efforts to control malaria are based on this class of compounds (7). To keep pace with the parasite's ability to develop resistance, researchers need to make a continuous effort to develop new drugs, especially in cases of severe malaria, for which only two alternative treatments (artesunate and quinine) are available (8).

Treatment and pharmacological requirements of drugs to treat uncomplicated or severe malaria differ fundamentally. Drugs for uncomplicated malaria should be available as an oral formulation, be easily administered (ideally once), and have a very good safety profile (e.g., wide therapeutic range; low toxicity, especially in children and pregnant or lactating women; and few unwanted side effects); ideally, they protect the individual for a prolonged time period and protract resistance development. In contrast, patients with severe and complicated malaria need fast-acting and usually parenterally administered drugs, which rapidly reduce the parasite burden and display activity against all parasite isolates within a narrow concentration range. Their pharmacokinetic profiles should allow parenteral administration in critically ill patients with organ failure. Since children are the most affected group, all antimalarials must be safe in this age group and must be available in pediatric formulations (9).

To control malaria on the epidemiological level and prevent the spread of resistant parasites, antimalarial drugs should act on gametocytes, the sexual stages of the parasite, to block transmission to mosquitoes. However, most current antimalarials do not act against gametocytes, and transmission of the parasite is not prevented (10). Nevertheless, gametocytocidal activity is highly desirable for new antimalarials to be used in elimination and eradication programs (11).

In the past, a particularly powerful way to find new chemotherapeutics against infectious diseases was by characterizing and developing natural compounds and their derivatizations (12). One reason for the success rate with natural products may be the long-lasting interaction of coevolving organisms, resulting in structural classes optimized for their respective targets. In malaria, most highly active compounds are plant derived and are thought to have a role in deterring herbivores (e.g., quinine) or to be potent herbicides (e.g., artemisinin) (13–16). Besides plants and fungi, bacteria, including the soil-dwelling myxobacteria (17, 18), are a rich source of biologically active compounds (19–23). We investigated the antimalarial properties of a chlorine-containing metabolite, chlorotonil A, whose antiplasmodial activity was primarily identified when screening a library of myxobacterial substances at the Swiss Tropical and Public Health Institute. Chlorotonil A, a

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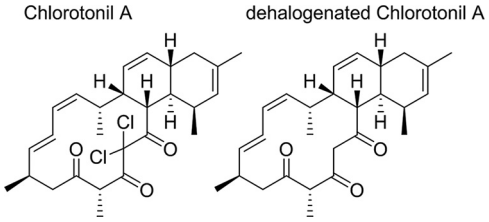

FIG 1 Chemical structures of chlorotonil A and its dehalogenated form.

tricyclic macrolide produced by *Sorangium cellulosum*, was first isolated and described by Gerth et al. (24). The total synthesis of this substance and its dehalogenated derivative was reported recently (Fig. 1) (25). In our study, we found chlorotonil A to exhibit potent *in vitro* and *in vivo* activity against *P. falciparum* and *P. berghei*, respectively. It acted against all erythrocytic stages of the parasite, showed a very rapid onset of action, and was active *in vitro* against the stages responsible for transmission.

MATERIALS AND METHODS

Parasite culture. *P. falciparum* strains 3D7 (chloroquine sensitive) and Dd2 (chloroquine, sulfadoxine, and pyrimethamine resistant) were maintained in continuous culture as previously described (26). In brief, parasites were kept in complete culture medium [RPMI 1640, 25 mM 4-(2-hydroxyethyl) piperazine-*N*′-(4-butanesulfonic acid), 2 mM L-glutamine, 50 μg/ml gentamicin, and 0.5% (wt/vol) AlbuMax] at 37°C and in 5% CO_2 and 5% oxygen at 5% hematocrit with daily changing of the medium. Synchronization was performed by sorbitol twice a week (27).

Chemicals. Chlorotonil A (molecular weight [MW], 479.44) was isolated as described before (24), and a compound without chlorine atoms (MW, 410.54) was obtained by dehalogenation (see the structures in Fig. 1). Stock solutions of chlorotonil A and its dehalogenated form were prepared at 2.1 mM and 2.4 mM in tetrahydrofuran (THF). The comparator drug artesunate (Shin Poong Pharmaceutical Co.) (MW, 384.4) was prepared in 70% ethanol at 15 mM, chloroquine diphosphate (Sigma) (MW, 515.86) in double-distilled water at 9.7 mM, and epoxomicin (Sigma) (MW, 554.7) and dihydroartemisinin (Shin Poong Pharmaceutical Co.) (MW, 284.35) in dimethyl sulfoxide (DMSO) at 1 mM and 20 mM, respectively. Further dilutions of all drugs were done in complete culture medium. None of the solvent dilutions used interfered with parasite growth in the pilot experiments. All results given are in nM.

***In vitro* drug sensitivity assay of laboratory strains.** Drug sensitivity assays were performed according to standard procedures (28). In brief, 96-well plates were precoated with a 2-fold serial dilution of the respective drug. Ring-stage parasites were diluted to a parasitemia level of 0.05% with ≥0 erythrocytes and seeded at a hematocrit level of 1.5% in a total volume of 225 μl per well. After 3 days, the plates were freeze-thawed twice and analyzed by measuring histidine-rich protein 2 (HRP2) with an enzyme-linked immunosorbent assay (ELISA). To measure the delayed activity of the drug, we incubated parasites for 6 days (29); on days 2 and 4, the medium was changed (140 μl) without replacement of the drug. All experiments were done in duplicate and repeated at least three times.

***In vitro* drug sensitivity assay of clinical isolates.** We tested the activity of chlorotonil A compared to those of standard drugs (artesunate and chloroquine) against *P. falciparum* clinical isolates from patients with uncomplicated malaria in Lambaréné, Gabon, between February and May 2009. The investigations of the *in vitro* drug sensitivity of the clinical isolates were approved by the ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné. Assent and informed consent were obtained from each child and his or her legal representative, respectively. A venous blood sample was taken into a lithium heparin tube and processed within 4 h. The assays were performed as for lab strains with minor modifications. Plates were precoated with a 3-fold dilution of each drug, and the parasites were incubated in a candle jar for 3 days at 37°C. Assays were performed only once, directly after the blood draw. Only samples in which the amount of detected HRP2 at least doubled within the 3 days were included in the analysis.

Stage-specific analysis. Synchronized parasites at the ring, trophozoite, and schizont stages were incubated with the respective drug at a 1.5% hematocrit level and a parasitemia level between 1% and 4% in a 96-well plate for a total of 40 h. Samples were taken every 8 h to determine parasite development microscopically by Giemsa (Sigma)-stained thin blood smears. Artesunate (20 nM) was used as a positive control for stage-specific action against rings and trophozoites. For action against the schizont stage, epoxomicin (500 nM) and artesunate (500 nM) were used as controls. Chlorotonil A was used at a concentration of 40 nM for the determination of action against the ring and trophozoite stages and also at 500 nM against the schizont stage.

Evaluation of onset of action. Assays were performed as for the *in vitro* standard drug sensitivity assay with laboratory strains but with removal of the drug after 1 h. In detail, synchronized ring-stage 3D7 parasites were seeded at 0.05% parasitemia level and 1.5% hematocrit level on precoated 96-well plates (chlorotonil A, artesunate, and chloroquine in a 3-fold serial dilution). After 1 h of incubation, the drugs were removed by washing with complete medium 3 times. A control plate, for which the washing step was omitted, was incubated in parallel to compare the results. Subsequently, incubation of the plates was continued for 3 days as for the standard assay before the HRP2 ELISA was performed. The 50% inhibition concentrations (IC_{50}s) of the 1-h pulsed and standard plates of 3 independent experiments were compared. Differences in the IC_{50}s are presented as mean fold change increase ± standard deviation with respect to the control plates. All experiments were performed in duplicate.

***In vivo* efficacy.** The *in vivo* antimalarial activity of chlorotonil A was assessed against that of the rodent malaria strain *P. berghei* ANKA (provided by David Walliker, University of Edinburgh, United Kingdom) in BALB/c and Swiss CD1 mice in the 4-day suppression test (30, 31). In brief, 4 BALB/c mice and 5 Swiss CD1 mice were inoculated intravenously with 2×10^7 parasitized erythrocytes (diluted in phosphate-buffered saline [PBS]) obtained from a donor mouse. After being infected, the mice were treated at 2 h, 24 h, 48 h, and 72 h postinfection with different amounts of chlorotonil A powder (BALB/c, 36, 68, and 110 mg/kg of body weight [1 control]; Swiss CD1, 3 mice received 100 mg/kg [2 controls]) in 100 mg of peanut butter (Barney's Best) or placebo control (peanut butter only). Peanut butter with or without chlorotonil A was given to each mouse individually out of a syringe. Thin blood smears from tail blood were taken daily from days 1 to 5 and stained with 20% Giemsa stain (Sigma). A minimum of 1,000 erythrocytes per slide was counted microscopically to assess parasitemia. The activity is expressed as percent reduction of parasitemia in comparison to the control group according to the following equation: activity = 100 − (mean parasitemia treated/mean parasitemia control) × 100.

Activity was analyzed on day 4; additionally, we analyzed activity on day 5, which is recommended for slow-acting drugs (31). The mouse experiments were approved by the competent authority for animal experiments in Tübingen (no. T1/08) and performed according to German legislation.

Gametocytocidal activity. The gametocytocidal activity of chlorotonil A was evaluated by an ATP bioluminescence assay as described previously (32). As comparators, artesunate, epoxomicin, and dihydroartemisinin were tested. In brief, gametocyte culture was initiated from synchronized 3D7 parasites with an increased concentration (0.75% [wt/vol]) of AlbuMax II solution, starting with a 10% hematocrit level and a 0.5% parasitemia level. Culture medium was changed daily without parasite dilution throughout the entire process. When the parasitemia level reached 5%, the volume of the medium was doubled, and the concentration of AlbuMax II was reduced to 0.5% (wt/vol). Between days 11 and 14, the cultures were treated with 50 mM *N*-acetyl-D-glucosamine (MP Biomedicals GmbH) to remove the asexual stages, and on day 15, the culture

TABLE 1 Inhibitory concentrations of tested compounds against *P. falciparum* strains 3D7 and Dd2[a]

Compound	IC (mean ±SD) (nM) of indicated compound for strain:			
	3D7		Dd2	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Chlorotonil A	9.1 ± 3	13.34 ± 3.2	18.1 ± 8.6	28.5 ± 8.5
Chloroquine	5.2 ± 1.2	8.2 ± 2.3	160.4 ± 62.1	228.6 ± 66.4
Artesunate	2.4 ± 1.3	7.5 ± 4.6	1.3 ± 0.2	1.8 ± 0.2

[a] Each value is the mean inhibitory concentration of at least 3 independent experiments performed in duplicate.

TABLE 2 Median inhibitory concentrations of 25 (chlorotonil A and chloroquine) and 26 (artesunate) different clinical isolates against the indicated compounds[a]

Compound	IC_{50} (median [range]) (nM)	IC_{90} (median [range]) (nM)
Chlorotonil A	15.2 (3.7–32)	37.1 (6.8–76.2)
Chloroquine	47.2 (19.5–117)	111.6 (44.2–191.1)
Artesunate	0.6 (0.2–3.2)	1.8 (0.6–4.7)

[a] Each experiment was performed once.

was purified by a NycoPrep 1.077 cushion density gradient and magnetic column separation in order to remove erythrocytes and enrich the gametocyte population.

The compounds were precoated in a 3-fold dilution in 96-well plates before 50,000 gametocytes were added to each well in a final volume of 100 μl and incubated at 37°C in 5% CO_2 and 5% oxygen. After 48 h, ATP production of the gametocytes was measured by the BacTiterGlo assay (Promega) according to the manufacturer's protocol and recorded by a Victor^3 V multilabel reader (PerkinElmer, Inc.). Each experiment was repeated at least three times.

Statistics. Individual inhibitory concentrations were determined by nonlinear regression analysis of log-concentration-response curves using the drc-package v0.9.0 (33) of R v2.3.1 (34). The mean 50% and 90% inhibition concentrations and standard deviations are presented for each drug assayed in the laboratory strains. For clinical isolates, the median 50% and 90% inhibition concentrations and their ranges are given. Correlations between the IC_{50}s of clinical isolates of the three different drugs were calculated with Spearman's (nonparametric) test for paired samples in JMP v5.0.1.2 software.

RESULTS

Chlorotonil A acts against laboratory and clinical *P. falciparum* isolates *in vitro*. Chlorotonil A potently inhibits the growth of chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *P. falciparum* parasite strains *in vitro* (Table 1). As reference compounds, chloroquine and artesunate were analyzed. To assess if chlorotonil A shows signs of hysteresis (also called delayed-death phenomenon) similar to those in some other antimalarial antibiotics, we performed a 6-day assay covering two intraerythrocytic cycles of parasite replication. The IC_{50}s obtained in the 6-day assay were 10.6 ± 4.1 nM (3D7) and 23.5 ± 9.4 nM (Dd2), and the IC_{90}s were 13.8 ± 6.4 nM (3D7) and 32.4 ± 13 nM (Dd2), indicating that chlorotonil A acts directly upon first contact with the parasite. The dehalogenated form of chlorotonil A showed no measurable activity against Dd2 and 3D7, even at the highest concentration tested (540 nM).

To assess the variability of the activity in clinical isolates of *P. falciparum*, we measured the activity of chlorotonil A against parasites freshly isolated from patients with uncomplicated malaria in Lambaréné, Gabon. Of 28 collected clinical isolates, 25 (chlorotonil A and chloroquine) and 26 (artesunate) were successfully grown in culture and analyzed in the *in vitro* assay. Chlorotonil A was active against clinical isolates, with IC_{50}s comparable to those obtained against laboratory strains; in addition, the determined IC_{50}s for the clinical isolates showed narrow ranges (Table 2). No correlations between the activities of chlorotonil A and artesunate (rho, 0.208; $n = 25$) or chloroquine (rho, −0.046; $n = 25$) were observed.

Chlorotonil A acts against all blood stages of the parasite. To investigate the effects of chlorotonil A on the morphology of different stages, we incubated synchronous 3D7 ring, trophozoite, and schizont stages with the drug and stained thin blood smears by Giemsa stain after 8, 24, and 40 h. Chlorotonil A arrested parasite development in the ring and trophozoite stages. Morphologically, the parasites looked similar to artesunate-treated parasites, since their development stopped immediately after contact with the compound (Fig. 2A and B). When added to schizont-stage parasites, higher concentrations (>40 nM) were required. Incubation with 500 nM chlorotonil A led to an arrest in development, which is comparable to the activity of proteasome inhibitors (e.g., epoxomicin) that act against the schizont stage (Fig. 2C). Notably, artesunate added at the same concentration (500 nM) did not block the egress of parasites; furthermore, erythrocytes newly infected by merozoites were found (Fig. 2C, notice the presence of ring-stage parasites after 8 h). The chlorotonil A-induced arrest in the schizont stage was apparent after 8 h by thin blood smear. At later time points, arrested schizonts showed signs of degradation.

Chlorotonil A acts quickly. The standard *in vitro* susceptibility assay does not account for short drug pulses, but *in vivo* drug concentrations fluctuate, and high concentrations are maintained for limited time periods only. Therefore, we incubated ring-stage parasites with chlorotonil A or the comparator drugs (artesunate and chloroquine) for 1 h, removed the drug, and continued incubation as in the standard drug sensitivity assay. The IC_{50} of a 1-h pulse of chlorotonil A was only 1.3 ± 0.7-fold higher than that of the standard assay. In contrast, the IC_{50} of a 1-h pulse of artesunate was 15.6 ± 9.1-fold higher than that of the standard assay. As expected, chloroquine was not active when given as a short pulse at the ring stage, even at the highest concentration tested (160 μM).

Chlorotonil A is active *in vivo*. Chlorotonil A is not soluble in most commonly used solvents. Nevertheless, a pilot experiment to assess the therapeutic efficacy of nonsolubilized orally administered chlorotonil A was performed in *P. berghei* ANKA-infected BALB/c mice and Swiss CD1 mice using the Peters 4-day suppression test (31). The activity levels of chlorotonil A on day 4 were 97% in BALB/c mice and 98% in Swiss CD1 mice compared to that of the control mice. On day 5, the antiplasmodial activity levels were 93% (BALB/c) and 85% (Swiss CD1). However, the cure was not complete with the standard protocol, as none of the mice were cleared of their parasitemia completely (Fig. 3). In BALB/c mice, all the doses (36, 68, and 110 mg/kg) resulted in a substantial reduction of parasitemia. This reduction showed some dose dependency; however, due to the small sample size, this was not analyzed formally. The mice did not show obvious signs of toxicity due to chlorotonil A treatment at either dose.

Chlorotonil A is active against gametocytes. To assess the activity of chlorotonil A against stage IV to V gametocytes, we used

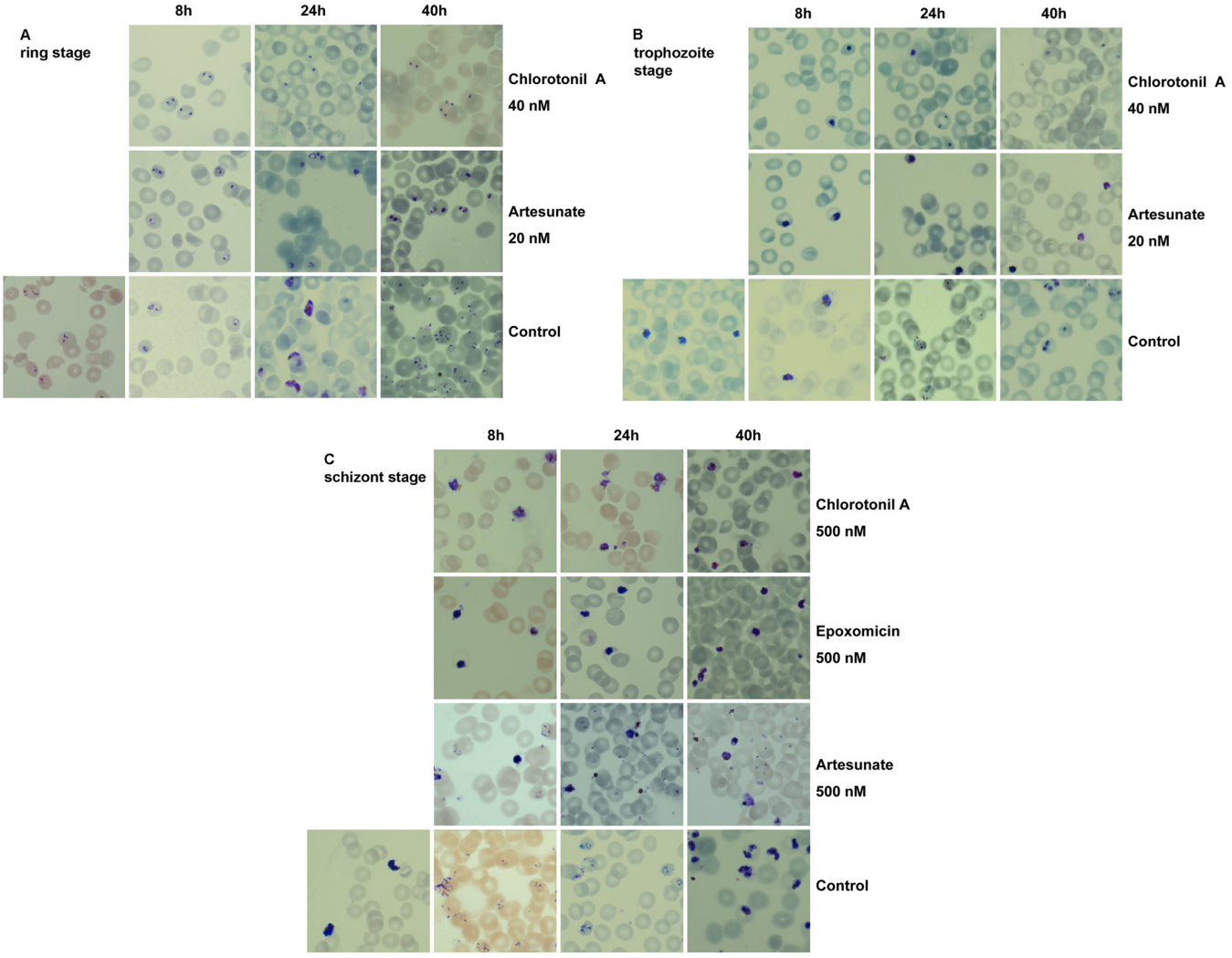

FIG 2 Stage-specific inhibition of *P. falciparum* by chlorotonil A. Giemsa-stained thin blood smears of synchronous ring (A), trophozoite (B), and schizont (C) stages. Shown are *P. falciparum* 3D7 parasites after different incubation times (8, 24, and 40 h) with chlorotonil A and artesunate (ring and trophozoite stages) and epoxomicin and artesunate (schizont stage) at the indicated concentrations compared to those of the drug-free control.

an *in vitro* bioluminescence assay (32). Chlorotonil A was active against late-stage gametocytes at concentrations similar to those against asexual blood stages. This is in contrast to artesunate and dihydroartemisinin, which act only when used in very high concentrations (Table 3). Epoxomicin served as an internal control for the assay, since it is known to be highly active against stage IV to V gametocytes.

DISCUSSION

So far, continuous development of new drugs remains the only option for keeping pace with the potential of *P. falciparum* to adapt to manmade interventions. Myxobacteria, especially those in the genus *Sorangium*, have proven to be a valuable source for new chemotherapeutic compounds, such as soraphens, sorangicins, thuggacins, and epothilones (35–37), some of which are in advanced preclinical and clinical development. For example, ixabepilone (BMS-247550), an analogue of epothilone B, was approved by the Food and Drug Administration in 2007 for the treatment of breast cancer (38). *Sorangium cellulosum* strain So ce1525 was identified as our main producer of chlorotonil A and yields several natural products exhibiting different chemical scaffolds. New scaffolds are of particular interest for the development of new antimalarials, since most compounds in the development pipeline belong to a restricted number of drug classes.

Chlorotonil A shows several remarkable features which characterize it as a promising lead compound. Apart from being active against chloroquine-sensitive and chloroquine-resistant strains at low nanomolar concentrations, it acts against all blood stages of the malaria parasite and does not show a hysteresis effect, which is a characteristic of several antimalarial antibiotics (39, 40). Chlorotonil A acts against early in the ring and trophozoite stages of the parasite and is therefore able to reduce parasite biomass and the generation of parasite toxins immediately upon contact with the parasite (41). This is notable since it is one characteristic that needs to be fulfilled by drug candidates for the treatment of severe malaria; also, artemisinin derivatives have a similar profile of action. The mode of action of chlorotonil A is not known, but the loss of activity observed for the dehalogenated derivative points

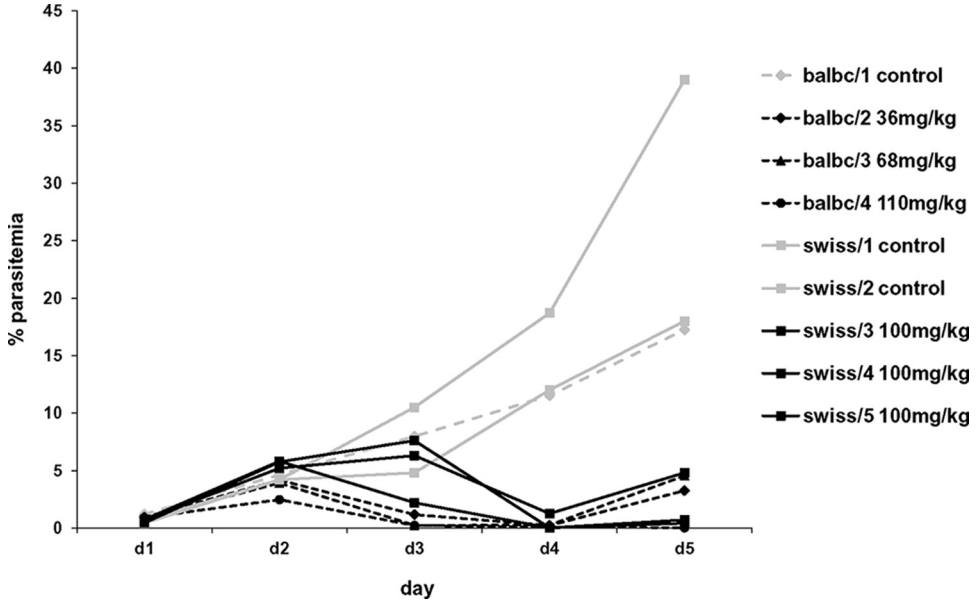

FIG 3 Parasitemia levels of *P. berghei* in chlorotonil A-treated mice. Parasitemia levels in four *P. berghei*-infected BALB/c (balbc/1 to balbc/4) and five Swiss CD1 (swiss/1 to swiss/5) mice from day 1 (d1) to d5 in the 4-day suppression test. Mice indicated by black lines received either 36, 68, or 110 mg/kg (BALB/c) or 100 mg/kg (Swiss CD1) chlorotonil A, and mice indicated by the light-gray lines received placebo control.

toward an important role of the chlorine-containing pharmacophore. It also acts against clinical isolates with a different genetic background, isolated from patients in Lambaréné, Gabon, an area of high-grade resistance against chloroquine and sulfadoxine-pyrimethamine (42, 43). The lack of correlation with its comparator drugs, chloroquine and artesunate, supports the assumption that chlorotonil A has a different mechanism of action than that of these two other drugs.

The first tests in the murine *P. berghei* model demonstrated that chlorotonil A is active *in vivo*, has low toxicity, and can be administered orally. Due to its poor solubility, it was given to the mice as a powder together with peanut butter because tetrahydrofuran, the solvent used for *in vitro* assays, is toxic for mice (44). Currently, we are exploring alternative formulations and chemical modifications to develop chlorotonil A from an early lead toward a drug development candidate. In addition, it would be interesting to test anthracimycin, a structural relative of chlorotonil A, and its dichloro derivative (45).

An exceptional property of chlorotonil A is its very rapid onset of action; 1 h of contact with the parasite is sufficient to exert its full activity. The two comparator drugs, artesunate and chloroquine, had strongly reduced activity levels when pulsed for only 1 h. Rapid onset, as seen for chlorotonil A, is especially important for drugs that are used to treat severe malaria, for which a rapid reduction of the parasitemia level is vital for the survival of the patient. In addition, rapid onset is important for drugs with a short half-life, like artesunate and dihydroartemisinin. These two drugs exhibit half-lives of <1 h (46), and thus, the time span of the therapeutic drug concentration above the antiparasitic threshold is short. This is one of the reasons why the cure rates of artemisinin derivatives are poor when given for <5 to 7 days as monotherapy (47). Its activity against late-stage gametocytes is an additional valuable feature of chlorotonil A. Late-stage gametocytes are especially difficult to target by drugs. Until now, primaquine has been the only licensed drug with known activity against them *in vivo*, but its hemolytic effect in individuals with glucose-6-phosphate dehydrogenase deficiency limits its use (48). Artemisinin derivatives decrease gametocyte carriage *in vivo* mainly by their rapid action, accompanied by the reduction of parasite biomass, but it has been shown that they cannot stop transmission completely (49, 50). The effect of artemisinin derivatives on gametocytes is still under discussion; they probably also act against early gametocytes, but only little activity against late-stage gametocytes has been reported (32, 51–53). This is also in concordance with our assay. Especially when compared side by side with chlorotonil A, the activity level of artemisinin is orders of magnitude lower. A new gametocytocidal drug would be a major step forward for the feasibility of elimination and resistance containment campaigns, especially in low-endemicity settings where transmission blocking is crucial for finally stopping the cycle.

Chlorotonil A exhibits the promising characteristics of a potential new lead compound, namely, its low toxicity, oral availability, rapid onset of action, and activity against all erythrocytic stages of the parasite. In addition, it is active against the transmission stages of the parasite. Improved derivatives and dose regimens are required prior to clinical development, but at this stage, it is al-

TABLE 3 Inhibitory concentrations of tested compounds against stage IV to V gametocytes of *P. falciparum* strain 3D7[a]

Compound	IC_{50} (mean ± SD) (nM)	IC_{90} (mean ± SD) (nM)
Chlorotonil A	29.6 ± 16.3	123.2 ± 36.9
Artesunate	8,917 ± 4,830	25,852 ± 19,563
Dihydroartemisinin	2,918 ± 964	16,603 ± 16,141
Epoxomicin	3.2 ± 2.6	14.3 ± 16.7

[a] Each value is the mean inhibitory concentration of at least 3 independent experiments.

ready evident that chlorotonil A has unique features that warrant its further assessment.

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