UNIVERSIDADE DE LISBOA

Faculdade de Medicina

TARGETING THE TRANSMISSION STAGE OF MALARIA PARASITES BY DRUG- AND VACCINE-BASED APPROACHES

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Orientadores: Prof. Doutor Miguel Prudêncio

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, especialidade de Microbiologia e Parasitologia

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2023

A impressão desta tese foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 17 de Abril de 2023

SUMMARY

Malaria remains a public health concern worldwide, despite significant efforts to reduce its case incidence and death rate. In recent years, due to increased funding and renewed efforts to eliminate malaria in endemic regions, interest in development of transmission-blocking strategies (TBS) gained momentum. These tools aim to reduce the prevalence of infection in a population by blocking the transmission of the parasite through the mosquito, consequently reducing the spread of drug-resistant parasites while prolonging the life span of antimalarial drugs. Moreover, targeting malaria transmission has additional advantages: antigens from mosquito stages are less genetically variant than blood and liver stage antigens; circulating parasite numbers in the bloodstream are significantly reduced once inside the mosquito; and parasites are extracellular for approximately 24 h in the mosquito compared to approximately 1 min during red blood cell invasion by merozoites.

Modelling studies have predicted the potential of targeting *Plasmodium* transmission in the context of malaria control, linking insecticide-treated nets and indoor residual spraying to the largest decrease in case prevalence in the African continent from 2000 to 2015. Hence, it seems logical to explore tools targeting the transmission of malaria parasites to complement currently available strategies, expanding the array of malaria control interventions, while addressing concerns raised by insecticide and drug resistance.

The present thesis addresses two independent but complementary TBS, transmission-blocking drugs (TBDs) and transmission-blocking vaccines (TBVs), unveiling the gametocidal and sporontocidal effect of avermectins beyond their known mosquitocidal effect, as well as the potential of antiretroviral (ARV) compounds and current first-line antiretroviral therapies (ARTs) to block malaria parasite transmission, and characterizing a new multistage whole-organism malaria vaccine candidate expressing a transmission-blocking target antigen.

Our results show that ivermectin and other avermectins target the sporogonic stages of *Plasmodium* whilst exerting no inhibitory activity on the parasite's sexual stages, lending further support to mass drug administration (MDA) of ivermectin in malaria-endemic regions to control the spread of disease. Our results also demonstrated that first-line ARTs used against

human immunodeficiency virus (HIV) commonly employed in the field impair the sporogonic development of *Plasmodium in vitro*, with the ART of zidovudine, lamivudine, lopinavir and ritonavir, the first-line treatment recommended for children under 3 years of age, reducing oocyst density *in vivo*. Finally, we show that a genetically modified *P. berghei* parasites can be engineered for expression of multiple *P. falciparum* (*Pf*) antigens, including the pre-erythrocytic *Pf* circumsporozoite protein (*Pf*CSP), the mosquito stage *Pfs*48/45 and the erythrocytic *Pf* reticulocyte-binding protein homolog 5 (*Pf*Rh5), thus creating a multistage malaria vaccine candidate. Our data confirm the correct expression of *Pf*CSP and *Pfs*48/45 by these parasites, as well as their immunogenicity in a rodent model of infection.

The continued development of new *Plasmodium* TBS is essential to diversify and extend our current array of malaria control interventions. Our work provides new insights into drug- and vaccine-based inhibition of *Plasmodium* transmission, paving the way for their further exploitation as part of the antimalarial toolbox.

Keywords: *Plasmodium*, malaria, transmission-blocking strategies, transmission-blocking vaccine, transmission-blocking drugs

RESUMO

A malária é uma doença parasitária responsável pela morte de mais de meio milhão de pessoas anualmente, apesar dos sucessivos esforços para controlar e eliminar esta doença. A malária é causada por parasitas do filo Apicomplexa e do genéro *Plasmodium*, do qual cinco espécies *P. falciparum* (*Pf*), *P. ovale, P. vivax*, *P. malariae*, e *P. knowlesi* infetam humanos. Recentemente, o interesse na eliminação da malária recebeu renovada atenção por parte da organização mundial de saúde (OMS), que incitou à intensificação do progresso de redução da mortalidade e morbilidade associadas à malária. No entanto, esse objetivo só será alcançado através da combinação de diferentes estratégias de controlo da infeção e da doença. Até ao presente, os maiores sucessos na redução do número efetivo de casos de malária foram alcançados através da combinação de diferentes estratégias de controlo do vetor e de terapias combinadas baseadas em artemisinina, que eliminam as formas do parasita na fase sanguínea do seu ciclo de vida. No entanto, o surgimento de parasitas resistentes a fármacos e de mosquitos resistentes a inseticidas, a par de uma redução no investimento associado a iniciativas de controlo da doença, representam uma ameaça aos progressos até agora alcançados. O desenvolvimento de um variado leque de ferramentas adicionais capazes de combater o parasita em diferentes fases do seu ciclo de vida, são determinantes para contornar estas limitações.

Estratégias visando a redução da prevalência do parasita em regiões endémicas, bloqueando a sua transmissão, são particularmente atrativas por diversas razões: o processo de transmissão do parasita do hospedeiro para o vetor corresponde a uma redução significativa no número de parasitas de aproximadamente 10^9 na fase sanguínea, para aproximadamente 10-100 oocistos no mosquito; os parasitas são extracelulares por aproximadamente 24 h, em comparação com cerca de 1 min durante o processo de invasão de eritrócitos pelos merozoítos; e a diversidade genética do parasita é significativamente inferior nas formas sexuais do parasita e nas formas parasitárias residentes no mosquito, comparativamente à que ocorre na fase sanguínea da infeção. Em conjunto, estes factos contribuem para que estratégias que atuem na transmissão do parasita diminuam a

persistência de parasitas mutantes, prolongando a sua suscetibilidade a fármacos direcionados para a fase sanguínea do parasita.

O potencial de estratégias que atuem na transmissão do parasita para reduzir a prevalência global da doença foi previamente demonstrado em diversos modelos que exploraram a dinâmica de transmissão do parasita tendo em conta diferentes fatores, incluindo a ecologia do vetor e o seu comportamento, tendo o decréscimo na incidência de casos de malaria entre 2000-2015 sido claramente correlacionado com utilização de redes mosquiteiras impregnadas com inseticidas e pulverizações de interiores com inseticidas.

O trabalho desenvolvido nesta tese debruça-se sobre duas estratégias de bloqueio da transmissão do parasita da malária, nomeadamente através da utilização de fármacos ou de vacinas capazes de a inibir.

Inicialmente foi investigado o impacto de avermectinas, uma classe de lactonas macro cíclicas com propriedades insecticidas e antiparasitárias, em parasitas na fase sanguínea e esporogónica. O derivado das avermectinas mais estudado, a ivermectina, é utilizada em regiões endémicas de malária para a redução de outras doenças parasitárias, nomeadamente filaríase linfática e sarna, através da administração em massa deste fármaco. Recentemente, a capacidade de a ivermectina em circulação tornar a refeição de sangue tóxica para o mosquito *Anopheles*, fez surgir o interesse na utilização desta estratégia como forma de controlo do número de casos de malária em regiões endémicas. Considerando a co-endemicidade do agente causador da malária *Plasmodium* com outros parasitas patogénicos, analisámos o impacto da ivermectina e de outras avermectinas nas fases sexuais e esporogónicas do parasita da malária *in vitro* e *in vivo*. Os resultados obtidos demonstram que as fases sexuais do parasita não são suscetíveis à ação da ivermectina *in vivo*. As restantes avermectinas testadas, com a exceção de emamectina, não são eficazes na formação de oocinetos in *vitro*. No entanto, os dados obtidos também mostraram claramente que a maioria destes compostos mostraram atividade contra as fases esporogónicas do parasita *in vitro*. Estes resultados reforçam a utilidade da administração em massa de ivermectina nas regiões endémicas para a malária como forma de controlar a incidência da doença, e clarificam o efeito da ivermectina nas diferentes formas do parasita durante o seu desenvolvimento no mosquito,

para além do seu amplamente conhecido efeito inseticida. Adicionalmente, sugerem que outras avermectinas possam ser incluídas em campanhas de administração em massa.

Tendo em conta a crescente resistência dos parasitas *Plasmodium* a fármacos para o tratamento de malária, a ideia de redirecionar compostos utilizados para tratar outras patologias para o tratamento de infeções por *Plasmodium*, e também para travar a sua transmissão, tem vindo a ganhar força. Desse modo, e dada a sobreposição geográfica da síndrome de imunodeficiência adquirida (SIDA) e malária na região da África subsariana, investigámos o impacto de tratamentos antirretrovirais, amplamente administrados nessa região para tratar infeções com o vírus da imunodeficiência adquirida (VIH), na transmissão do *Plasmodium*. Para esse efeito, recorremos a um ensaio *in vitro* baseado em luminescência para determinar a capacidade de compostos antirretrovirais e as suas combinações utilizadas como tratamentos de primeira linha, na inibição do desenvolvimento das fases esporogónicas do parasita. Os resultados obtidos *in vitro* foram posteriormente validados através da administração *in vivo* das terapias antirretrovirais, bem como de combinações de fármacos alternativas, e determinando o seu impacto na formação de oocistos no mosquito. Os resultados obtidos sugerem que as combinações de fármacos atualmente administradas como tratamentos de primeira linha em regiões tropicais inibem o desenvolvimento das fases do ciclo de vida do parasita no mosquito, e sugerem a possível inclusão de dois fármacos alternativos, rilpivirine e etravirine, nesses tratamentos.

Por último, considerámos o desenvolvimento de uma vacina de organismo inteiro utilizando parasitas da malária de roedores, *P. berghei*, como plataforma de administração de antigénios do parasita infecioso para humanos, *Pf*. A maioria das vacinas desenvolvidas contra a malária conferem proteção apenas contra uma das fases do ciclo de vida do parasita, como é o caso da vacina pré-eritrocitária aprovada pela OMS, a RTS,S. No entanto, existe a possibilidade de o parasita escapar às respostas imunitárias induzidas pela imunização com tais vacinas, levando à sua posterior multiplicação quando transita para uma nova fase do ciclo de vida. É por isso de extrema importância o desenvolvimento de uma vacina que atue contra todos os estados do ciclo de vida do parasita. Procurámos então otimizar um candidato a baseado em parasitas *P. berghei* geneticamente modificados para expressar o antigénio pré-

eritrócitário *Pf* circumsporozoite (*Pf*CSP), introduzindo-lhe novos antigénios da fase eritrocitária – *Pf* reticulocyte binding homolog 5, e de transmissão do parasita- *Pfs*48/45. Os resultados obtidos revelam a correta expressão de ácido ribonucleico mensageiro (mRNA) dos genes *Pfcsp* e *Pfs48/45* e das proteínas respetivas nas diferentes linhas de parasitas transgénicos, bem como a imunogenicidade destes parasitas em modelos de roedores. Estes resultados mostram a viabilidade da criação de uma parasita transgénico que expressa múltiplos antigénios de diferentes fases do ciclo de vida do parasita, demonstrando a correta expressão dos antigénios *Pf*CSP e *Pfs*48/45 inseridos no genoma do parasita de roedores. Os dados obtidos revelam ainda que a imunização com os parasitas transgénicos gerados leva à produção de anticorpos contra aqueles antigénios, abrindo caminho para a utilização desta estratégia de vacinação como forma de espoletar respostas imunitárias contra fases distintas do ciclo de vida do parasita humano.

Um investimento continuado em estratégias visando o bloqueio da transmissão do parasita da malária é essencial para o desenvolvimento de novas ferramentas de controlo desta doença. O nosso trabalho vem alargar o conhecimento atualmente existente acerca da inibição da transmissão de parasitas *Plasmodium* por fármacos e vacinas, abrindo a porta à sua exploração enquanto componentes de um conjunto alargado de ferramentas de combate à malária.

Palavras-chave: *Plasmodium*, malaria, ivermectina, antirretrovirais, vacina.

ACKNOWLEDGEMENTS

I'm writing this part of my thesis as I'm sitting on my desk, on my last day at IMM. These acknowledgements are not only for the people who supported me throughout this chapter of the PhD, but those who I've come across on my journey at IMM. As I've recently said in a job interview, opportunities are not something that come very often. So, to my supervisor, Miguel, thank you for taking a chance with me, for putting up with my daily complaints, and for being someone I will always look up to. I can only wish for the future that my new bosses will be as respectful and considerate of my opinion as you were. To Blandine, thank you for accepting to be my supervisor half the way through the PhD, it's been an honor to collaborate with you in this project.

To all my colleagues, past and current members of Figueirêncios and Motas, I have to say there is really something special about working here. I've laughed and cried with you, I've made lifelong friendships and I've mostly learned that, sometimes, it's ok to trust and open up to others. Thank you for never complaining about my never-ending babbling or the good mornings openings with "I'm pissed, you cannot believe what happened!", and the support in this PhD ride. I'm always a phone call away (Yes, I can come to the next Town Hall).

To my family, hell yeah! We made it. The success, if, or when it comes, will forever be yours. Nanna, when some poet wrote that time heals everything, it's because they never knew you. I still miss you to this day, I still go back to your advice and stories, and I will forever talk about you. May this achievement be a sign that our family will have a better future and that the next generations will have the same educational opportunities that you fought for us to have. When I have my house, with that picket fence and a dog, there will be thousands of pictures of you, because there, you will forever be a queen. Mom and dad, I wish to one day be as selfless as you are, I won't forget the vacation you didn't take so I could attend those private schools, and the extra hours you worked so I could study abroad and take English classes. I hope I'm doing you proud. Little sis, just remember that your journey is yours alone, and that there is always one place you are free at: your art.

To all of you, thank you

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2 PROGRESS ON TRANSMISSION-BLOCKING CHEMOTHERAPY

3 PROGRESS ON TRANSMISSION-BLOCKING VACCINES

ABBREVIATIONS

3TC	lamivudine
6C	C-terminal 6-cys domain
Ab	abamectin
ABC	abacavir
ABSL2	animal biosafety level 2
ACT	artemisinin combination therapy
AIDS	acquired immune deficiency syndrome
AMA1	apical membrane antigen 1
An. darlingi	*Anopheles darlingi*
An. dirus	*Anopheles Dirus*
An. minimus	*Anopheles Minimus*
An. stephensi	*Anopheles stephensi*
AnAPN1	anopheline alanyl aminopeptidase N1
APV	amprenavir
ART	antiretroviral therapy
ARV	antiretroviral
At	atovaquone
ATP	adenosine triphosphatase
ATV	atazanavir
Az	azithromycin
AZT	zidovudine
BC	before Christ
BSA	bovine serum albumin
BSG	basigin
BSL1	biosafety level 1
BSV	blood stage vaccine

CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDPK3	calcium dependent protein kinase 3
CelTOS	cell traversal for ookinetes and sporozoites
Ch	chloroquine
ChAd63	chimpanzee adenovirus serotype 63
CHMI	controlled human malaria infection
COVID-19	coronavirus disease 2019
CPS	chemoprophylaxis with sporozoites
CSP	circumsporozoite protein
CT	cycle threshold
CTRAP	circumsporozoite and thrombospondin-related adhesive protein
Cy	cycloheximide
CyRPA	cysteine rich protective antigen
D. melanogaster	*Drosophila melanogaster*
DA	dihydroartemisinin
DAPI	4’,6-diamidino-2-phenylindole
DDT	dichlorodiphenyltrichloethane
DEPC	diethylpyrocarbonate
DMEM	Dulbecco’s modified eagle medium
DMFA	direct membrane feeding assay
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Do	doramectin
DRV	darunavir
EC_{50}	half maximal effective concentration
EEF	exoerythrocytic form
EFV	efavirenz
EGF	epidermal growth factor

ELISA	enzyme-linked immunosorbent assay
Em	emamectin
EMP1	erythrocyte membrane protein 1
Ep	eprinomectin
EPA	exoprotein A
ETV	etravirine
FBS	fetal bovine serum
FELASA	federation of European laboratory animal science associations
FTC	emtricitabine
FW	forward
G6PD	glucose-6-phosphate dehydrogenase
GAP	genetically attenuated parasites
GFP	encoding green fluorescent protein
GIMO	gene insertion/marker out
GPI	glycosylphosphatidylinositol
HA	halofantrine
HAP2	hapless 2
HBsAg	hepatitis B surface antigen
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HIV	Human immunodeficiency virus
HPRT	hypoxanthine phosphoribosyl transferase
HRP2	histidine-rich protein-2
HSP70	heat shock protein 70
HSPG	heparan sulfate proteoglycan
IC50	half maximal inhibitory concentration
IDV	indinavir
Ig	immunoglobulin
IL	interleukin

IMM JLA	instituto de medicina molecular João Lobo Antunes
INSTI	integrase strand transfer inhibitor
IPT	intermittent preventive treatment
IPTi	intermittent preventive treatment in infants
IPTp	intermittent preventive treatment in pregnancy
IRS	indoor residual spraying
ITN	insecticide-treated bed nets
Iv	ivermectin
LDH	lactate dehydrogenase
LLIN	long-lasting insecticidal nets
LPV	lopinavir
LPV/r	lopinavir and ritonavir
LSA	liver-stage antigen 3
Lu	lumefantrine
luc	luciferase
LUMC	Leiden university medical center
MAOP	membrane-attack ookinete protein
MDA	mass drug administration
ME	multiple epitopes
Mei2	meiosis inhibited 2
MMV	medicines for malaria venture
Mo	moxidectin
mRNA	messenger ribonucleic acid
MS	mass spectra
MSP1	merozoite surface protein-1
MVA	modified vaccinia virus Ankara
NFV	nelfinavir
NIH	national institutes of health
Nluc	nano luciferase

NNRTI	non-nucleoside reverse transcriptase inhibitors
NRTI	nucleoside reverse-transcriptase inhibitor
ns	non-significant
NVP	nevirapine
OMS	organização mundial de saúde
P. cynomolgi	*Plasmodium cynomolgi*
PABA	para-aminobenzoic acid
Pb	*Plasmodium berghei*
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PCR	polymerase chain reaction
PEM	peritrophic matrix
PEV	pre-erythrocytic vaccine
Pf	*Plasmodium falciparum*
PFA	paraformaldehyde
PFG	pulsed field gel
Pg	*Plasmodium gallinaceum*
PI	protease inhibitor
Pk	*Plasmodium knowlesi*
PM	plasmepsin
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
Po	pyronaridine
Pr	*Plasmodium relictum*
PSM	peptide-spectrum match
PV	parasitophorous vacuole
Pv	*Plasmodium vivax*
Py	*Plasmodium yoelii*
Py	pyrimethamine

qRT-PCR	quantitative real-time polymerase chain reaction
RAL	raltegravir
RAS	radiation attenuated sporozoites
RBC	red blood cell
RDT	rapid diagnostic tests
RFP	red fluorescent protein
Rh5	Reticulocyte-binding protein homolog 5
RIPR	rh5-interactive protein
RLU	relative luminescence units
RNA	ribonucleic acid
RON2	rhoptry neck protein 2
RPMI	Roswell Park memorial institute
RPV	rilpivirine
rRNA	ribosomal ribonucleic acid
RT	room temperature
RTV	ritonavir
RV	reverse
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIDA	síndrome de imunodeficiência adquirida
SIL	*silent intergenic locus*
SM	selectable marker cassette
SMC	seasonal malaria chemoprevention
SMFA	standard membrane feeding assay
SOAP	secreted ookinete adhesive protein
SP	sulfadoxine-pyrimethamine
SPZ	sporozoite
SQV	saquinavir

SRP	signal recognition particle
TB	transmission-blocking
TBA	transmission-blocking activity
TBD	transmission-blocking drug
TBS	transmission-blocking strategies
TBV	transmission blocking vaccine
TDF	tenofovir
Th	thiostrepton
TMB	tetra-methyl-benzidine
TNF	tumor necrosis factor
TRAP	thrombospondin-related adhesive protein
UIS4	upregulated in sporozoite 4
USA	United States of America
UTR	untranslated region
VAR2CSA	variant surface antigen 2-condroitin sulphate A
VIH	vírus da imunodeficiência adquirida
WHO	world health organization
WSV	whole-sporozoite vaccines

1 GENERAL INTRODUCTION

1.1 HISTORY OF MALARIA

1.1.1 DISCOVERY OF THE MALARIA PARASITE

Malaria has been around since the Paleogene period, with *Plasmodium* parasites being first found in mosquitoes preserved in amber for approximately 30 million years (1). The first written reports about this disease date back to ancient history, with multiple records mentioning an illness characterized by “intermittent” and “malignant” fevers, consistent with malaria symptoms (2–7).

The earliest reference of a possible *Plasmodium* infection was in the Chinese canon of medicine from 2700 BC (8), but others followed: clay tablets in Mesopotamia from 2000 BC, Egyptian papyri from 1570 or even Hindu texts from the sixth century have described malaria-like symptoms (9). However, Hippocrates was the first to correlate the fevers with stagnant bodies of water (7), to classify fevers as tertian (*P. vivax* (*Pv*) and *P. ovale* (*Po*)) or quartan (*P. malariae* (*Pm*)) (10,11) and to associate the disease with the seasons (7). The term mal’aria (meaning ‘spoiled air’), commonly used by Italian folk medicine, was later introduced in the English scientific literature by John MacCulloch as malaria (12). Charles Laveran, considering it vulgar, coined the French term paludisme, in reference to swamps, as it was thought that malaria fevers were caused by miasmas rising from swamps (13). It was this French surgeon, who, in 1880, first discovered the malaria parasite, by observing the process of exflagellation in a blood sample from a febrile soldier. In this work, he also described the female and male gametocyte, trophozoite and schizont forms of the parasite’s life cycle (**Fig. 1.1.1**) (14). This discovery was supported by the observations of Johann Meckel in 1847, who had already observed black pigmented granules (hemozoin) in brain and spleen samples of an autopsied patient, but wrongly identified them as a blood product, and of Rudolf Virchow, who, in 1849, correlated the pigment with hemozoin (5,12,15). In 1907 Laveran was awarded the Nobel Prize of medicine for his discovery, having named the parasite *Oscillaria malariae*. The term *Plasmodium* was introduced by Marchiafava and Celli in 1885, and was the one that eventually would prevail to this day (16).

From 1886-1892 Camillo Golgi expanded the knowledge on *Plasmodium* by describing the erythrocytic stages of the life cycle, and by correlating the recurrent chills and fever with

the parasite release in the blood (17,18). However, the question remained regarding the transmission of the disease.

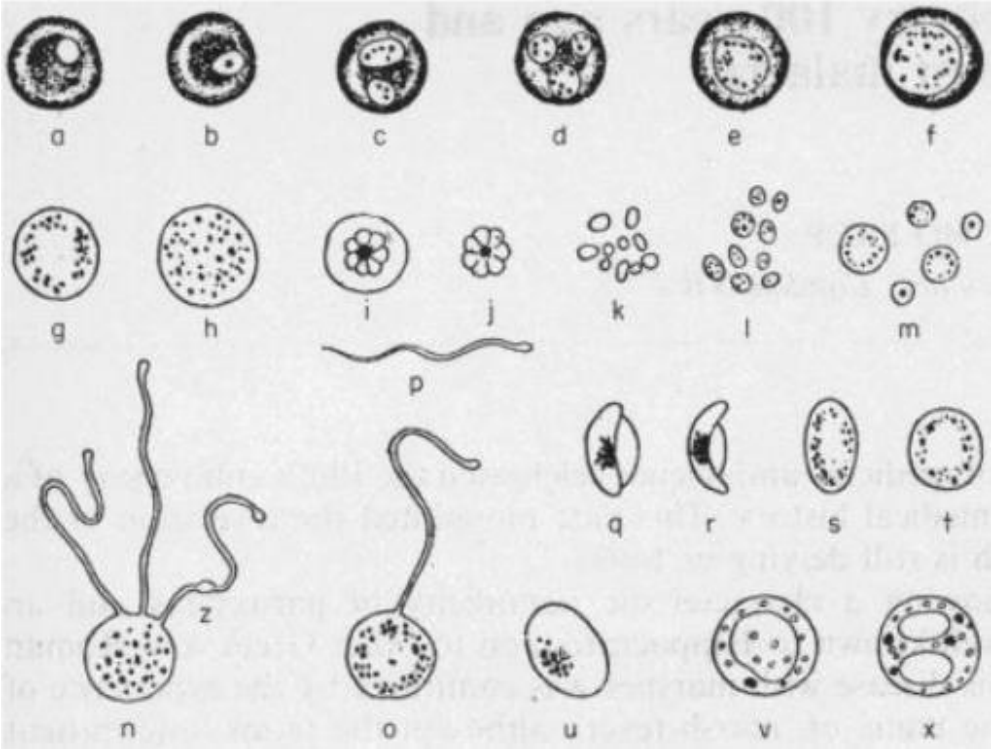

FIGURE 1.1.1 - Laveran's drawing of *Oscillaria malariae*. (Lalchhandama, 2014)

It was only in 1898 that Bignami and Grassi elucidated the complete cycle of *Plasmodium* transmission (16) . In the two subsequent years, both of them showed that the parasite was transmitted through the bites of female infected *Anopheles* mosquitoes, and described the life cycle from the erythrocytic to the mosquito stages of *Pv*, *P. falciparum* (*Pf*) and *Pm* (19). However, no knowledge existed regarding what is now known to be the liver stage of the parasite's life cycle. For 40 years, the scientific community believed that sporozoites could directly infect red blood cells (RBCs). This belief was based on the works of Fritz Schaudinn, who, in 1903, claimed that sporozoites from *Pv* invaded RBC (20). This was only contradicted by MacCallum in 1898, when he identified *P. relictum* (*Pr*) in the liver and spleen of infected birds (21). In 1947, Henry Shortt and Cyril Garnham showed that the parasite passed through an obligatory phase in the liver before entering the bloodstream (22), and, shortly afterwards, these two scientists also found pre-erythrocytic forms in volunteers infected with *Pv* (23), *Pf* (24) and *Po* (25). It remained to be understood what happened in patients where parasites

would reappear in the blood even after treatment until 1982, when Wojciech Krotoski discovered the dormant liver stages of the parasite – hypnozoites (26).

1.1.2 HISTORY OF ANTIMALARIALS

The discovery of antimalarials walked different paths in Eastern and Western medicine since its genesis. While 400 years ago the Western world reported the discovery of Chinchona, brought to Europe from Peru, 2000 years earlier, traditional oriental medicine records mention *Artemisia annua* (**Fig. 1.1.2**) , known at the time as qinghao (27). Qinghao's first known mention was in an ancient Chinese medical text "Recipes for 52 kinds of diseases" from 168 BC, in which it is employed as a remedy to treat haemorrhoids (28). The first description for its use in the treatment of fever appeared in "The handbook of prescriptions for emergency treatments" by Ge Hong in the Eastern Jin dynasty (28).

In the 19th century, during the war in Vietnam, the Northern Vietnamese forces were suffering great losses due to malaria cases amongst soldiers, and requested the help of the Chinese (29).

FIGURE 1.1.2 - Professor Tu Youyou in the 1980 and *Artemesia annua* (Adapted from Perlez,2015 and Tu, 2011)

On the 23rd of May of 1967 project 523 was born (30), aiming at building a collaborative task-force of several laboratories, to unravel new antimalarial drugs that might have been described in ancient Chinese traditional medical texts or by screening synthetic

chemicals, to treat the troops (29–31). Initially, the project suffered major setbacks, due to the challenge posed by the extraction of the active principle from *Artemisia annua*. Tests performed on the antimalarial activity on the extract of the plant with alcohol or heated water showed low inhibitory activity (32). Professor Tu Youyou (**Fig. 1.1.2**) suggested that the extraction process might have been degrading the active principle and, together with her team, went back to the written ancient texts in search of an answer (31). Based on Ge Hong's recipe for the preparation of a medicinal beverage, Tu Youyou decided to combine extraction by ether and low temperature, which, after further optimizations, resulted in an increase in antimalarial activity (29,31,33). The results were validated in clinical trials in 1972, with the administered dosage curing all *Pv* malaria cases, and seven out of nine *Pf* malaria cases (34). This discovery led to Professor Tu Youyou being awarded the Nobel Prize in Physiology or Medicine in 2015 (35). Meanwhile, across the globe, quinine was being explored to tackle the emergence of malaria cases. Although the exact moment of the discovery of quinine is not known, is it thought that the south American indigenous people used infusions of cinchona bark to combat shivering when exposed to the cold (36).

Due to a previous malaria outbreak that caused several cardinals to perish in 1623, Pope Urban, who was one of the afflicted, instructed Jesuit missionaries to search for new medicines against the disease (37). In their exploits for new medicines in the Americas, they discovered the bark of the cinchona tree (38) which was tested for its medicinal properties in 1643 when Cardinal Juan de Lugo instructed the Pope's physician, Gabriel da Fonseca, to freely distribute the bark to the poor people of Rome and the patients of Hospital of Santo Spirito, where it gained popularity as Jesuits' Bark (39). By 1712 Francesco Torti demonstrated the importance of cinchona to treat intermittent fevers (40). Carl Linnaeus would coin the name cinchona in 1742 when describing the plant, in honor of Lady Chinchón (39). In 1812, a Portuguese surgeon named Bernardino António Gomez isolated a crystalline substance, which he called cinchonino (41), but it was only 8 years later that quinine was isolated by Pierre Pelletier and Joseph Caventou (41). This discovery was essential during European colonialism, which had been severely impacted by the devastation caused by malaria deaths (38). The development of synthetic antimalarials became necessary in light of the scarcity of quinine during the two

World Wars (42), and the synthesis of artemisinin would later revolutionize the treatment of malaria.

1.2 EPIDEMIOLOGY AND SOCIOECONOMIC BURDEN

Malaria remains a public health problem in the regions where it is endemic. The World Health Organization (WHO) estimated 247 million cases of malaria and 619 000 deaths worldwide in 2021 alone (43). These numbers represent an increase of 15 million cases from 2019, that could partially be due to the negative impact of the coronavirus disease 2019 (COVID-19) pandemic on malaria programs, thus influencing prevention, diagnostic and treatment of malaria cases (44).

Most cases (94.7%) and deaths (95.7%) are concentrated in the WHO African Region, followed by the WHO South-East Asia Region (2.2% and 1.5%), the Eastern Mediterranean Region (2.5% and 2.2%) and the West Mediterranean Region (0.6% and 0.4%) (43). Infections in these and in the WHO Western Pacific regions, are predominantly due to *Pf*, with the *Pv* burden being the highest in the WHO South-East Asia region, mostly in Myanmar and India, accounting for 4.9 million cases worldwide (43).

Children under the age of 5 are the most vulnerable group affected by malaria; in 2021 they accounted for 76% (approximately 470 000) of all malaria deaths worldwide (43). In the WHO African Region, pregnant women have the highest prevalence of exposure to malaria during pregnancy, with 961 000 children in this region having low birthweight – a strong risk factor for neonatal and childhood mortality (43). Work towards malaria elimination is progressing slowly, but steadily. The total number of malaria endemic countries reporting less than 10 000 cases increased from 27 to 46 in the last 21 years (43).

The prevalence of malaria is closely related to poverty. Hence, it is of no surprise that the geographical boundaries that delimitate malaria transmission overlap with those of poverty worldwide, with the most affected being the population of the African continent (45). The debilitating impact on these countries' economy happens through many fronts. On the one hand, malaria is suggested to impact the countries' trade with other nations, tourism, and foreign investment (46,47). On the other hand, the disease burden might negatively impact

the microeconomy of the household. Evidence shows that high infant and children mortality rate is associated with high fertility rates (47,48). In malaria-endemic regions, where the income of many households is low, a high birth rate represents a reduction of investment on education of children, especially on that of girls, which will spend the majority of their future dedicated to household and child care (49). Moreover, children that become infected will decrease their school attendance, which in turn will affect their school performance (50,51). It is also hypothesized that children afflicted by malaria can have impaired cognitive abilities (46,52). This is supported by the observation that children with malaria often have a poorer nutritional status than non-malarial children, which can impair brain development (53,54). Altogether, malaria seems to impact every aspect of the social and economic life of endemic countries and its inhabitants.

1.3 SYMPTOMS AND DIAGNOSIS

Symptoms of malaria start between 10 to 16 days after a bite from an infected female *Anopheles* mosquito (55). In uncomplicated *Plasmodium* infection, besides fever, patients develop mild anemia and, after several days, a palpable spleen (55). In endemic regions, patients, particularly children, can develop an enlarged liver and thrombocytopenia (56–58). They also develop, more frequently than adults, abdominal pain and vomiting (59). The progression of the disease will depend on the patient's immune system, as well as on the time of initiation of treatment. Severe malaria symptomatology differs in children and adults (55,60). In adults, renal failure, pulmonary oedema, coma (cerebral malaria), and hyperlactatemia are common, but severe anemia, contrary to what is observed in children, is rare (55,61). Children will develop the aforementioned symptoms, but oedema and renal failure are rare (61). Early diagnostic is critical in malaria case management and can be critical for reduction of malaria transmission (62). More importantly, the correct diagnostic of malaria cases can prevent the unnecessary administration of antimalarials and, consequently, prevent the proliferation of drug resistance (62). The gold standard for parasite detection relies on microscope examination of Giemsa-stained blood smears, by observation of thick drop and thin blood films (63,64). However, in a field setting, detecting malaria parasite infections can

be challenging (63). Many factors can influence the correct diagnostic of a *Plasmodium* infection: the availability of a light microscope with a 100× magnification objective, trained staffed that can differentiate between different *Plasmodium* species and can prepare patient's blood smear slides, lysis of RBC that elicit changes in parasite morphology, and lastly, the level of parasitemia (63,65,66). To tackle the lack of availability of electricity or health services in remote rural areas, alternatives must be set in place.

Rapid diagnostic tests (RDT) use a small amount of blood on a test strip, where by lateral flow immunochromatography, monoclonal antibodies bind to target antigens in malaria parasites such as lactate dehydrogenase (LDH), adolase and histidine-rich protein-2 (HRP2), resulting in a colored line (64,67). They are easy to use, as they do not require electricity, can provide results within 20 minutes, and are not dependent on the technician's expertise (68). The main limitations of this method are the ability to detect infections by all *Plasmodium* species (*Pm* and *Po*), low sensitivity (65,68), and, in the case of *Pf*HRP2-based RDTs, which are specific for *Pf*, to distinguish between recent and effectively treated infections, due to the persistence of *Pf*HRP2 in the blood of treated patients for 1-5 weeks, and the presence of HRP2 deletions in *Pf* parasites in the Amazon region, resulting in false negatives (67,69). In low density *Plasmodium* infections, nucleic acid amplification tests enable sensitive parasite detection (65). There are three type of tests that can be included in this group: polymerase chain reaction (PCR) – includes nested, quantitative, or real time reverse transcription PCR; loop mediated isothermal amplification; and quantitative nucleic acid sequence-based amplification (70). These tests provide information on the species and parasite load, and enable detection of asexual or sexual parasites (70). Moreover, this is the only method available that can detect asymptomatic, sub-microscopic infections (65). These methods, although useful for drug resistance and epidemiological studies, are expensive and highly complex (71) deeming them inappropriate for a field setting (65,67,72).

1.4 PARASITE LIFE CYCLE

Malaria is a disease caused by a parasitic protozoan of the genus *Plasmodium* (73). The parasite goes through a complex life cycle that alternates between sexual reproduction in the

mosquito and asexual reproduction in a vertebrate host (74). In addition to mammals, reptiles, birds and amphibians are also hosts of malaria parasites (75,76).

The life cycle begins when a mosquito takes a blood meal and injects sporozoites into the skin of the mammalian host (77). Around 15–123 sporozoites reach the host's skin (78–81) and remain there for approximately 5 minutes, eventually entering the bloodstream while using their gliding motility to migrate through cells (82–84). Once in the circulatory system, sporozoites reach the liver sinusoids and invade their target cells – the hepatocytes (85) (**Fig. 1.4.1**).

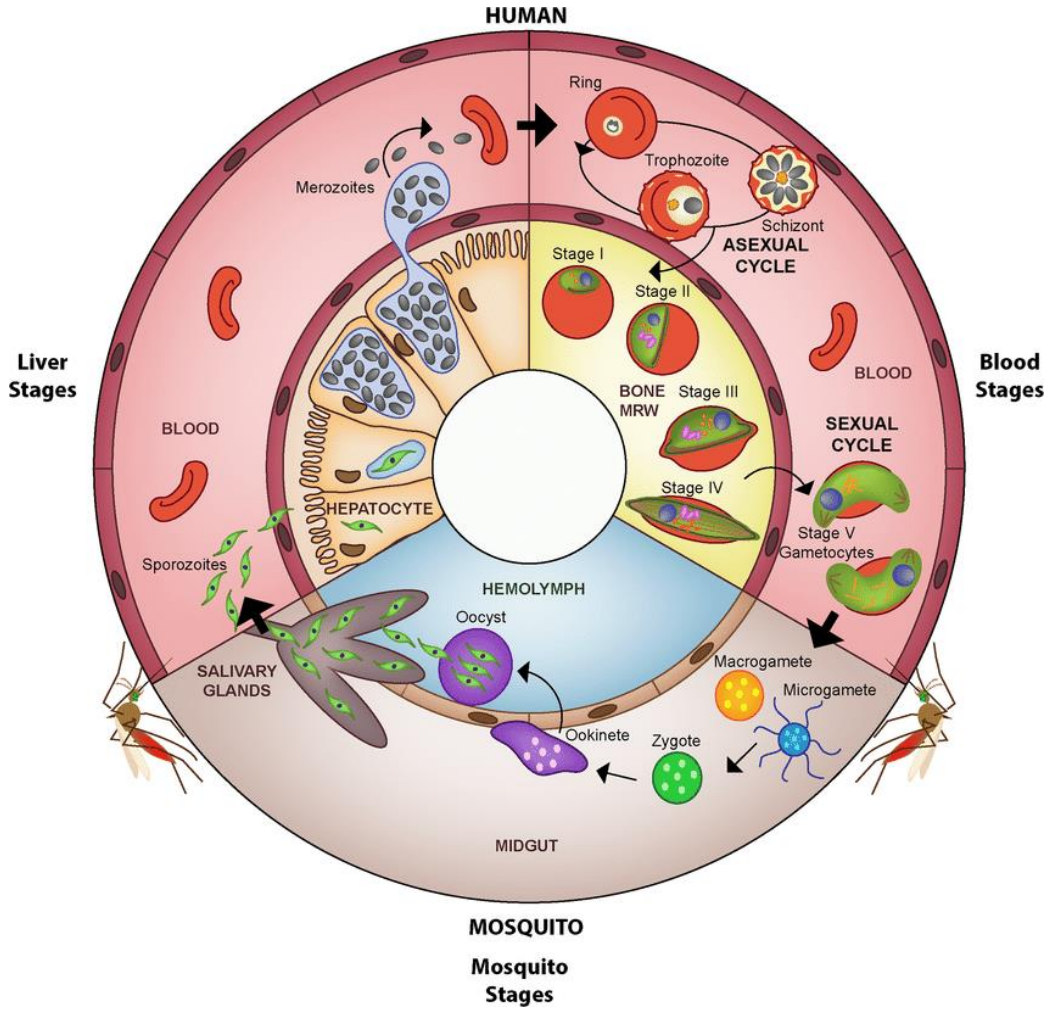

FIGURE 1.4.1 – Life cycle of *Plasmodium.* (Nilsson *et al*., 2015)

The arrest of the sporozoites in the liver sinusoid is thought to be mediated by interactions between parasite-encoded surface proteins and host molecules (78) namely *Plasmodium* sporozoite circumsporozoite protein (CSP) and thrombospondin-related adhesive

protein (TRAP) that bind to heparan sulfate proteoglycans (HSPGs) protruding from the hepatocytes into the liver sinusoids (86–88). The liver sinusoidal cell layer is composed of endothelial cells and resident Kupffer cells (84), which are the two proposed routes of hepatocyte invasion. While some suggest that sporozoites migrate through fenestrated or between the discontinuous endothelial cells (89,90), others have observed sporozoites inside Kupffer cells (85,90,91). Supporting evidence of the latter shows preference of *P. berghei* (*Pb*) and *P. yoelii* (*Py*) sporozoites to enter Kupffer cells as opposed to endothelial cells *in vitro* (85). Subsequently, a nonfusogenic parasitophorous vacuole (PV) is formed inside the Kupffer cells protecting the parasite from being phagocytized (85,91,92). After crossing the sinusoidal layer, the sporozoite traverses several hepatocytes, by disrupting the cell plasma membrane or by forming a transient non replicative vacuole (92,93). Eventually the parasite switches to productive invasion, entering a moving junction dependent PV in the hepatocyte cytoplasm and gliding into it (84,92,94). The PV is remodeled by secreting components of the rhoptries organelles (95,96) and integrates parasite derived proteins, such as UIS3 and UIS4 (93,97). Inside the PV, the sporozoite will differentiate into a replicative form, the liver stage trophozoite (98) (**Fig. 1.4.1**). Trophozoite formation starts with the breakdown of the cytoskeletal structure beneath the parasites' plasma membrane, where the invasion motor is located, leading to cytoplasm expansion followed by contraction of its distal ends (98). Organelles such as micronemes and the inner membrane complex are disassembled, followed by their discharged in the vacuolar space, while a minimal set of organelles, essential for parasite replication, is retained in the cytoplasm (99). Afterwards, the parasite's nuclei divide repeatedly without cell division, in a process termed schizogony, and the parasite replicates its organelles and transforms the PV into a multi nucleated compartment (99,100).

The transition to schizogony does not always occur in infections with *Pv* and *Po*. After entering the hepatocyte, the sporozoite may dedifferentiate into a latent trophozoite, the hypnozoite (100). This parasite form can remain dormant from months to years and reactivate its development by yet unknown triggers, causing a malaria relapse (100). Due to the nutritional demands of the trophozoite-to-schizont replicative stage, the parasite upregulates the expression of host genes responsible for metabolite transport and anabolism (101–103),

creating highly permeable channels in the PV membrane that allow the passage of small metabolites from the host's cytosol (103). During the schizont maturation process, the infected hepatocyte expands to accommodate the replicating parasite (113).

Merozoite formation starts with repeated invagination of the parasite plasma membrane, in parallel with coordinated fission of the nuclei and organelles, and their subsequent individual compartmentalization (104). Once the merozoites are fully formed, the PV membrane breaks down, releasing merozoite-packed vesicles called merosomes into the sinusoidal lumen (105,106) (**Fig. 1.4.1**). The merosomes escape elimination by Kupffer cells by deriving their membrane from the host hepatocyte's plasma membrane (104). They then travel in the bloodstream through the heart, eventually rupturing and releasing thousands of merozoites inside pulmonary capillaries (100,107). Merozoites subsequently invade erythrocytes (*Pv* shows a preference for reticulocytes (108)) within approximately 1 min, in a multi-step process involving (109,110) merozoite binding to the erythrocyte, reorientation of its apical end onto the host cell surface, and formation of a moving junction that allows the parasite to pass through and enter the erythrocyte, powered by the parasite's actin-myosin motor (111,112).

After invasion, the parasite transforms into a biconcave shape inside a PV in the RBC (113), feeding on hemoglobin through its cytostome, while also taking other nutrients from the plasma (114). As the parasite continues to increase in size it modifies the RBC membrane, which starts to adhere to the linings of visceral and other blood vessels, such as those of the placenta (115). Eventually, the trophozoite is formed, the most active form in terms of parasite growth (114,116) (**Fig. 1.4.1**). At this stage, the intensive digestion of hemoglobin leads to the formation of a dark crystalized pigment – hemozoin, which is released in the food vacuole (114,116). Export of new parasite proteins to the RBC surface leads to the formation of knobs (117) and adherence to the endothelium of blood vessels, which can cause the development of cerebral malaria, in case of adherence of RBCs to blood vessels in the brain, or low fetal growth, in case of adherence to the placenta (118–120).

The trophozoite form will undergo DNA synthesis, repeated mitosis and nuclear division, producing a multi nucleated schizont (121). After 48 hours, 16-32 merozoites will egress, resulting in destruction of the RBC and release of the parasites to reinvade a new RBC (122).

The clinical symptoms of the disease emerge at this stage of the parasite's life cycle, with the rupture of mature schizonts (11,123). However, a proportion of the parasites commit to develop into transmissible sexual forms – the gametocytes (124). Some stimuli, such as high parasitemia, chloroquine, anemia or antiparasitic responses are associated with parasite differentiation into micro (male) and macro (female) gametocytes (74,122) (**Fig. 1.4.1**). Ingestion of gametocytes during a blood meal activates the formation of male and female gametes in the mosquito midgut lumen (125). Environmental triggers have been described as inducers of this process: a decrease in temperature of infected blood, a rise in pH, and the presence of xanthurenic acid (126,127). As a consequence, microgametes exflagellate and fuse with macrogametes within the mosquito bloodmeal, forming diploid zygotes (128,129) (**Fig. 1.4.1**). After fertilization, a banana shaped ookinete is formed, that migrates through the bloodmeal and traverses the midgut epithelium by invasion of the epithelial cells (125). The ookinetes exit through the basal side of the epithelium and cease movement, rounding up beneath the basal lamina of the midgut epithelium, forming an oocyst (130–132) (**Fig. 1.4.1**). The process of sporogony starts within each oocyst, with the parasite's massive growth and its nuclei undergoing endomitosis (133,134), leading to the formation of several thousand mature sporozoites inside each oocyst (135). Eventually, the basal lamina that covers the oocyst ruptures, and the oocyst capsule becomes thinner, culminating with the release of sporozoites from the oocyst (134,136,137). Sporozoites will then glide to the mosquitoes' salivary glands, reinitiating the parasite's life cycle (137).

1.5 MALARIA CONTROL MEASURES

1.5.1 VECTOR CONTROL

Malaria is amongst the group of mosquito-borne diseases that are a public health problem in many countries around the globe, with vector control being a vital component of their control and elimination. In the nineteenth century malaria was endemic is most of the

world, affecting approximately 90% of its population (138). Since there were not always drugs available for the prevention and treatment of the disease, early preventive efforts included screening of houses, the use of mosquito nets, drainage and filling of water bodies used by mosquitoes for breeding, and the application of oil or Paris green to mosquito breeding zones (139). In the 1940s, dichlorodiphenyltrichloethane (DDT) started to be used as an insecticidal, showing promising results in reduction of malaria cases by 1945 (138). This led to the decision of the World Health Assembly in 1955 to deploy a massive administration of DDT as a vector control tool in all malaria endemic countries (with the exception of Madagascar) in the form of indoor residual spraying (IRS) (140). However, the success of this initiative was short-lived. Although malaria was successfully eliminated from southern Europe, parts of North Africa and the Middle East, insecticide-resistant mosquitoes, and drug-resistant parasites began to emerge (141). Even so, vector control still remains the most effective measure in preventing malaria transmission, successfully reducing malaria mortality and morbidity worldwide (142).

In 2015, the Global Technical Strategy for malaria 2016-2030 aimed to reduce malaria incidence and mortality by at least 90%, eliminate malaria from at least 35 countries, and prevent malaria re-establishment from malaria free countries by 2030 (143). The intervention strategy relied greatly on integrated vector management based on local epidemiological and entomological data (143), in order to increase cost-effectiveness of vector control and to reduce the spread of drug and insecticide resistance, determinant in under developed countries with limited financial resources (143). The two core interventions for elimination of malaria are IRS and insecticide-treated bed nets (ITN) (144).

1.5.1.1 INDOOR RESIDUAL SPRAYING

IRS has been employed as the main vector control strategy and a major contributor to malaria elimination and the reduction of malaria burden in many countries (145–148). Previous success with this intervention relied on the fact that most malaria vectors are endophilic (149). Therefore, when a female *Anopheles* vector enters the household to bite, the vector will come into contact with a sprayed surface, reducing the mosquito's life span (149). This might not directly protect the household residents, but can prevent subsequent transmission of the

parasite (149). The efficacy of the spraying varies not only with species susceptibility, but also the dosage and coverage of the spraying, its residual efficacy and population adherence (144). On the downside, IRS can be costly, and poses more risks to human health and the environment than other interventions (150). For example, despite showing early promising results, DDT has been banned in some countries due to environmental concerns (150). There has also been growing reports of resistance to pyrethroids, raising the need for new insecticides and new approaches to IRS (151). Most IRS is now conducted with organophosphate and neonicotinoid insecticides, and alternative methods include combination of insecticides, use of insecticides with different modes of action in neighboring geographical areas – mosaic spraying, rotation of insecticides, and deploying multiple interventions simultaneously (139,152). However, the best practice so far, relies on annual rotation of the used insecticides to manage vector resistance (153).

1.5.1.2 INSECTICIDE TREATED NETS

ITNs have a double protective effect. They act as a physical barrier between the mosquito and the human, and the vector is killed by the insecticide present on the bed net (151). They include conventionally treated nets, with insecticides that remain active for up to 12 months, and long-lasting insecticidal nets (LLINs), in which the insecticide stays active for up to 3 years (150). It is estimated that 68% of 668 million cases of malaria averted in Sub-Saharan Africa from 2000 to 2015 were due to the use of ITNs (154). ITNs are most effective against late-night and indoor-biting vectors – endophagic (151). All ITNs recommended by WHO are treated primarily with a pyrethroid insecticide, because of its safety, low cost, and fast insecticidal and increasing residual activity (155,156). Due to the emergence of resistance, alternative insecticides have been sought. However, field studies show that new types of ITNs have differential performance, and lose their effectiveness before the end of their physical lifespan (156,157).

1.5.2 TREATMENT

Malaria case management is a vital component of malaria control strategies (158). The main objective of treatment is to ensure swift and complete elimination of parasites from the patient's bloodstream, thus preventing progression into severe disease and death, as well as chronic infection, which leads to malaria-related anemia (158). Following WHO's centralized guidelines for malaria treatment, all *Pf*-endemic countries have updated their treatment policies from monotherapy with drugs such as chloroquine, amodiaquine and sulfadoxine-pyrimethamine (SP) to artemisinin combination therapies (ACTs) (159). Combining antimalarials is crucial for therapy efficiency and to prevent the emergence of resistant parasites that could be exposed to high selective pressure through the widespread and indiscriminate use of antimalarials (158).

The following ACTs are currently recommended by WHO for the treatment of uncomplicated malaria cases (160):

- Artemether - lumefantrine
- Artesunate - amodiaquine
- Artesunate - mefloquine
- Dihydroartemesinin - piperaquine
- Artesunate + sulfadoxine – pyrimethamine
- Artesunate-pyronaridine

In cases of severe malaria, administration of intravenous or intramuscular artesunate during 24 hours and until oral medication is tolerated is recommended (160). Afterwards, medication should be switched to ACTs combined with primaquine in low transmission settings (except pregnant women, infants under 6 months and women breastfeeding infants under 6 months of age) (160).

1.5.3 CHEMOPREVENTION

Chemoprevention relies on the use of antimalarials for prophylaxis and preventive treatment, that will inhibit infection and malaria disease (160). The WHO currently recommends the following chemoprevention strategies: intermittent preventive treatment in

pregnancy (IPTp), perennial malaria chemoprevention (PMC), seasonal malaria chemoprevention (SMC), intermittent preventive treatment of malaria in school-aged children (IPTsc), post-discharge malaria chemoprevention (PDMC) and mass drug administration (MDA) (160,161).

IPT involves the delivery of treatment, without previous diagnostic and knowledge of possible infections (162). In malaria-endemic regions, the WHO recommends administration of SP-IPTp to all pregnant women in regions of moderate-to-high *Pf* transmission (160). PMC with antimalarials is recommended to all children belonging to age groups at high risk of contracting severe malaria, while SMC with antimalarials is advised in regions of high seasonal transmission for children of the above-mentioned group (160). The goal of this therapy is to maintain therapeutic antimalarial drug concentrations in the blood during the period of highest risk, with clinical trials indicating that high protection is achieved against uncomplicated malaria for 4 weeks after each administrated dosage (160,163). Recently, the WHO extended their chemoprevention guidelines to include IPTsc in moderate-to-high perennial or seasonal malaria transmission settings. However, since young children (≤ 59 months) are most susceptible to developing severe malaria, interventions targeting this age group should be prioritized over those directed at school-aged children (160). Moreover, the WHO introduced PMDC with a full therapeutic course of an antimalarial at specific times following hospital discharge as a strategy to reduce re-admission and death of children admitted with severe anemia (160). All the strategies above should be deployed with precaution, considering that SP resistance is spread in the African continent (164).

MDA consists on treating massively a population with a drug without previous knowledge of possible positive infection cases (161). Previously, MDA was advised against, due to concerns about efficacy, the logistical feasibility and the risk of accelerating drug resistance (165–167). However, its potential for malaria elimination was demonstrated in several interventions: MDA with artemisinin-piperaquine and primaquine lead to elimination of malaria in children in 17 Cambodian villages (168); MDA with SP combined with IRS achieved high levels of control of malaria in Northern Nigeria (169) and the combination of primaquine with chloroquine in Nicaragua was estimated to prevent 9200 malaria cases in the region (170).

In recent developments, MDA with ivermectin, which is an endectocide, proved to be a powerful tool in malaria control (171–174). The administered drug remains in the bloodstream for approximately 6 days, rendering the blood meal toxic to the mosquitoes upon feeding, and consequently decreasing their survival rate (175,176).

Recently, the WHO changed its position on MDA in specific settings, due to the need for new strategies that could contribute to malaria elimination, and taking in account previous contribution of MDA in this context, as well as the availability of new antimalarial drugs (167,168,177). Currently, the WHO recommends MDA for short-term reduction in disease burden in regions of high-to-moderate *Pf* transmission; interruption of transmission of *Pf* malaria in regions of low to very low transmission; to reduce *Pv* transmission (chemoprevention with an 8-aminoquinoline is not advised), during emergencies or periods of health service disruption in defined geographical areas to reduce disease burden (160,178).

Finally, chemoprophylaxis is recommended for people traveling from non-endemic countries to countries with local malaria transmission, preferentially in combination with other personal protective measures such as the use of insect repellents, ITNs, long sleeves and long pants (179). The prophylactic drugs of choice for travelers are atovaquone/proguanil (Malarone), doxycycline, and mefloquine (179).

1.6 VACCINES

The fight against malaria relies on a variety of approaches that would benefit immensely from the development of an effective vaccine. The difficulty in development of such a vaccine arises, at least partly, from the complexity of the malaria parasite (180), which raises an extremely difficult issue to solve for the development of a vaccine: immunization with stage-specific vaccines will only elicit immune responses against antigens expressed on that stage, preventing either infection, disease or transmission (181). Additionally, malaria vaccine development has struggled with designing a vaccine capable of eliciting long-lasting effective immunity (182).

In theory, a pre-erythrocytic vaccine (PEV) would prevent infection early on, before the appearance of clinical symptoms. However, if a single sporozoite would escape the host's

immune response, it could result in progression to disease and transmission (181). Moreover, mutations and sexual recombination lead to further genetic variation in response to the selective pressure of the host's immune system, drugs, and other malaria interventions (183). In individuals living in endemic regions, naturally acquired immunity to malaria is achieved by recurrent infection and subsequent acquisition of anti-malarial antibodies, which is reflected in a reduction of parasite density and prevalence of disease with age. In other words, naturally acquired immunity is achieved against the symptoms of disease, but sterile immunity likely is not (184) [reviewed in (185)]. Thus, the disease is specially threatening to children under five, which have not yet acquired any immunity to the parasite and may develop severe symptoms (186). It is therefore crucial that an effective vaccine against malaria is developed. The Malaria Vaccine Technology Roadmap defined two goals to be met by 2030: the development of a vaccine with 75% efficacy over 2 years against *Pf* and/or *Pv*, and the development of mass administrable vaccines that would reduce transmission, therefore reducing incidence of infection (187).

Malaria vaccines can be divided into three types: PEVs, erythrocytic and transmission-blocking vaccines (TBVs) (188) (**Fig. 1.6.1**).

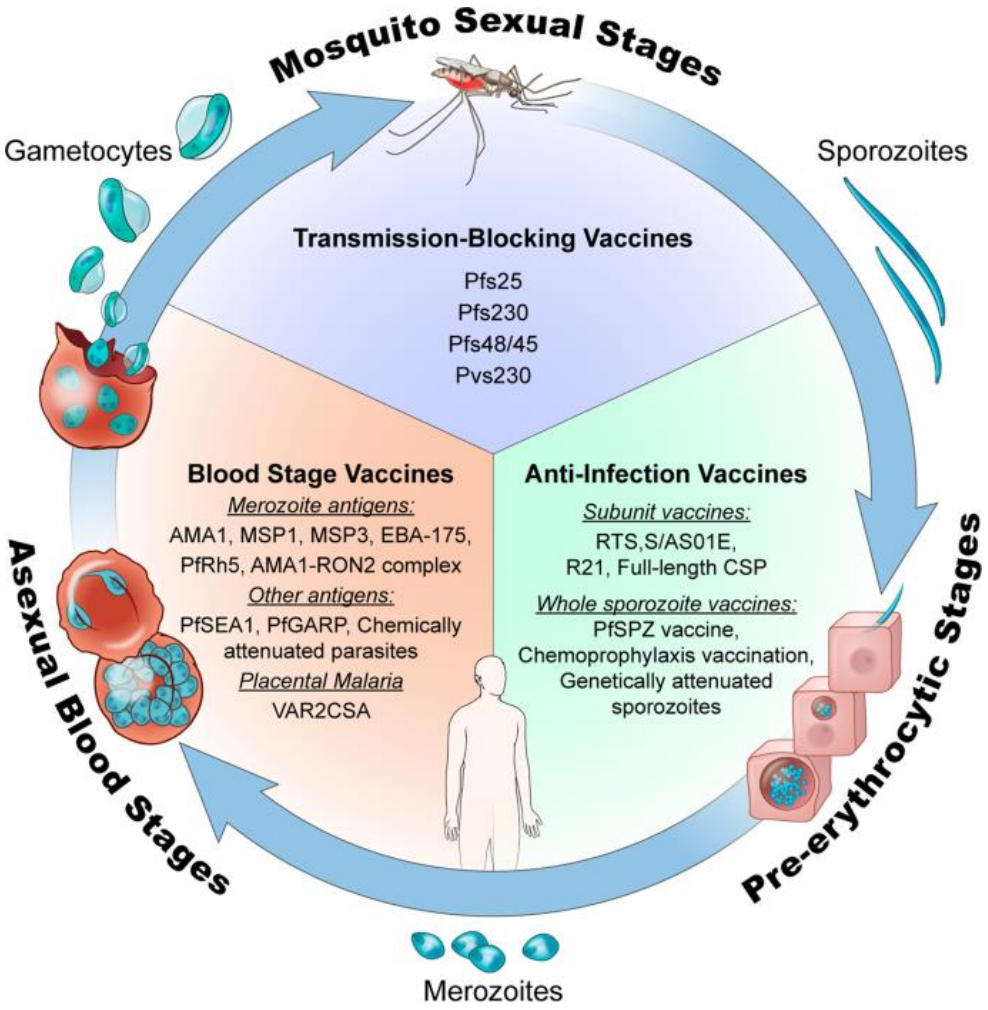

FIGURE 1.6.1 - Life cycle stages of *Plasmodium* and vaccine candidates that target each stage (Duffy and Gorres, 2020)

1.6.1 PRE-ERYTHROCYTIC VACCINES

PEVs target antigens expressed on the *Plasmodium* sporozoite and liver stages (189). At this point of development, the parasite's metabolism is highly active, but the infection is symptomatically silent, while the parasite multiplies inside hepatic cells (190). Designing a vaccine to target the parasite at this stage is thus quite attractive: hepatocyte infection represents a parasite's life cycle bottleneck, with only dozens to hundreds of liver cells becoming infected (191); it takes approximately seven days for *Pf* and *Pv* to complete

development in the liver (192,193); infected hepatocytes can present the parasite's antigens to immune effector cells, contrary to what is observed with infected RBC (194); and eliminating the parasite at this stage of the life cycle would prevent the emergence of clinical symptoms, and the occurrence of transmission (195).

PEVs act by either inducing antibodies against surface antigens of the sporozoite, preventing it from reaching the hepatocyte and invading it, or by inducing T cell responses that target infected hepatocytes (189). Two types of PEVs have been investigated and developed until present: subunit vaccines and whole-sporozoite vaccines (WSV) (**Fig. 1.6.1**).

1.6.1.1 SUBUNIT VACCINES

Subunit vaccines are designed to contain a fragment of a parasite protein in order to elicit immune responses against *Plasmodium* parasites (**Fig. 1.6.1**) (196). They are relatively easy to produce and administer in the field (197). They can be composed of peptides, multi-peptide constructs or recombinant proteins, that can be arranged in a one or multiple antigens vaccine, with the possibility of incorporating adjuvants to boost protection (197). In the past, antigens with low immunogenicity and high genetic variation were selected, leading to underwhelming protection (198). The first malaria gene to be cloned encoded the *Pf* CSP (199). CSP is the immunodominant surface antigen of sporozoites, and is crucial for host hepatocyte invasion (199–202). Early malaria vaccine research was mostly focused on this immunogen, with several approaches for protein delivery being explored (203). The most advanced CSP-based malaria vaccine is RTS,S/AS01 (195). It consists of a fusion protein of the CSP 19 NANP (N, asparagine; A, alanine; P, proline) central repeats and C-terminal regions containing T cell epitopes, fused to the hepatitis B surface antigen (HBsAg) (204). Phase III clinical trials revealed that, over the 4 years follow-up period, 36% protection against clinical malaria was achieved in children 5-17 months with a booster dose of RTS,S/AS01 (205). In infants 6-12 weeks old the vaccine had a 25.9% efficacy over 38 months (205). Vaccine efficacy against severe malaria after a booster dosage in children was 32.2% and 17.3% in young infants (205). Although the answer for lower protective efficacy of RTS,S/AS01 in young infants is not completely clear, it has been hypothesized to be the result of infants' exposure to maternal antibodies (206). The

presence of anti-CSP antibodies prior to immunization in these infants is postulated to have led to lower post-vaccination anti-CSP response, which has been previously correlated to protection and might explain the lower degree of protection (206). Another explanation is the timeline of administration coinciding with other mandatory vaccines, resulting in immune interference (206).

Although RTS,S/AS01 was recently endorsed by WHO for prevention of *Pf* in children living in endemic regions with moderate to high transmission, its protective efficacy is far from ideal. One explanation is that some CSP T cell epitopes used in the vaccine are highly polymorphic in endemic regions, thus evading immune responses elicited by immunization (207). Further improvements of RTS,S/AS01 are currently under development (195). R21 is an upgraded version of RTS,S/AS01, where virus-like particles contain a fusion of CSP-HBsAg, the sole protein component (208). R21 has been shown to induce high anti-CSP titers when combined with adjuvants, with minimal anti-HBsAg antibody response (208). Results from a phase IIb clinical trial showed a protective efficacy of 77% with R21 compared with 44% for RTS,S/AS01 at the same site in Burkina Faso, over 12 months of follow-up (210).

Liver-stage antigen 3 (LSA-3), which is expressed on the surface of sporozoites, in the PV of liver stage parasites and in merozoites, has been shown to induce protection against sporozoite challenge in chimpanzees and *Aotus* monkeys (203,210–212). Daubersies *et al*. reported that sterile protection could be achieved by immunization with the LSA-3 plus adjuvants in 8/9 immunized chimpanzees against successive heterologous challenge in early preclinical studies, later reporting similar results with a LSA-3 DNA vaccine (210,213). *Aotus* monkeys were also reportedly protected by immunization with the N-terminus region of the LSA-3 alone or in combination with the repeat region of LSA-3, inducing effector B and T-cell responses capable of conferring sterile immunity (211). This vaccine candidate has yet to be tested in humans.

Alternative approaches to enhance the efficacy of subunit vaccines have been explored using viral vectors with pre-erythrocytic stage *Pf* antigens (195,214,215). A delivery platform using the chimpanzee adenovirus serotype 63 (ChAd63) and a modified vaccinia virus Ankara (MVA) in a prime-boost administration regimen was employed, in attempts to increase the

efficacy of CSP-based vaccines (216). Although it was able to induce high level T cell responses, the ChAd63-MVA CSP vaccine performed poorly in a controlled human malaria infection (CHMI) clinical trial, protecting only 1/15 test subjects (217). Another vaccine insert consists of the ChAd63-MVA multiple epitopes (ME)-TRAP viral vector vaccine candidate (214). ME is composed of 20 pre-erythrocytic stage epitopes that are fused to the TRAP (218). Prime-boost immunization with this vaccine candidate in phase I/IIa clinical trials had a protective efficacy of 21% against challenge with heterologous sporozoites and a pre patency delay of 36% in volunteers (219). In a phase IIb clinical trial in Kenyan adults a 67% reduction in the risk of *Pf* infection was observed (218), but in Senegal the vaccine candidate showed no protective efficacy (220). These results suggest that ChAd63-MVA ME-TRAP does not confer protection against heterologous parasites frequently found in malaria-endemic regions. Although viral vector malaria vaccines performed poorly in clinical trials, their potential should not be discarded. Due to their ability to induce high T cell responses, optimizations regarding the addition of several antigens to the viral vector, and combinations with RTS,S, might be considered (195).

Another vector-based approach uses bacteria to present *Plasmodium* antigens to the host's immune system. This strategy has been employed in the construction of a pre-erythrocytic and mosquito stage vaccine, using the *Plasmodium* protein Cell Traversal of Ookinetes and Sporozoites (CelTOS). Its role is essential in infection of liver cells by the sporozoite and in ookinete traversal of the mosquito midgut (221). Using a codon harmonization approach, full-length CelTOS was successfully expressed in *E. coli* (222) and immunization of mice with this vaccine candidate, coupled with an adjuvant (Montanide ISA-720), evoked strong humoral and cellular responses, resulting in 75% protective immunity against viral challenge with homologous *Pb* sporozoites (222).

Sub-unit vaccination approaches benefit greatly from extensive research on the CSP, which has led to the development and recent endorsement by WHO of RTS/S, AS01. However, it should be considered that other antigens might bare greater immunogenic potential than the CSP, and research directed to the identification of new *Plasmodium* proteins and new subunit vaccine strategies should not decline.

1.6.1.2 WHOLE-SPOROZOITE VACCINES

WSV rely on the use of live-attenuated sporozoites that are eliminated or arrest their development during, or shortly after the liver stage of development (223). Contrary to subunit vaccines, they benefit from expressing correctly folded and post-translationally modified parasite proteins, that could induce the production of a wider array of antibody responses against human malaria sporozoites, allowing the development of different types of immunes responses (197). This strategy aims to induce sterilizing immune responses, without the emergence of clinical symptoms and remains the most effective to date, with protection ranging from approximately 35% to 100% (223,224). The degree of protection was shown to be correlated with the timing of parasite development arrest in rodents, to be dependent on the immunization regimen and to follow a dose-dependency in humans (214,225,226). However, there are still some limitations to the strategy, namely, the production and administration of large quantities of sporozoites for vaccination (224). Currently, this vaccination strategy includes: radiation-attenuated sporozoites (RAS), genetically attenuated parasites (GAP) and chemoprophylaxis with sporozoites (CPS) (**Fig. 1.6.1**) (224).

1.6.1.2.1 RADIATION ATTENUATED SPOROZOITES

RAS, as its name implies, requires the use of sporozoites that have been attenuated with a dose of radiation strong enough to cause DNA damage in the parasites, leading to the early arrest of their development in the liver (197,223). Although this results in inhibition of parasite's DNA replication and modifications in the parasite's ultrastructure and gene expression, there is limited to no impact on hepatocyte infectivity (227–230). RAS vaccines are the most clinically advanced PEVs (197). During the 1970's several studies established that protection could be achieved through immunization with bites of irradiated mosquitoes infected with *Pf* and *Pv* sporozoites (231–238). Moreover, *Pf* RAS-immunized volunteers were protected from subsequent challenge with homologous and heterologous *Pf* sporozoites (231–234). These studies showed that a vaccine against malaria offering sterile immunity was achievable. Yet, taking in account that more than 1000 mosquito bites are required to achieve

a high level of efficacy, this strategy was not considered suitable for global vaccination (197). Another drawback of this strategy is the possibility of different degrees of irradiation of parasites resulting in incomplete attenuation and consequently ineffective immunity and breakthrough infections (239).

In 2010, the development of a platform for harvesting sporozoites from aseptic mosquitoes and subsequent cryopreservation by Sanaria, brought new hope for WSVs, allowing to explore the administration of cryopreserved sporozoites by needle and syringe (239). In the first clinical trial with Sanaria's *Pf*SPZ vaccine, subcutaneous and intradermal administrations were poorly protective, and inferior to that of intravenous injection in studies with non-human primates (240). In 2013, sterile immunity was reported in 6/6 test subjects with Sanaria's RAS vaccine candidate, *Pf*SPZ, administered intravenously against mosquito bite CHMI in the United States (225). A factor shown to be critical for the efficiency of RAS vaccines was the route of inoculation, as demonstrated by Seder *et al.* (225). In the first clinical trial with Sanaria's *Pf*SPZ vaccine, subcutaneous and intradermal administrations were poorly protective, and inferior to that of intravenous injection in studies with non-human primates (240). It was subsequently demonstrated that delivering RAS intravenously was crucial to the induction of circulating and liver-resident *Pf*-specific CD8+ T cells in macaques (240). More recently, a clinical trial of *Pf*SPZ in Malian adults showed a 29% efficacy against heterologous strains during the following 6 months post-immunization (241). This reduction in efficacy, when compared to what was observed previously with CHMI in United States adults (225,241), could be justified by a pre-exposure to malaria parasites in individuals living in endemic regions, leading to hypo-responsiveness to vaccination (197,214). Although there are fundamental differences between the two studies, such as field strains being different from that employed in CHMI challenge, these results suggest that improvements need be made to RAS vaccination strategies. Moreover, technical aspects involved in the administration of RAS vaccines in endemic countries need to be considered. RAS require the maintenance of a cold chain from transport to storage, which can be challenging in developing countries (195).

1.6.1.2.2 GENETICALLY ATTENUATED PARASITES

The availability of genomic libraries of several *Plasmodium* species (242–246), identification of stage-specific gene expression (247–249), and the development of genetic modification techniques (250,251), has enabled the generation of GAP (252). In GAP immunization approaches, genes essential for parasite development are modified or deleted, preventing progression to the blood stage of infection, without compromising parasite viability (252). They were initially postulated to elicit a higher protection than RAS due to their ability to arrest later during liver stage development, exposing the host's immune system to a wider range of antigens (253). It was demonstrated in rodents that a late-arresting GAP vaccine conferred higher protection than an early-arresting GAP sporozoite vaccine, as well as cross-species and cross-stage immune responses (254). Currently, GAPs can be engineered to arrest at different time points during liver stage development (255). When compared to RAS, they offer more flexibility for additional genetic modifications, that could increase protection potency, and more precise attenuation (223).

In the first clinical trial with a GAP vaccine, *p52-*/*p36-* genes, involved in the formation of the PV, were knocked-out without success in impairing parasite replication, resulting in a breakthrough infection in one volunteer (256). To date, few late arresting GAPs have been generated, with only one viable *Pf* GAP currently available, a knockout of *Pf* meiosis inhibited 2 (*Mei2*) (257). Still, considering the ability of late liver stage-arresting GAPs to provide superior protection than early arresters and RAS, this vaccination strategy would greatly benefit with the development of *Pf* late arresting parasites.

1.6.1.2.3 CHEMOPROPHYLAXIS WITH SPOROZOITES

The CPS immunization strategy relies on the administration of wild-type, non-attenuated sporozoites under the cover of a blood stage-specific drug to eliminate erythrocytic parasites before they can lead to disease symptoms (197). This approach offers the added advantage of letting parasites reach the blood, therefore allowing completion of liver stage development, before eliminating them and preventing the development of clinical illness (195). Although it has been suggested that humoral immune responses against blood-stage malaria antigens are generated with parasite release in the blood, Bijker *et al.* showed that test

subjects that received CPS and were subsequently challenged by blood stage parasites became infected (258). This indicates that protection by immunization with CPS is mostly conferred by pre-erythrocytic stage immune responses (259).

CPS was first explored as a vaccination strategy in murine malaria models in the late 1970s using infection under chloroquine prophylaxis (260–262). Its protective efficacy in a CHMI setting was shown in 6 immunized volunteers where 100% protection was observed 8 weeks after final immunization, with 4/6 volunteers still protected approximately 2 years later (263). It was later shown that this strategy did not convey protection against challenge with heterologous parasites (264). Even so, CPS remains a powerful tool in understanding malaria immunity (214). Mordmüller *et al.* showed that immunization with *Pf*SPZ-CVac, combining Sanaria's PfSPZ vaccine with chemoprophylaxis, conferred 100% protection against CHMI (226). Remarkably, the number of sporozoites required for immunization was 10 to 100 fold lower than those required for RAS vaccination (226).

Vaccination with CPS still faces many hurdles: the continuous administration of antimalarial drugs is impractical, making it difficult to envision the implementation of CPS in endemic countries (265), achieving parasitological cure with drug treatment might be challenging due to incorrect administration and emergence of drug resistance (197,266), and close monitoring of vaccinated subjects is required (195). To tackle the issue of drug resistance, other drugs continue to be tested for CPS, such as primaquine (267), azithromycin (267,268), pyrimethamine (269) and mefloquine (270). Moreover, controlled released drug formulations have been under development to address the issue of non-compliance and logistic constraints associated with frequent drug-dosing regimens (271–274). To enhance the efficacy of this vaccination strategy, optimizations of immunization regimens and pharmacological alternatives for prophylaxis need to be explored.

1.6.1.2.4 *Pb*Vac

In recent years an alternative to the WSV immunization strategies outlined above has emerged – *Pb*Vac (275). This vaccine candidate employs *Pb* parasites that have been genetically modified to function as a delivery platform of human malaria antigens, more

specifically the *Pf* CSP, placed under the *Pb* pre-erythrocytic stage-specific promotor UIS4 (275,276) (**Fig. 1.6.2**). Sir Edward Jenner was the first to coin the concept of cross-species protection with the hypothesis that inoculation with cowpox protected from subsequent infection with smallpox (277). The confirmation of his observation laid the foundations for the development of whole-organism vaccines and, with them, *Pb*Vac.

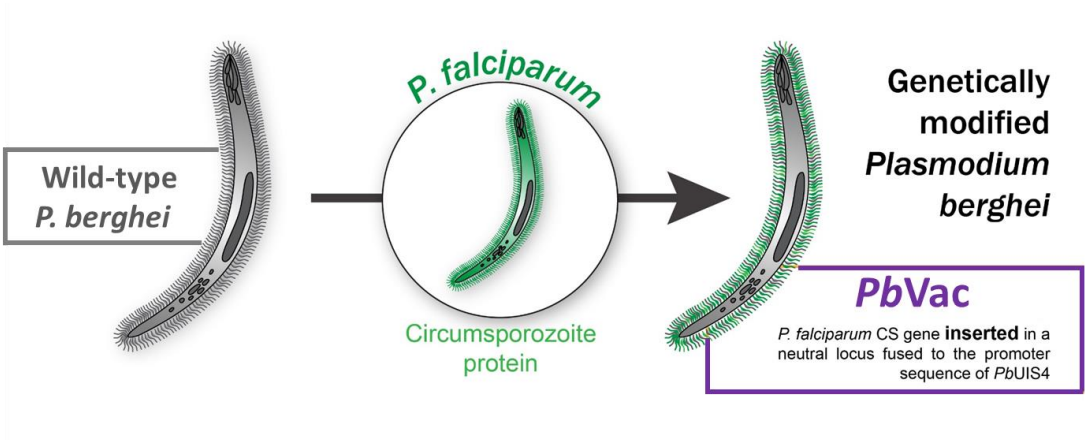

FIGURE 1.6.2 – Schematic representation of the construction of *Pb*Vac

Pb is a murine malaria species that shares large blocks of synteny with human malaria species in its core genome, with several *Pb* ANKA proteins having orthologs in *Pf* clone 3D7 (246). Moreover, due its amenability to transfection (278), non-pathogenicity to humans (279), promiscuous cell infectivity (280) and wide availability, it has frequently been used in studies of *Plasmodium* infection, and in the understanding of its cellular and molecular biology (280–284). Its potential, however, goes beyond that of basic biology. *Pb* sporozoites can infect human hepatic cells, a crucial step for the induction of pre-erythrocytic immune responses, without developing an erythrocytic stage infection (275). With the development of transgenesis methods, these parasites can be engineered to incorporate human malaria immunogens, eliciting cross-species immune responses and answering to safety issues previously posed by GAP and RAS (197,256,266,278). Mendes *et al.* report that safety of *Pb*Vac is further ensured by the integration of genes by double crossover recombination, a permanent and stable method that prevents reversion to wild-type genotypes (276,285). Pre-clinical studies showed that *Pb*Vac immunization in rabbits was capable of eliciting high antibody titers, mostly targeting *Pf*CSP, that can prevent subsequent infection by human-infective

parasites in liver-humanized FRG mice (275). In a phase I/IIa clinical trial, although sterile protection could not be achieved after CHMI in immunized volunteers with *Pb*Vac, a delay in patency of approximately 2 days was observed as well as an estimated 95% reduction in parasite density when compared to non-immunized controls (286). Additionally, the study revealed that immunization with *Pb*Vac elicits dose-dependent cross-species cellular immune responses, and that anti-*Pf*CSP antibodies elicited by immunization are able to functionally inhibit *Pf* invasion of liver cells *in vitro* (286).

Further optimizations on immunization regimen and target antigens need to be performed to improve the potential of *Pb*Vac as a vaccination tool. Although this strategy has had limited success, these results demonstrate the potential of this platform for the development of a next-generation malaria vaccine.

1.6.2 ERYTHROCYTIC VACCINES

Blood stage vaccines (BSVs) focus on asexual parasites, either by targeting the merozoite or infected RBCs (287). They act by eliciting antibodies that bind to merozoite antigens, inhibiting RBC invasion (287), or they induce the production of antibodies that recognize parasite antigens expressed on the infected RBC (**Fig. 1.6.1**) (196). The ultimate goal of BSVs is to prevent the emergence of clinical symptoms, and consequently, reduce disease-associated morbidity and mortality (196). The development of pre-erythrocytic vaccines faces many hurdles: blood stage parasites are briefly extracellular (~ 1 min) when merozoites egress and infect RBC, narrowing the window of action; antigen polymorphism limits immune protection with heterologous strains; redundant invasion pathways allow the parasite to use different routes to invade RBCs; and PEVs and TBVs benefit from life cycle bottlenecks that lead to extreme reduction in parasite numbers, which is not in the case during the blood stage of infection (189).

The first surface merozoite proteins identified were *Pf* merozoite surface protein-1 (*Pf*MSP1) and *Pf* apical merozoite antigen 1 (*Pf*AMA1) (288). *Pf*MSP1 is a surface antigen involved in merozoite invasion of RBC (289), which has had limited success in conferring protection against clinical malaria (290,291) *Pf* apical merozoite antigen 1 (*Pf*AMA1) is localized

in the micronemes and is secreted to the merozoite surface during invasion (288), and is also expressed in the sporozoite before hepatocyte invasion (292). Although it was shown that immunization with *Pf*AMA1 did not elicit protection against *Pf* challenge (293–296), combination with adjuvanted rhoptry neck protein 2 (RON2), which interacts with AMA1 in the process of invasion, improved vaccine efficacy in non-human primates (87.5% AMA1-RON2 vs. 25% AMA1) (297). Future vaccines containing AMA1 may be improved by redesigning antigens, to include different epitopes of this protein (189).

Pf reticulocyte-binding protein homolog 5 (*Pf*Rh5) is part of the superfamily of erythrocyte ligands, which has at least one member in every *Plasmodium* genome. It is secreted from the rhoptries forming a heterotrimeric complex with the cysteine rich protective antigen (*Pf*CyRPA) and the Rh5-interactive protein (*Pf*RIPR) (298–301). This complex interacts with the erythrocyte receptor basigin (BSG), during parasite invasion of the RBC (302). Antibodies elicited by immunization with *Pf*Rh5 and natural infection with *Pf* in non-primate models can block RBC invasion at a lower EC_{50} than that of anti-AMA1 and anti-MSP1 antibodies (303–305). Moreover, when combined with other antibodies, a synergistic effect is observed, with RBC invasion inhibited at lower antibody concentrations (306). *Pf*Rh5 is also highly conserved, and antibodies elicited in the aforementioned experiments induced cross inhibition of all *Pf* field isolates tested (307–309). *In vivo* efficacy studies in non-human primates showed that protection could be achieved using a viral-vectored platform of *Pf*Rh5 delivery followed by challenge with heterologous *Pf* parasites (310). Nonetheless, mild or no boosting of vaccine-induced antibodies was observed upon natural infection, which may limit the duration of protection (310).

Other BSV antigens have been explored, besides those involved in erythrocyte invasion. *Pf* erythrocyte membrane protein 1 (*Pf*EMP1) is a parasite surface antigen involved in sequestration encoded by *var* genes (311), with limited success in pre-clinical trials due to its sequence being highly polymorphic, large, and rich in cysteines (189,312,313). One *Pf*EMP1 family member, known as variant surface antigen 2-condroitin sulphate A (VAR2CSA), which has been suggested to mediate sequestration of infected RBC in the placenta (314), has been investigated as a syndrome-specific vaccine candidate (203). Due to VAR2CSA's large size (>300

kD), vaccine development has focused on the induction of immune responses using individual or combined protein domains (315). PAMVAC and PRIMVAC, two candidates that incorporate the VAR2CSA N-terminal fragments, have completed phase I clinical trials, showing their ability to induce functional active antibodies against homologous parasites, but cross-reactivity was limited (256,316). As in other vaccine strategies and candidates, placental malaria vaccines efficacy might be increased by combination with other antigens and schedule and dosage adjustments (189).

1.6.3 TRANSMISSION-BLOCKING VACCINES

TBVs employ surface antigens of the mosquito and sexual stages of *Plasmodium* to control transmission of parasites from the invertebrate to the human host (188,189) (**Fig. 1.6.1**). TBVs are termed altruistic vaccines since immunization of individuals with these vaccines does not result in a direct benefit for the individual, but rather contributes to protection at the population level through herd immunity (317). The deployment of such a vaccine is not without challenges: for reduction of case incidence to be observed, mass vaccination needs to be carried out on the entire target population of a given endemic area, including infants, children and adults, which could be impractical and logistically challenging (317). Even so, as new target antigens are unraveled, interest remains in the development of TBVs that could eventually integrate a multi-stage vaccine (317).

The potential of TBVs was first demonstrated in the 1970s by immunizing chickens with attenuated infected RBCs or purified gametes of the avian parasite *P. gallinaceum* (*Pg*) to induce the production of antibodies that impair parasite development in the mosquito (318,319). The four leading TBV candidates are the gamete surface proteins *Pfs*230 and *Pfs*48/45 (320) and the zygote surface proteins *Pfs*25 and *Pfs*28 (321,322). TBVs will be addressed in further detail in section 1.8.6.

1.7 MALARIA COINFECTIONS

Malaria is widespread in tropical and subtropical regions, where people are frequently exposed to several pathogens simultaneously (323). It is not uncommon that other bacterial,

viral or parasitic diseases are found concomitantly with malaria (323). Due to the restricted access to diagnostic tools and their intrinsic limitations, the identification of the infectious agent(s) underlying a given clinical condition might be complex (324). Moreover, coinfections with malaria parasites might be challenging to study due to the possibility of repeated acute, or asymptomatic chronic infections, as is frequently observed in previously exposed individuals with a certain degree of naturally acquired immunity (325). In the case of a fever diagnostic where coinfection with *Plasmodium* is involved it is particularly difficult to understand if the parasite is the cause of the illness, a contributor, or a passerby (325). Coinfections with multiple pathogens can worsen the clinical outcome or one infection may protect against the other (323). For example, the distribution of malaria and acquired immune deficiency syndrome (AIDS) overlaps geographically, particularly in sub-Saharan Africa (152,326). Evidence suggests that Human immunodeficiency virus (HIV) infection might increase the risk and severity of *Plasmodium* infection, and that increased parasitemia might provide an ideal environment for HIV replication (327).

In the African continent, the geographical overlap between helminths and *Plasmodium* also leads to frequent co-infections (328). Some studies show that helminthic infection leads to an increase on *Pf* parasite load, incidence and worsen the clinical outcome of malaria in patients (329–338) while others show their protective effect (339–347), or simply no effect (348). Here, it seems that several factors are responsible for the outcome of interaction between *Plasmodium* and helminth coinfections: the type and intensity of helminthic infection (328,340,342,347), the age group of the study population (328,342), the timing of infection (349), and the target endpoint (infection or disease) (335).

1.8 THE IMPORTANCE OF BLOCKING *PLASMODIUM* TRANSMISSION

In the beginning of the 21st century, malaria was renewed as a global health priority worldwide (152). Although many successes were achieved in the fight against the disease, mostly due to ACTs, IRS and ITN, more recently progress has stalled (350,351). In recent years it was acknowledged that in order to reach malaria control and elimination, both the impact of the disease on an individual and the emergence of new infections in a population must be

reduced (352). Due to the severe bottleneck in parasite numbers upon transmission to the mosquito, targeting *Plasmodium* at this stage would potentially contribute to the reduction of transmission (**Fig.1.8.1**) (352). Moreover, target antigens from mosquito stages are less genetically variant when compared to blood and liver stage antigens (353–360) and parasites are extracellular for approximately 24 h in the mosquito compared to about 1 min it takes the merozoite to invade the RBC (361,362).

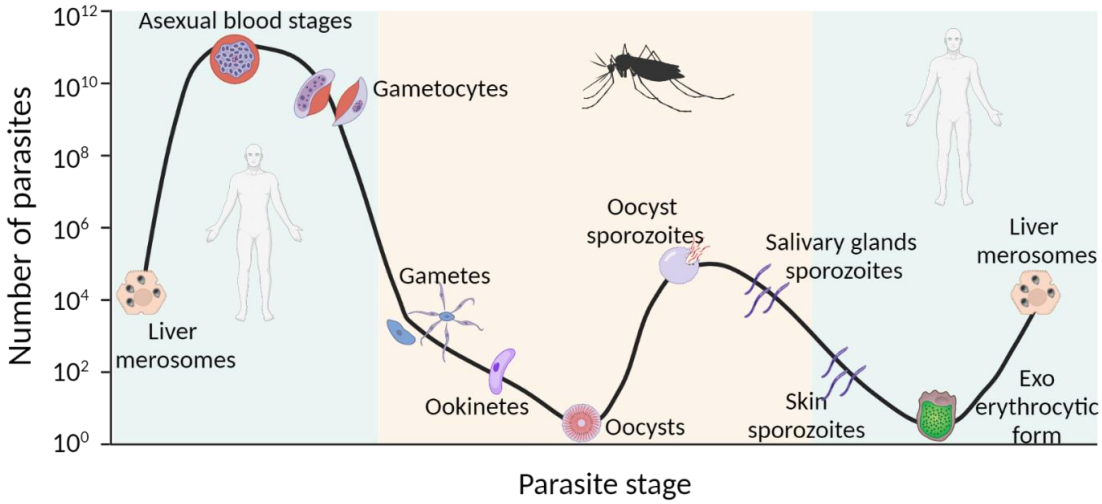

FIGURE 1.8.1 - Bottlenecks of the *Plasmodium* life cycle. (Adapted from Graumans *et al.*, 2020)

In particular, transmission-blocking strategies (TBS) could address two major challenges of malaria elimination: the emergence of parasite and mosquito resistance to antimalarials and insecticides respectively, and asymptomatic and undiagnosed parasite infections that contribute to the cycle of transmission (143). Here we will explore how drug repurposing and TBVs can be explored to impact *Plasmodium* transmission.

1.8.1 THE SPOROGONIC STAGE OF THE *PLASMODIUM* LIFE CYCLE

The transmission stages of the parasite's life cycle are highly conserved across the genus (124), with differences in length of the developmental stages and morphology between lineages (124). Gametocytogenesis in *Pf* and *Pv* takes between 7-15 days and 2-3 days, respectively, and goes through several different stages (74,363,364). Morphologically, while *Pf* gametocytes have a crescent shape, gametocytes of other *Plasmodium* species are spherical (363). Commitment to gametocyte development in the blood stage of infection is thought to

happen before schizogony (365). The parasite is hermaphrodite, i.e., each sexually committed schizont has the possibility of transforming into either a microgametocyte (male) or a macrogametocyte (female) (366). Therefore, the eventual differentiation of the parasite into one of the sexes is likely determined early in gametocytogenesis (367) (**Fig. 1.8.2**).

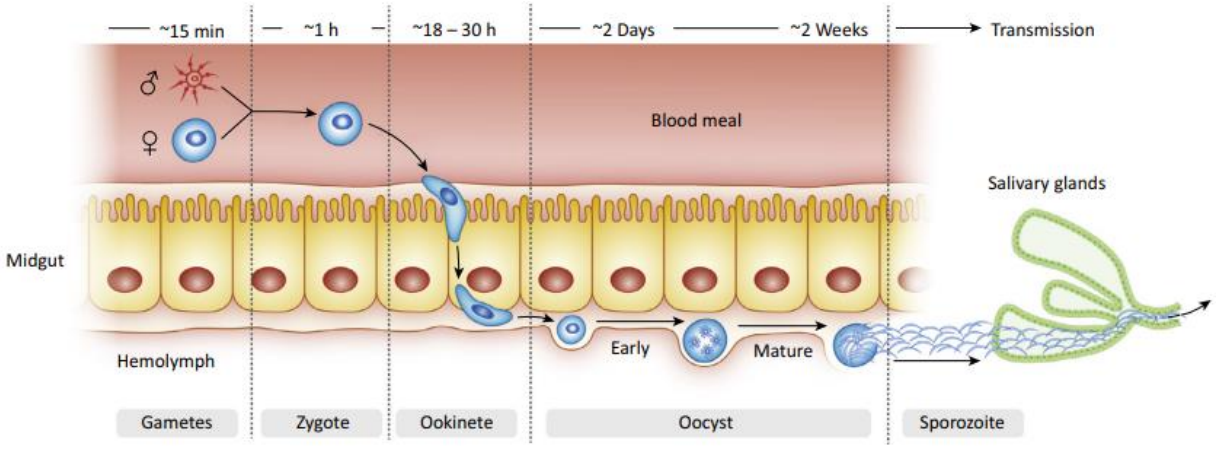

FIGURE 1.8.2 – *Plasmodium* development in the mosquito host (Smith and Barillas-Mury, 2016).

Initially, a few microtubules appear beneath the parasite's cell membrane, increasing in number and doubling the nuclear DNA content as the membrane layer expands, suggestive of mitotic replication (368,369). As the parasite continues its development, sexually dimorphism becomes apparent (137,368). The female parasite presents more mitochondria, cytoplasmatic vesicles, membrane bound osmiophilic bodies and ribosome density in the cytoplasm than the male parasite, and the nucleus of the former is smaller than that of the latter (137,368).

The male parasite develops large nuclear lobes, suggesting higher DNA replication than in female parasites, and few cytoplasmic organelles and synthetic apparatus, indicators of pre-synthesis and storage of proteins essential for gamete formation (137,368,370). When a female mosquito takes a blood meal, it ingests gametocytes. Gametogenesis is induced within minutes in the mosquito midgut lumen (125) as a result of a drop in temperature of the infected blood, a rise in pH, and the presence of gametocyte activation factors, such as xanthurenic acid (127,371–373). After activation, *Plasmodium* species with non-spherical gametocytes round up, followed by egress from the RBC and gamete formation (128) (**Fig.**

1.8.2). Escape of the RBC is achieved by the fusion of the osmiophilic bodies to the gametocyte plasma membrane, and release of its contents in the PV through small ducts (368,374). It is thought that this process may be mediated by proteases derived from the RBC or the parasite (375). Macrogametocytes do not go through any apparent morphological changes besides the emergence of the RBC as a single extracellular and non-motile spherical female gamete (137,368). However, microgametocytes go through three rounds of endomitosis, and intracytoplasmic axoneme assembly, resulting in the release of eight motile haploid daughter genomes in approximately 10-15 minutes – a process known as exflagellation (128,376).

Male gametes are single membrane-bound nucleated flagella, without cytoplasm, apicoplasts or mitochondria, the latter two organelles being solely inherited from macrogametes (137,377–379). The male gamete swims through the mosquito bloodmeal while adhering to neighbor RBC, forming a rosette (380). The microgamete swims to find a macrogamete and, upon fertilization, the gamete membranes fuse and the male axoneme and condensed nucleus enter the female's cytoplasm (129). Nuclear fusion follows gamete fusion, and meiosis occurs for approximately 3 hours, leading to the formation of a tetraploid zygote (369,381) (**Fig. 1.8.2**). The nucleus of the parasite elongates forming a cone shape apical complex, with the apex extended away from the nucleolus, which sits at the base of the cone (128). The parasite is enveloped by a triple-membrane pellicle, that protects it against mosquito midgut proteases (129), and the maturing apical complex includes a polar ring and several micronemes that contain proteins involved in motility, tissue traversal and invasion (125,382). Several cytoplasmic crystalloid form, consisting of small lipoprotein particles, and are thought to function as energy stores or oocyst capsule precursors (383). The apex continues to extrude from the plasma membrane, while microtubule formation starts from the apical end of the cone towards its base (382). The process of elongation of the microtubules is essential for the formation of the ookinete. As the structures elongate further, the cell begins to elongate, passing through an intermediate development stage called the retort, until it forms a banana-shaped ookinete (381) (**Fig. 1.8.2**).

In the midgut of an adult female mosquito, a chitinous structure termed peritrophic matrix (PEM) surrounds the ingested blood meal (384). Several proteases pass through the

PEM and digest the erythrocytes as well as asexual erythrocyte parasite forms and gametocytes that did not fuse (385,386). The ookinete escapes this digestion by migrating through the bloodmeal, and penetrating the PEM (125,137). The micronemal proteins calcium dependent protein kinase 3 (CDPK3), and circumsporozoite and TRAP (CTRAP) are involved in motility and infectivity of the ookinete (387–390). It has been proposed that ookinetes enter the midgut epithelial cells through their apical lateral membrane, following the crevices between adjacent midgut epithelial cells (391). It seems that the parasite exerts mechanical force in penetrating the midgut epithelium, but the micronemal protein membrane-attack ookinete protein (MAOP) is also involved in this process by inducing the formation of pores in the apicolateral plasma membrane (392). It is possible that the parasite enters the host cell through a moving junction created between the ookinete and the midgut cell plasma membrane (84,392). The ookinete traverses several epithelial cells before exiting through the basal side of the epithelium (125). This exit is thought to be promoted by midgut epithelial cells that are expelled upon parasite invasion, and contract their basolateral plasma membrane, causing constriction of the ookinete (391,393). According to the ticking bomb model (394,395), after exiting the invaded midgut cells, intercellular parasites move directly to the basal surface of the midgut epithelium, or firstly migrate within the midgut epithelium between adjacent midgut cells via an intra or extracellular route (130,396). After reaching the basal lamina of the midgut epithelium, the ookinete initiates the transformation into a sedentary round form – the oocyst (125) (**Fig. 1.8.2**).

Although the triggers for this differentiation are not known, it is hypothesized that laminin and other host factors of the basal lamina induce the differentiation into an oocyst (397). An extracellular capsule is formed around the oocyst, as well as new basal lamina at its point of attachment (131,132,398,399). The oocyst capsule is formed by an outer layer, formed mainly of mosquito laminin, parasite transglutaminase and Cap380, and an inner oocyst plasma membrane (400–402). The organelles involved in motility and host-cell invasion are absorbed into the oocyst cytoplasm (383,399), and the parasite undergoes multiple rounds of endomitosis to produce thousands of genomes (137), while it increases in size from 2-3 µm to 40 µm in diameter within 10-13 days (125,403). The nucleus develops into a syncytium-like

compartment, eventually differentiating into small ovoid nuclei, while the apicoplast and the mitochondria proliferate (133,134,383). Beneath the oocyst capsule, sporoblast segregation begins with the appearance of vacuoles that fuse to form large clefts that divide the oocyst cytoplasm into sporoblasts (404). The CSP plays an important role in oocyst development, beginning its expression a few days after oocyst formation, and accumulating on the growing plasma membrane and sporoblasts (405). The nuclei undergo a final step of mitotic division, and the microtubule organizing centers lead the formation of the apical complex and reorganization of nuclei positioning for incorporation into daughter sporozoites (383). Each sporozoite acquires an apicoplast and a mitochondrion, and micronemes and rhoptries develop within the apical region of the sporozoites (134,383).

Sporozoites eventually bud-off from the sporoblast and exit the oocyst either through holes within the oocyst wall and basal lamina, or oocysts rupture to release thousands of mature, haploid sporozoites simultaneously (134–136). Sporozoites migrate to the salivary glands, either by passing through the hemolymph or the internal organs of the mosquito, using gliding motility to finally invade the mosquito salivary glands (82,406,407) (**Fig. 1.8.2**). This process is thought to happen initially through sporozoite attachment and penetration of the basal lamina of the salivary glands (135,136), followed by the formation of a moving junction between the parasite pellicle and the plasma membrane of the salivary gland cells (408). A transient PV is formed, allowing sporozoites to emerge into the salivary gland duct (408). When a female adult mosquito takes a blood meal, the next phase of the malaria parasite life cycle is initiated (137).

1.8.2 TRANSMISSION-BLOCKING CHEMOTHERAPY

Transmission-blocking (TB) chemotherapy traditionally relies on the use of antimalarial drugs to reduce the transmission of gametocytes to the human host, thus interrupting the cycle of parasite transmission (143). However, the concept should be expanded to include compounds that could inhibit parasite development in the mosquito, such as ookinetes and oocysts. ACTs, the first line of treatment against uncomplicated malaria, eliminate the parasite's asexual stages, and have limited effect on the sexual transmissible forms, which

remain in circulation for 1-3 weeks after treatment (159,351,409). Nonetheless, the WHO recommends using primaquine, a strong gametocytocidal, in combination with ACTs, to reduce *Pf* transmission in low transmission settings (160). Despite previous concerns of toxicity of this intervention for individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency [reviewed in (410,411)], it has recently been considered that at the recommended single administration dose of 0.25 mg/kg, serious toxicity is highly unlikely to occur (160,412). This strategy has been previously employed in 17 Cambodian villages where administration of artemisinin-piperaquine was combined with a low dose of primaquine to attempt rapid parasite elimination, and consequently, control malaria (168). Although this mass administration scheme resulted in a decrease of 96.2% of *Pf* infection rates in children, it is not clear weather this reduction was due to the effect of primaquine on the sexual stages of the parasite or the elimination of asexual parasites by ACTs (168). Additionally, a review of eight randomized controlled clinical trials on the efficacy of adding a single dose of primaquine to an ACT regimen has shown a reduction of 14% to 2% in mosquito infectiousness on days 3-4 and 8 after treatment, and its relative safety for most tested individuals (413).

With such limited options for targeting the transmission stages of the parasite, in recent years many high throughput transmission screening assays aiming to identify new drugs and small molecules, as well as to explore the TB activity (TBA) of current antimalarials, have been developed (414–422). Atovaquone, either alone or in combination with proguanil was shown to reduce parasite transmission by inhibiting ookinete formation and oocyst development (414,415,423). Contrary to primaquine, which has a short half-life (424), atovaquone demonstrated a long-lasting TB effect in a standard membrane feeding assay (SMFA) with serum from drug treated volunteers (423). In fact, when selecting compounds with TBA, their half-life, as well as the rate and the timing at which parasites are cleared or neutralized should be taken into account (425).

The TBA of methylene blue has also been thoroughly investigate *in vivo* and *ex vivo*, and its activity against late-stage gametocytes has been demonstrated (419,426–431). One study also reported its gametocytocidal effect in field isolates *in vitro* (432). Other compounds remain in study and are currently undergoing clinical development. Cipargamin is a fast

antimalarial, which acts on the *Pf* adenosine triphosphatase 4 (*Pf*ATP4), disrupting the sodium homeostasis in the parasite (433). It is active against all the intra-erythrocytic stages of *Pf* and *Pv* and has gametocytocidal activity (433,434). In a phase IIa clinical trial, Cipargamin was shown to have a parasite clearance half-life superior to that of artesunate, however, its effect on sexual parasites in the field remains unclear (435). Other *Pf*ATP4 inhibitors, MMV048 and SJ733 are currently undergoing phase II clinical trials (436,437).

One of the most concerning issues regarding the current path for discovery of TB compounds relates to dosing. In addition to TBA, all the compounds mentioned above present an effect on the erythrocytic stages of the parasite. However, it has been shown that the *in vitro* dosing required to have TBA is higher than that required to eliminate the parasite's erythrocytic stages, highlighting a recurrent concern in the treatment of malaria: resistance (438). Since the administration of sub-effective dosing may lead to the emergence of resistant sexual parasites, alternative drugs with effect only on the sexual and mosquito stages of *Plasmodium*, which could be administered with ACTs, should be considered.

1.8.3 *IN VITRO* SPOROGONIC CULTIVATION

The mosquito stage of *Plasmodium* is the most enigmatic phase of the parasite's life cycle. Previous research has explored the interaction between the invertebrate host and the parasite in an attempt to better understand the dynamics of the parasite's sporogonic development. However, research has been limited by the inherent difficulties in studying the parasite in a complex model such as the mosquito, which limits the availability of biologically pure material. Consequently, the development of *in vitro* systems for the cultivation of the parasite's sexual and mosquito stages became essential to further our knowledge of the parasite's life cycle and unravel new TBS against malaria.

Cultivation of the *Plasmodium* parasite *in vitro* has been hindered by the parasite's physiological, metabolic and nutritional requirements during this stage of development (439). An *in vitro* system would have to mimic the conditions outlined above for each of the transformation steps during parasite development: gametocyte formation inside the RBC, ookinete formation within the mosquito midgut, oocyst development on the basal lamina of

the midgut epithelium, and sporozoite maturation in the salivary glands (440). Even so, complete *in vitro* development from gametocyte to sporozoite was achieved for *Pf* (441), *Pg* (442), *Pb* (443) and *Py* (444). Despite these achievements, it is still not possible to produce high amounts of sporozoites of most *Plasmodium* species *in vitro* guaranteeing their viability and sufficient purity for biological studies. Sanaria has, in recent years, reported on the development of a method to manufacture pure *Pf* sporozoites *in vitro* (445).

1.8.3.1 HUMAN MALARIAS

Trager and Jensen (446) and, subsequently, Haynes *et al*. (447), described the continuous *in vitro* culture of *Pf* parasites in RBC, with the platform developed by the former supporting the formation of gametocytes. In 1977 Carter and Beach observed exflagellating *Pf* gametocytes produced *in vitro*, when parasites were exposed to an increase in pH (448). Phorbol diesters (449) and hypoxanthine (450) were also suggested to be involved in the formation and maturation of gametocytes, although the effect of the former on gametocyte numbers is hypothesized to be an indirect result of its effect on the RBC. In 1992 Warburg and Schneider successfully produced *Pf* sporozoites *in vitro* using Matrigel as a replacement of the mosquito basal lamina, and *Drosophila melanogaster* cells, as sources of extracellular matrix molecules, which are crucial for the formation of oocyst cultures (441). Wheat germ agglutinin was also used to boost the transformation of zygotes into ookinetes. Recently, Eappen *et al.* reported on the production of millions of infective sporozoites from a matrix free platform using Schneider's insect medium, chemically defined medium for high density cell culture, a cocktail of different sugars, hypoxanthine, *N*-acetyl glucosamine, glucosamine and sodium hydroxide to promote oocyst and sporozoite formation in vitro (451). However, *in vitro* culturing of *Pv* remains limited and, so far, only production of ookinetes from blood of infected patients has been achieved (452).

1.8.3.2 NON-HUMAN MALARIAS

As previously mentioned, access to non-human malaria parasites has greatly propelled the investigation on *Plasmodium* and its interactions with its hosts. The complete *in vitro*

development from gametocyte to oocyst of *Pr*, an avian parasite, was achieved by Ball and Chao, using culture medium composed of a mix of phosphate and bicarbonate buffered basic solutions, supplemented with glucose, amino acids, B vitamins, purines and pyrimidines plus chick serum and chicken embryo extract (453). Although oocysts were formed, their development ceased after 5 days. Only when oocysts were extracted from mosquitoes, and cultured in medium containing saline and pupae extract, were infective sporozoites produced (454). Warburg and Miller, using HEPES-buffered RPMI medium with 15% FBS, supplemented with trehalose, hypoxanthine, and a lipoprotein-cholesterol mixture co-cultured with *D. melanogaster* L2 cells and Matrigel, could induce transformation of ookinetes up to sporozoite release of *Pg* (455). Sporogonic cultivation of *Py* was first achieved in 2005 with the addition of *D. melanogaster* cells conditioned medium, which contains soluble factors essential for ookinete to oocyst transformation (444). Sporozoites produced *in vitro* showed far lower infectivity than those produced *in vivo* (444).

1.8.4 *IN VITRO* CULTURING OF *P. BERGHEI*

Pb has been used as a surrogate for studies on the biology of *Pf* for many years. With the development of whole-sporozoite vaccines employing *Pb* genetically modified parasites, and due to the inherent difficulties of working with *Pf*, the complete *in vitro* sporogonic development of *Pb* became widely investigated. Janse *et al.* could successfully achieve *in vitro* production of *Pb* gametocytes in 1985 (456), followed by the identification by Billker *et al.* of a gametocyte activation factor, produced in the mosquito, identified as xanthurenic acid (127) . The latter showed that increased levels of xanthurenic acid could act together with an increase of pH of 0.2-0.3 and a decrease in temperature, to induce gametocyte activation (127).

Weiss and Vanderberg studied how different medium compositions could lead to the formation of *Pb* ookinetes *in vitro*. Although differentiation could be achieved with a minimum essential medium, the conversion rate from gametocyte to ookinete was 1% or less (457). An increase on ookinete formation to 44% was achieved by Janse and colleagues employing HEPES-buffered RPMI medium instead of minimum essential medium (456). Oocyst to sporozoite transformation of *Pb* was first reported by Al-Olayan *et al.* (443), who obtained

mature oocysts *in vitro* by culturing the parasite in Schneider's medium at pH 6.8. As described before, both Matrigel and *Drosophila* cells were essential for parasite transformation (441,455). It was later demonstrated that ookinetes bind to the annexin produced by these cells in the process of invasion of the mosquito midgut (458) and that laminin, present in Matrigel, binds to the ookinete surface proteins P25 and P28 as well as to the secreted ookinete adhesive protein (SOAP) (459). Carter *et al.* were, however, able to elicit the transformation of ookinetes to oocysts in the absence of a substrate, hypothesizing that this process may be driven from soluble factor derived from the mosquito hemolymph. These authors also showed that increasing concentrations of p-aminobenzoic acid (PABA) enhanced oocyst yield and speed of growth, and that an environmental trigger, bicarbonate, was necessary to induce initial ookinete to oocyst transformation. Full transformation was, however, dependent on a combination of several ingredients, including 15% heat-inactivated FBS, 0.2% lipid/cholesterol, Schneider's medium and a pH of 7-8 (460).

1.8.5 *IN VITRO* DRUG SENSITIVITY ASSAYS

Most drug discovery efforts have focused on the asexual erythrocytic (symptomatic) stages of the parasite's life cycle, largely disregarding the parasite's sexual and sporogonic stages. However, TB drugs (TBDs) can potentially impair parasite transmission, thereby contributing to elimination of the disease. Hence, reliable *in vitro* screening assays that can identify the TBA of large libraries of compounds against the parasite's gametocyte and mosquito stages could represent an alternative to the time-consuming and cost demanding SMFAs.

1.8.5.1 GAMETOCYTE STAGE ASSAYS

Currently available *in vitro* assays to identify drugs that are active against the sexual and sporogonic stages of *Plasmodium* have focused on the evaluation of compound activity up until the development of female and male gametes. This limitation arises from the difficulties of *in vitro* culturing *Pf* mosquito stages. Several gametocyte viability assays have been developed to assess the TBA of multiple compounds (418,421,426–428,431,461,462). These

assays rely on the expression of metabolic markers or reporter genes, or even on the assessment of *in vitro* activation of female and male gametes (418,421,426–428,431,461,462). Tanaka and Williamson reported the TBA of compounds in stage III-V gametocytes by measuring an oxireduction indicator, AlamarBlue, to assess the parasite's metabolic activity (418). Alternatively, the assessment of the TBA of compounds has been assessed by Buchholz *et al.* (462) employing a targeted cell-based assay, using genetically modified parasites, and by Lelièvre *et al.* (426), employing flow cytometry- or bioluminescence-mediated detection of ATP. D'Alessandro *et al.* reported the development of a simple and cheap screening method measuring the parasite's lactate dehydrogenase activity by optical density to determine gametocyte viability (428).

Due to constraints in the cultivation of *Pf* sporogonic stages *in vitro*, Delves *et al.* and Azevedo *et al.* described the use of *Pb* parasites to evaluate the effect of compounds on the ookinete formation (463), and ookinete to oocyst maturation, respectively (415). The *in vitro* activity of compounds on parasite development was determined by microscopy analysis of cultures for the further and measurement of luminescence expression of genetically modified parasites for the latter (415,463).

1.8.6 TRANSMISSION-BLOCKING VACCINES

In recent years, malaria elimination from endemic regions has received renewed attention, but currently available tools have systematically proven to be insufficient to eliminate the disease from regions where transmission is stable (464). Vaccines have been previously shown to be safe, cost-effective, and amenable to mass administration, having played a vital role in the control or elimination of diseases that previously threatened humankind. Nonetheless, only in 1974 was vaccination widespread in low and middle-income countries (465). Traditionally, vaccines aim to prevent infection and disease. However, a crucial step for elimination and eradication of a disease from a population is the prevention of transmission (466). In the specific case of malaria, TBVs would prevent the spread of disease by eliminating the parasite in the mosquito vector (**Fig. 1.8.3**) (466). Initially, malaria vaccine development efforts were solely focused on the pre-erythrocytic stage of the parasite's life cycle (467). Nevertheless, the development of vaccines that could interrupt malaria

transmission became increasingly attractive in order to fulfil the goal of malaria eradication (468). Although previously overlooked in favor of vaccines targeting other stages of the parasite's life cycle, TBVs present several attractive features mentioned above, common to other TB interventions (see section 1.8).

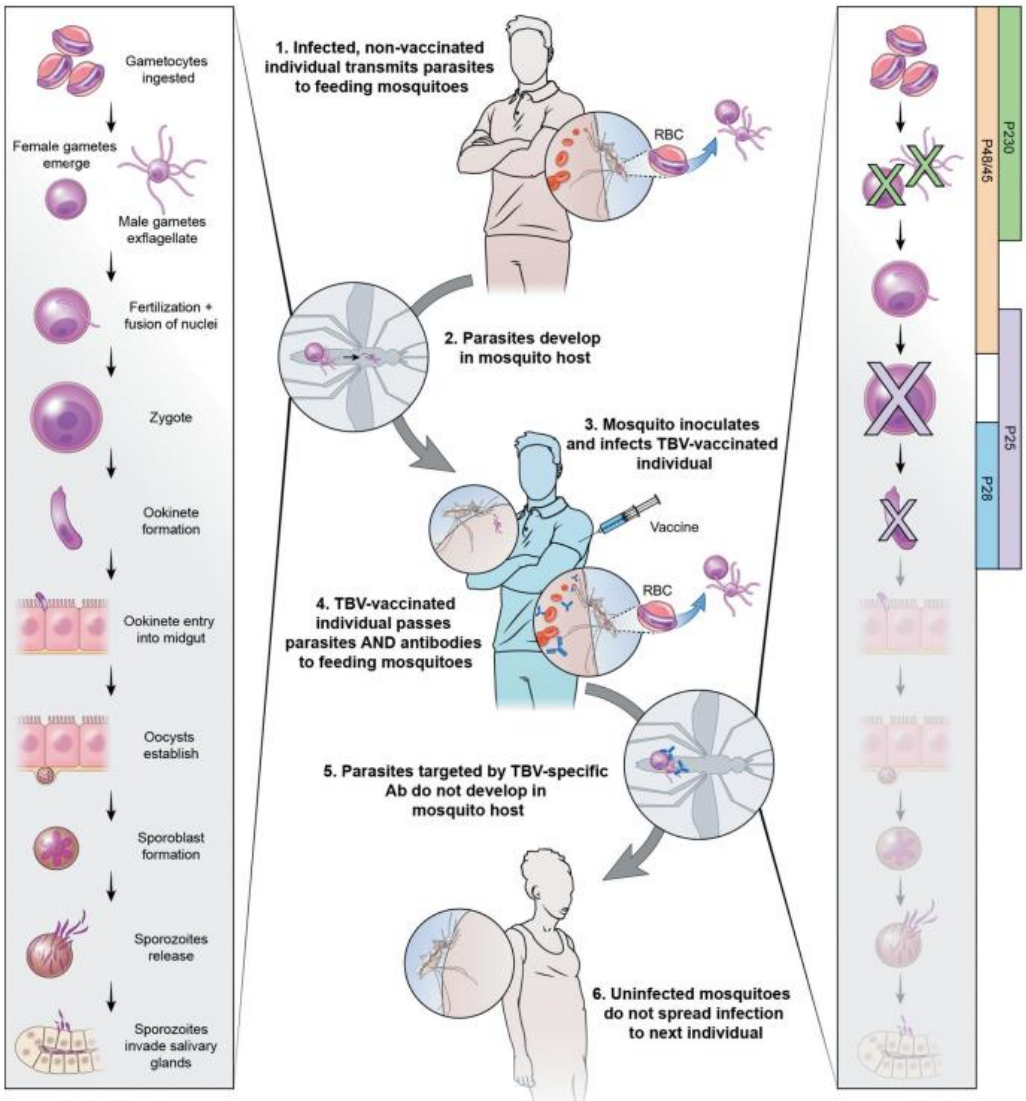

FIGURE 1.8.3 -Transmission-blocking vaccines and their mechanism of action (Duffy, 2021)

Despite their numerous advantages, TBVs are not without their disadvantages too. The boosting of immunity elicited by natural infection that is achieved with pre-erythrocytic and erythrocytic vaccines is limited in TBVs, since they induce immune responses against either mosquito stage parasite antigens or sexual blood stage parasite antigens, which are present in much lower numbers than other blood stage parasite forms (469). Moreover, as previously

mentioned, these vaccines would likely be administered only in malaria-endemic regions, and therefore, logistics and production limitations need to be taken in consideration (317). TBVs incorporate antigens expressed by gametocytes, *Plasmodium* mosquito stages – gametes, zygotes and ookinetes - and, more recently, from the mosquito, in order to induce antibodies that inhibit parasite development (466). The most advanced TBV antigens in clinical studies have remained the same since they were first identified (466,469,470). They are typically rich in cysteines with multiple 6-cys (*Pfs*230 and *Pfs*48/45) or epidermal growth factor (EGF)-like domains (*Pfs*28 and *Pfs*25), that so far, have been challenging to prepare as correctly folded recombinant proteins and have been poorly immunogenic (**Fig. 1.8.3**) (466). In addition, anopheline alanyl aminopeptidase N1 (AnAPN1) (471,472), *Pf* hapless 2 (HAP2) (473,474), *Pf*CelTOS (475–477), *Pfs*47 (478) and *Pf* enolase (479,480) are currently undergoing preclinical trials.

*Pfs*230 is expressed in both male and female gametocytes, forming a complex with *Pfs*48/45, and has previously been suggested to be involved in gamete fusion (**Fig. 1.8.3**) (481,482). Due to difficulties in expressing correctly folded *Pfs*230, vaccines have focused in incorporating immunogenic fragments rather than the full-length protein (483). Preclinical studies indicate that the C fragment spanning the N-terminal prodomain and the first three 6-Cys domains have the highest TBA when compared to other protein fragments (483). Subsequently, the N terminal *Pfs*230 fragment containing domain 1 (*Pfs*230D1) was shown to induce higher TBA than *Pfs*230D1-2 in a SMFA (484). Early results from a clinical trial with a *Pfs*230D1 vaccine candidate suggest that it is able to induce functional serum activity after two vaccine doses in some individuals (485).

*Pfs*28 and *Pfs*25 are associated with ookinete attachment to the mosquito midgut (**Fig. 1.8.3**) (486–488). Duffy *et al.* showed that a combination of both antigens' antisera had a synergetic TBA in a SMFA, in comparison to the isolated activity of the antiserum from each antigen (489). This was confirmed by the co-expression of both antigens as a fusion protein in *Saccharomyces cerevisiae*, which was shown to require fewer vaccinations and doses than *Pfs*25 and *Pfs*28 alone to elicit antibodies that block oocyst development (490). However, the same was not observed using a dual protein adjuvanted delivery system (491). Individually,

antibodies against *Pfs*28 were shown to have TBA (490,492), and the immunogenicity of *Pfs*25 conjugated to outer-membrane protein complex of *Neisseria meningitidis* (493) or the ExoProtein A (EPA) of *Pseudomonas aeruginosa* was shown to be superior to that of *Pfs*25 alone (494–496). In a CHMI Phase I clinical trial with *Pfs*25-EPA, serum form immunized individuals was shown to have dose-dependent TBA (497). Subsequently, a clinical trial in Mali indicated that, even though significant TBA could be achieved after a complete immunization regimen with *Pfs*25-EPA, antibody titers decreased rapidly after the fourth immunization dosage (498).

*Pfs*48/45 is expressed on the surface of late stage II gametocytes until fertilization (**Fig. 1.8.3**) (499,500) and plays an essential role in male gamete fertility (501), forming a glycosylphosphatidylinositol (GPI)-anchored complex with *Pfs*230 (499,502). P48/45 is part of a family of proteins in which the 6-cys domains share a conserved structure across *Plasmodium* species (503,504). *Pfs*48/45-based vaccine development has been hampered by the difficulties in expressing the full length correctly folded protein, including the C-terminal domain 3, known to be a target for potent rodent transmission-blocking monoclonal antibodies (mAbs) (505). In a field setting, it has been shown that low anti-*Pfs*48/45 antibody titers are present in the serum of individuals naturally exposed to *Plasmodium* infection, and their titers correlate with TBA, although association between sexual stage immune responses and gametocytemia was not always observed (505–507). This illustrates the potential of using this vaccine candidate, since individuals immunized with *Pfs*48/45 could benefit from immune boosting through exposure to natural infection (508).

The TB potential of *Pfs*48/45 was previously demonstrated in mouse immunization and SMFA studies that showed its ability to elicit the production of TB antibodies (509). More recently, it was demonstrated by SMFA that antibodies against *Pfs*48/45 present in the serum of individuals from endemic regions could inhibit parasite development in mosquitoes (510). The correctly folded expression of the domain 3 of *Pfs*48/45 was eventually achieved in a vectorial delivery system with *Lactococcus lactis* (511,512). In preclinical animal studies, this fusion protein was able to induce TB antibodies (511,512). A chimera of the prodomain of *Pfs*230 and the C-terminal 6-cys domain (6C) of *Pfs*48/45 was later shown to elicit antibodies

that have a >80% reduction activity by SMFA with sera from immunized mice and superior TBA activity than each antigen induced alone (513).

Additional sexual and mosquito stage antigens that might constitute TBV candidates have been a subject of interest. Among them, AnAPN1 is expressed in the mosquito midgut wall, and is associated with ookinete attachment (514). This mosquito stage antigen eliminates the problem of addressing antigenic polymorphisms in parasite antigens, and has the advantage of being effective in all *Plasmodium* species parasitizing a specific mosquito species (317). Studies showed that serum from AnAPN1-immunized mice can lead to >90% reduction in oocyst intensity, whit a similar (80-90%) TBA observed by direct membrane feeding assay (DMFA) in Cameroon with total IgG purified from immunized mice (472). This vaccine candidate contributed to a renewed interest in the development of mosquito stage TBVs.

THESIS AIMS

The experimental work developed throughout this thesis aimed to explore new *Plasmodium* transmission-blocking strategies that directly target the parasite, through either transmission-blocking drugs or a multi-stage malaria vaccine.

Transmission-blocking drugs could represent an additional tool in malaria elimination by reducing the parasite population to be targeted by other malaria control strategies. However, currently available drugs with transmission-blocking activity are not suitable for widespread and systematic administration in a field setting, and the drug development pipeline is a time demanding and costly proceeding. Thus, drug repurposing represents a possible solution to this issue since compounds used to treat other diseases which are co-endemic with malaria could possibly have *Plasmodium* transmission-blocking activity. To assess the transmission-blocking potential of compounds commonly employed in malaria-endemic regions, we proposed to:

I. Evaluate the effect of ivermectin on the blood and sexual stages of *P. berghei*;
II. Screen the activity of several avermectins against *P. berghei* sporogonic stages *in vitro*;
III. Assess the activity of antiretroviral compounds and first-line antiretroviral therapies against *P. berghei* sporogonic stages *in vitro*;
IV. Determine the *in vivo* activity of first-line antiretroviral therapies and alternative drug combinations selected based on the *in vitro* screen results.

A highly effective vaccine against malaria has been a long-term goal of the world health organization that could have a major public health impact in malaria-endemic regions. However, the most advanced malaria vaccine, RTS,S, has limited efficacy, partly due to the high polymorphism of liver stage antigens found in field parasites. Therefore, a multi-stage malaria vaccine that would confer protective immunity against different phases of the parasite's life appears as an attractive approach to malaria vaccination. With this in mind, we aimed at developing and characterizing a new multi-stage malaria vaccine. To this end, we proposed to:

I. Design and generate *P. berghei* transgenic parasite lines expressing the liver stage antigen *Pf*CS, the blood stage antigen *Pf*Rh5 and the transmission-blocking antigen *Pfs*48/45;
II. Assess the fitness of the newly generated transgenic parasite lines;
III. Assess the expression of *Pf*CS and *Pfs*48/45 by the transgenic parasite lines at the mRNA and protein levels, both *in vitro* and *in vivo*;
IV. Assess the ability of the transgenic parasites to elicit immune responses against *Pf*CSP, and *Pf*s48/45 upon immunization of rodent models of infection.

2 PROGRESS ON TRANSMISSION-BLOCKING CHEMOTHERAPY

2.1 A BIOLUMINESCENCE METHOD FOR *IN VITRO* SCREENING OF *PLASMODIUM* TRANSMISSION-BLOCKING COMPOUNDS

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NOTE: This section is included in the present thesis for the purpose of providing adequate context to the experimental work described in sections 2.2 and 2.3. The results in this section were obtained by the candidate during her master's thesis research project, prior to the initiation of her PhD project.

AUTHOR CONTRIBUTIONS:

Azevedo R., Markovic M., Mendes A.M., and Prudêncio M. designed the experiments. Azevedo R., Markovic M., Machado M., Franke-Fayard B. and Mendes A.M. performed the experiments. Azevedo R. and Prudêncio M. wrote the paper. Mendes A.M. and Prudêncio M. supervised the work and revised the text.

INTRODUCTION

Malaria remains a formidable public health threat, accounting for the deaths of more than half a million people annually (43). It is caused by apicomplexan parasites of the genus *Plasmodium*, five species of which, *Plasmodium falciparum* (*Pf*), *P. ovale* (*Po*), *P. vivax* (*Pv*), *P. malariae* (*Pm*), and *P. knowlesi* (*Pk*), can infect humans.

The complex life cycle of the *Plasmodium* parasite starts when an infected female *Anopheles* mosquito vector bites and injects sporozoites under the skin of a mammalian host (515). Sporozoites enter the bloodstream, reach the liver, and eventually invade a hepatocyte, where they undergo an extensive replication process that culminates in the formation of thousands of merozoites (107). Following the asymptomatic liver stage of infection, released merozoites infect erythrocytes, thus initiating the symptomatic blood stage of infection. A small proportion of parasite blood stages differentiates into female and male gametocytes, which can be taken up by the mosquito vector upon a subsequent blood meal (516). The sporogonic phase of the *Plasmodium* life cycle occurs inside the mosquito vector. Ingested gametocytes mature and form female and male gametes, a process induced by environmental triggers such as a drop in the temperature of the infected blood, a rise in pH, and the presence of gametocyte activation factors such as xanthurenic acid (127). Gamete fertilization results in the formation of a zygote, which then transforms into the motile, banana-shaped ookinete. After 24 h, these parasite forms leave the mosquito midgut, migrate toward the midgut wall, traverse the epithelial cells, and eventually arrest between the midgut epithelium and the basal lamina. Here, ookinetes round up and develop into oocysts, where sporogony, a process of asexual replication driven by mitotic divisions, takes place, leading to the development of numerous sporozoites. After 15 to 21 days in the mosquito vector, the oocyst wall bursts, releasing the sporozoites, which are transported through the hemocoel toward the salivary glands, where they remain until they are transmitted to another mammalian host (517,518).

The complex life cycle of *Plasmodium* parasites significantly contributes to the enormous challenge posed by malaria eradication. The compounds most commonly used for treatment and prophylaxis nowadays target the asexual blood stages of infection (519).

However, to support eradication of the parasite, combination therapies should address three major issues: transmission of the pathogen, radical cure of *Pv* malaria, and the emergence of compound resistance (463). Thus, transmission-blocking strategies (TBS) emerge as a crucial component of a multifaceted antimalarial strategy (425). Such strategies aim at reducing the prevalence of infection in communities where malaria is endemic by targeting *Plasmodium*'s mosquito stages, impairing the transmission of the disease from an infected host undergoing treatment to the vector, and clearing the parasite from the vector (520). In this context, an increased understanding of the invertebrate vector stages of the malaria parasite, as well as effective methods to identify transmission-blocking (TB) compounds, is urgently required.

Efforts to cultivate *Plasmodium* parasites *in vitro* have been hindered by the complexity of the parasites' hosts, the human host and the arthropod vector, each having its own physiological, metabolic, and nutritional requirements, which are difficult to reproduce *in vitro* (439). Nevertheless, *in vitro* culturing of *Plasmodium* mosquito stages has been reported for different species of these parasites, including *P. gallinaceum* (*Pg*) (442), *Pf* (441), *P. berghei* (*Pb*) (443), and *P. yoelii* (*Py*) (444). Early studies of *in vitro* production of *Pb* ookinetes employed minimum essential medium for cultivation of gametocytes produced *in vivo*. In these studies, 1% or less of the initial macrogametocytes were found to originate ookinetes (457). The *in vitro* production of *Pb* gametocytes and their infectivity toward mosquitoes were first demonstrated by Janse *et al.*, who achieved the transformation of 44% of macrogametocytes into ookinetes (456). Later, transformation of *Pb* zygotes into ookinetes (521) and ookinete transformation into early oocysts (522) were also achieved in the absence of cells. However, studies of *Pg* showed that zygote-to-ookinete transformation rates of up to 75% and substantially increased ookinete longevity were observed in the presence of insect cells (523). *Pb* oocyst-to-sporozoite transformation *in vitro* was first described in 2002 (443). In that study, mature oocysts were obtained in 15-day cultures employing Matrigel and cocultures with *Drosophila* cells to mimic the mosquito's basal lamina, which is expected to provide the necessary cues for oocyst transformation (524,525). Under these conditions, *Drosophila* cells produce laminin, which has been suggested to coat ookinetes as they pass through the mosquito's midgut epithelium (401), and annexin, which was shown to

bind to ookinetes during the invasion of the mosquito midgut (458). Nevertheless, Carter *et al.* subsequently showed that no basal lamina components are required to trigger *Pb* ookinete-to-oocyst transformation *in vitro* and defined a minimal medium that supports transformation and oocyst growth in the absence of Matrigel or cocultured cells (460). However, maintenance of the parasites in minimal medium did not permit complete sporogonic development, and the duration of oocyst viability was reduced to 7 days (460).

The complexity of currently available methods of *in vitro* cultivation of *Plasmodium* sporogonic stages renders their reproducibility very challenging. Nevertheless, a suitable *in vitro* culturing system for such stages could represent an important tool for the development of TB interventions, as well as sporozoite-based vaccines, the sole immunization strategies shown to convey sterile immunity to malaria (526). Here, we have optimized an *in vitro* system for culturing *Pb* mosquito stages and screening TB compounds, employing a bioluminescence-based output of increased simplicity. Our results show that this system can be employed for effective and fast screening of TB compounds and for identification of the specific stages of parasite development upon which these compounds act. Finally, this method may contribute to a better understanding of the sporogonic stage of the parasite's life cycle.

MATERIALS AND METHODS

Experimental animals and *Pb* ANKA reference line. BALB/cByJ (6 to 8 weeks old) from Charles River were used. The cloned reference line cl15cy1 of the ANKA isolate of *Pb* (48) as used to obtain the transgenic *Pb* line that expresses a fusion of the genes encoding green fluorescent protein and luciferase (*gfp-luc*), placed under control of the circumsporozoite (*csp*) promoter (PBANKA_0403200) integrated into the silent *230p* gene locus (PBANKA_0306000).

Generation and characterization of *Pb* csp-GFP-luc expressing line. The *csp* promoter was polymerase chain reaction (PCR) amplified using primers 2590 (5' CCGGATATCACATAAAAGGGAATATGGAATATACTAGC) and 2591 (5' CGCGGATCCAAATATATGCGTGTATATATAGATTTTG) and cloned as a BamHI/EcoRV fragment into plasmid pL1141 (exchanging the *apical membrane antigen 1* (*ama1*) promoter for the *csp* promoter) to create pCSGFP203p. Then the NcoI/ScaI fragment of pL1156 was introduced to create pL1161. The final DNA construct was linearized with SacII before transfection. Transfection, selection and cloning of transformed *Pb* parasites were performed using standard genetic modification techniques (527) with *Pb* ANKA cl15cy1 as the parent parasite line. Cloned parasite lines (**Fig. 2.1.1,** *Pb*CSPGFP-Luc) were obtained by the limiting dilution method. Correct integration of DNA constructs and disruption of genes were verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes (527). PFG-separated chromosomes were hybridized with the 3'UTR of *Pb dihydrofolate reductase-thymidylate synthase* (*dhfr/ts*) gene recognizing the endogenous *dhfr/ts* locus on chromosome 7 and the *GFP-Luc-csp* expression cassette on chromosome 3.

Ookinete production. *Pb* ANKA expressing GFPand luciferase under the control of the CSP protein promoter (line 784cl1, RMgm-152, *Pb*CSPGFP-Luc) was maintained in *Anopheles stephensi* mosquitoes and BALBc/byJ mice. To maintain gametocyte infectivity, only up to six passages of parasites from infected to naïve mice were performed. Briefly, BALBc/byJ mice were treated with 0.1 ml phenylhydrazine (Sigma) (25mg/ml) 3 days prior to infection with 10^7 *Pb*-infected red blood cells (iRBC) obtained from a donor mouse, which was infected either

from a previously infected mouse or from a frozen vial of iRBC. Parasitaemia and gametocytaemia were followed by the analysis of Giemsa-stained tail blood smears and the presence of exflagellating gametocytes in ookinete medium (1:4 dilution) was monitored. Three days after infection, blood collected by heart puncture was pooled from 2 mice and washed with Roswell Park memorial institute (RPMI; Invitrogen) medium at 37°C, followed by centrifugation at 1100g for 10 minutes at 37°C. After washing, 5, 10 or 15 µL of blood containing exflagellating gametocytes were mixed with medium supplemented for ookinete formation (RPMI1640; Invitrogen), 25 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES; ThermoFisher Scientific), 0.4 mM hypoxanthine (Merck), 100 mM xanthurenic acid (Fluka; 85570), 10% (vol/vol) foetal bovine serum (FBS; Invitrogen), pH 7.6) in a final volume of 200 µl and cultured in 96-well plates for 24 h at 19 °C. Additionally, blood containing exflagellating gametocytes was mixed with the ookinete medium in 1:20 ratio and cultured in T75 flasks for 22 to 24h at 19 °C. Following incubation, ookinete enrichment was performed as previously described (463), with some modifications. Briefly, cultured blood was collected, and erythrocytes were lysed for 15 minutes on ice with 30 volumes of ice-cold 0.17M ammonium chloride (VWR). Lysed erythrocytes were removed by washing with RPMI, and ookinetes were purified by centrifugation on a 63% Nycodenz (Axis-Shield) cushion at 650×g at 4°C for 30 min. Following centrifugation, the ookinete-containing interface was collected, washed in ice-cold RPMI and resuspended in 0.5-1 ml of oocyst medium (see paragraph on oocyst cultures below). Ookinete conversion rates were determined by counting all *Pbs*21-positive cells 22 to 24 h after *in vitro* incubation of infected blood in ookinete culture medium after staining with 3.3 µg/ml rabbit polyclonal antibody 13.1 for 2 h (kindly provided by Leiden University Medical Center). After washing, samples were further incubated with 6.66 µg/ml Alexa Fluor 488 (Invitrogen)-conjugated goat anti-rabbit IgG for 1 h. The ookinete conversion rate is the percentage of ookinetes (mature and retort forms) of the total number of *Pbs*21-positive cells (ookinetes and unfertilized gametes).

Oocyst cultures. Purified ookinetes were seeded with *Drosophila melanogaster* S2 cells (*Drosophila* Genomics Resource Center, Bloomington, IN) in a 1:10 ratio (10^4 ookinetes and 10^5 S2 cells) in Schneider's medium (Sigma) supplemented with 15% FBS (Invitrogen), penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively; ThermoFisher Scientific) and gentamicin (50 µg/ml; ThermoFisher Scientific) to promote oocyst development. Oocysts were cocultured with *D. melanogaster* S2 cells in flat bottom 96-well plates (Corning) for 21 days at 19°C. One-quarter of the medium was changed 3 times per week (every 48 to 72 h), and 10^5 S2 cells were added once or two times per week. In parallel, S2 cells were maintained at 27°C in Schneider's medium (Sigma) supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively; ThermoFisher Scientific).

Bioluminescence assay. A bioluminescence assay was used to assess the development of the mosquito stages of the parasite. To monitor the development of gametocytes into ookinetes, firefly luciferase-expressing parasites (*Pb*CSPGFP-Luc) were collected from the ookinete culture at five time points over a period of 24 h. Parasites were then collected from oocyst cultures every day for 21 days of culture to assess the dynamics of firefly luciferase expression during oocyst development. Based on the results obtained, in subsequent experiments performed to evaluate the effect of compounds on the development of different *Plasmodium* mosquito stages, parasites were collected at three different time points (see paragraph on evaluation of anti-plasmodial compounds). The bioluminescence assay was performed using the firefly luciferase assay kit (Biotium) in accordance with the manufacturer's instructions, with some modifications. Briefly, the whole well contents were collected, washed with PBS, frozen in 75 µl of lysis buffer and stored at -20°C until further use. Collected samples were lysed, and 30 µl of the resulting supernatant were transferred into white 96-well plates. Fifty microliters of D-luciferin in firefly luciferase assay buffer (1:50 ratio) were added to the samples and parasite load was determined by measuring luminescence intensity using a microplate reader (Infinite M200).

Live imaging of oocyst cultures. Live imaging of *Pb*CSPGFP-Luc oocyst cultures was performed in black 96-well plates (Corning) on either a Zeiss Axiovert 200M or a Zeiss Cell Observer WF fluorescence microscope, using a 40x objective. Oocyst development and sporulation were monitored on days 3, 5, 8, 10, 15, 18 and 21 of the culture. For quantification of oocyst numbers and areas, 50 images were acquired per well and analysed using the Fiji software (528).

Immunofluorescence microscopy. Blood was collected from mice infected with *Pb*CSPGFP-Luc 3 days after the infection as described above. Fifty microliters of the blood containing gametocytes was centrifuged immediately, while the remainder was centrifuged after incubation for 24 h in the medium to promote ookinete growth. After centrifugation at 4000 rpm for 5 min, samples were first fixed with 4% Paraformaldehyde (PFA; ThermoFisher Scientific) and 0.0075% glutaraldehyde (Sigma) in PBS (NZYTech) for 30 min, then permeabilized by incubation with 0.1% Triton X-100 (Roth)-PBS (NZYTech) for 10 min, and then blocked with 3 % bovine serum albumin (BSA; VWR)-PBS (NZYTech) for 1 h. To characterize the expression of the *Pbs*21 protein, gametocytes were first incubated with 3.3 µg/ml of mouse 13.1 anti-*Pbs*21 primary antibody for 2 h (kindly provided by Jorge Santos). After washing, samples were further incubated with 6.66 µg/ml Alexa Fluor 555 (Invitrogen)-conjugated goat anti-mouse IgG, 6.66 µg/ml Alexa Fluor 488 (Invitrogen)-conjugated rabbit anti-GFP tag antibody, and 5 µg/ml of Hoechst-33342 (Invitrogen) for 1 h. To characterize CSP expression, ookinetes of *Pb*CSPGFP-Luc and *Pb*GFP-Luc_{con} were incubated with 5 µg/ml of mouse anti-CSP 3D11 antibody (50) for 2 h, and then incubated with 6.66 µg/ml Alexa Fluor 555 (Invitrogen)-conjugated donkey anti-mouse IgG, 6.66 µg/ml Alexa Fluor 488 (Invitrogen)-conjugated rabbit anti-GFP tag antibody, and 5 µg/ml of Hoechst-33342 (Invitrogen) for 1 h. All antibodies were diluted in 3% BSA (VWR)-PBS (NZYTech) and all steps were performed at room temperature. Finally, samples were mounted with Fluoromount-G (SouthernBiotech) on polylysine coverslips (Corning) on microscope slides and images were acquired on Zeiss LSM 880 and Zeiss LSM 710 fluorescence microscopes. To characterize CSP expression and to image parasite nuclei, oocysts were cultured in 8-well chambers (IBIDI), air dried overnight, fixed for 10 min

with 4% PFA-PBS, and permeabilized and blocked for 1 h with 0.1% Triton-X100 (Roth)-1%BSA (VWR)-PBS (NZYTech). Samples were then incubated with 10 µg/ml mouse anti-CSP 3D11 antibody (529) for 1 h, and subsequently with 4 µg/ml Alexa Fluor 594 (Invitrogen)-conjugated goat anti-mouse IgG secondary antibody for 1 h. All incubations were performed at room temperature and all washing steps were performed with 0.05% Triton-X100 (Roth)-1%BSA (VWR)-PBS (NZYTech), which was also used to prepare antibody dilutions. Nuclei were stained with 5 µg/ml Hoechst-33342 (Invitrogen). Samples were mounted in Fluoromount-G (SouthernBiotech) and images were acquired on either a Zeiss LSM 880 or a Zeiss LSM 710 fluorescence microscope and processed using the Fiji software (528).

To characterize CSP expression *in vivo*, BALB/cJ (Charles River) mice were infected by intraperitoneal injection of 1×10^7 *Pb*CSPGFP-Luc and *Pb*GFP-Luc_{con}-iRBCs. Parasitemia was monitored by analysis of Giemsa-stained tail blood smears, and when it reached around 3% and after the confirmation of male gametocyte exflagellation, these mice were used to infect *An. stephensi* mosquitoes raised in the Instituto de Medicina Molecular João Lobo Antunes (IMM-JLA) insectary facility. Following their blood meal, mosquitoes were starved and kept at 20°C and 80% humidity under a 12-h light-dark cycle for 24 h. After 24 h, approximately 20 midguts per parasite strain were collected, fixed with 4% PFA for 1 min, and then washed in PBS. Midguts were opened longitudinally, and the contents were removed in PBS, fixed for 30 min at room temperature, and permeabilized and blocked for 1 h with 0.1% Triton X-100 (Roth)–1% BSA (VWR)–PBS (NZYTech). Samples were then incubated with 5 µg/ml mouse anti-CSP 3D11 antibody and 6.6 µg/ml 13.1 rabbit anti-*Pbs*21 polyclonal antibody overnight at 4°C and subsequently with 6.66 µg/ml Alexa Fluor 594 (Invitrogen)-conjugated goat anti-mouse IgG secondary antibody and 6.66 µg/ml Alexa Fluor 488 (Invitrogen)-conjugated goat anti-rabbit IgG secondary antibody. Midguts were mounted on a microscope slide with Fluoromount-G (SouthernBiotech, 0100-01), and images were acquired with a Zeiss LSM 880 fluorescence microscope and analyzed with the Fiji software.

Western blot analyses. To evaluate *Pb*CSP expression throughout the transformation of parasites from gametocytes to ookinetes *in vitro*, cultured blood of parasite cultures

of *Pb*CSPGFP-Luc parasites produced as described above in 96-well plates was collected at 0, 6, 12, 18, and 24 h post-incubation. After collection, samples were lysed with firefly luciferase lysis buffer from the firefly luciferase assay kit (Biotium) in accordance with the manufacturer's instructions. Samples were prepared by adding 4× Laemmli sample buffer (Bio-Rad; diluted in β-mercaptoethanol) to the lysed parasite solution (1:3) and boiled at 95°C for 5 min. Lysates were then separated by gel electrophoresis on a precast Mini-PROTEAN TGX gel (Any Kd; Bio-Rad), and lysates of 1,000 *Pb*CSPGFP-Luc sporozoites dissected in lysis buffer (25 mM Tris-HCl (Sigma, VWR; pH 7.4), 1% Triton X-100 (Roth), 10% glycerol (VWR)) with protease inhibitor (1:50) were used as a positive control. The approximate molecular weights of the proteins were deduced by comparison with a prestained protein ladder (Bio-Rad). The gel was transferred to a nitrocellulose membrane with iBlot (Invitrogen). Membranes were blocked for 30 min in PBS (NZYTech)–0.1% (vol/vol) Tween–5% (wt/vol) milk, and then incubated with mouse anti-CSP 3D11 antibody (1:1000) overnight at 4 °C. Membranes were washed five times for 5 min in PBS (NZYTech)–0.1% (vol/vol) Tween (Sigma) and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000) and washed as described above. Membranes were developed with the Immobilon Western blot kit (Millipore) and developed with the ChemiDoc XRS+ chemiluminescence detection system (Bio-Rad). The blots were analyzed with Image Lab software (Bio-Rad).

To confirm CSP expression in ookinete samples produced *in vitro*, purified ookinete samples of *Pb*CSGFP-Luc and *Pb*GFP-Luc$_{con}$ were collected at 24 and 48 h post incubation and lysed has described above. Samples were treated with Benzonase and $MgCl_2$ (1:100 dilution) to degrade nucleic acids. In parallel, midguts of *An. stephensi* mosquitoes infected with both parasite strains as previously described were collected at 24 and 48 hpi and lysed in lysis buffer (25 mM Tris-HCl (Sigma, VWR; pH 7.4), 1% (vol/vol) Triton X-100 (Roth), 10% (vol/vol) glycerol (VWR)) with protease inhibitor (1:50). Samples were prepared by adding 4× Laemmli sample buffer (Bio-Rad; diluted in β-mercaptoethanol) to the lysed parasite solution (1:3); boiled at 95 °C for 5 min; and separated, transferred, and developed as described above.

Evaluation of anti-Plasmodial compound activity *in vitro*. The effects of azithromycin (Az), atovaquone (At), chloroquine (Ch), cycloheximide (Cy), dihydrortemisinin (DA), halofantrine (Ha), lumefantrine (Lu), pyrimethamine (Py), pyronaridine (Po) and thiostrepton (Th) on the *Plasmodium* mosquito stages were evaluated. The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma) and the amount of DMSO equivalent to that present in the highest compound concentration was used as control. To assess the effects of the compounds on the ookinetes, a 10 µM concentration of each compound was added to the blood containing mature gametocytes after 1 h of incubation to evaluate their effect on ookinete formation and development when the zygote is already formed. To determine the intensity of the bioluminescence signal, parasites were collected from the ookinete cultures after 24 h of incubation. To assess the effect of the compounds on oocyst development, compounds were mixed with the mature ookinetes or added to the oocyst culture after 3 days of culture. Az, Ch, DA, Ha, Lu, Py, Po, Th, At and Cy were assayed at 10 µM. Compounds were replenished when the medium was changed to maintain the appropriate final concentration. When the compounds were mixed with the ookinetes, parasites were collected after 3 days of culture to assess the effects of the compounds on the early stages of oocyst development. When the compounds were added after 3 days of oocyst culture, parasites were collected after 15 days of oocyst culture to evaluate the compounds' effect on oocyst development when added to already formed young oocysts. IC_{50}s for oocyst growth were calculated for Cy (assayed at 0.005, 0.025, 0.05, 0.5, 1, 5, and 10 µM), Py, and Th (assayed at 0.05, 0.5, 1, 5, 10, 25, and 50 µM). IC_{50}s were estimated by nonlinear regression analysis with the sigmoidal dose-response equation in GraphPad Prism (version 5.00; GraphPad Software, La Jolla, USA).

Evaluation of anti-Plasmodial compounds *in vivo*. To assess the *in vivo* activities of compounds previously identified *in vitro*, BALB/cJ mice (Charles River) were infected by intraperitoneal injection of 10^7 *Pb*GFP-Luc_{con}-iRBCs. Parasitemia was monitored by the analysis of Giemsa-stained tail blood smears, and when it reached around 3% and after the confirmation of male gametocyte exflagellation, these mice were used to infect *An. stephensi* mosquitoes raised in the IMM-JLA insectary facility. Following their blood meal,

mosquitoes were starved and kept at 20 °C and 80% humidity under a 12-h light-dark cycle for 2 days. A solution of 100 mg/ml glucose and 2 mg/ml *p*-aminobenzoic acid mixed with selected compounds, At, Cy, DA, and Th, at a 50 µM concentration was provided to mosquitoes for 8 days in 384-well plates and replaced every 2 days. On day 10 after mosquito infection, approximately 30 midguts per experimental condition were collected and fixed with 4% PFA for 20 min. To determine oocyst numbers and development, midguts were mounted on a microscope slide with Fluoromount-G (SouthernBiotech) and images were acquired with a Leica DM5000B and analyzed with Fiji software (528)). To assess *in vivo* hepatic infection, the remaining mosquitoes were kept under standard diet conditions and on day 21 of infection, approximately 20 mosquitoes were allowed to feed on naive BALB/cJ mice (Charles River) for 30 min. On the following day, salivary glands were dissected and salivary gland sporozoite numbers were determined.

Quantification of *in vivo* hepatic infection. Liver parasite burdens of infected mice was quantified by quantitative real-time PCR (qRT-PCR) as previously described (530). Briefly, livers were collected at 46 h postinfection and immediately homogenized in a denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.7% β-mercaptoethanol in diethyl pyrocarbonate-treated water). Total RNA was extracted with the TripleXtractor directRNA Kit (GRiSP), in accordance with the manufacturer's protocol. One microgram of total RNA was converted to cDNA (NZY First-Strand cDNA Synthesis Kit, no oligos, (NZYTech)) and parasite load was quantified by qRT-PCR with primers specific for *Pb* 18S rRNA (5'-AAGCATTAAATAAAGCGAATACATCCTTAC-3' and 5'-GGAGATTGGTTTTGACGTTTATGTG-3'). Primers for the well-established housekeeping gene for hypoxanthine-guanine phosphoribosyltransferase (5'-TTTGCTGACCTGCTGGATTAC-3' and 5'-CAAGACATTCTTTCCAGTTAAAGTTG -3') were used for normalization of infection loads in all experiments. The qRT-PCRs reactions were performed in a total volume of 20 µL in a ABI Prism 7500 Fast system (Applied Biosystems) with the iTaqTM Universal SYBR® Green kit (BioRad) as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 sec and 60 °C for 1 min, melting stage was done at 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 30 sec. The delta-

delta cycle threshold (ΔΔCT) relative quantification method was used for analysis of qRT-PCR results.

Statistical analysis. Statistically significant differences between control and treated conditions were analyzed with the Mann-Whitney nonparametric test by using a 95% confidence interval. Differences were considered not be significant at a *P* value of >0.05. Under this value, all differences were considered to be statistically significant. All statistical tests were performed by GraphPad Prism (version 5.00; GraphPad Software, La Jolla, USA).

Ethics statement. All work with laboratory animals was performed in accordance with national and European regulations. All protocols were approved by the animal experimentation ethics committee of the Instituto de Medicina Molecular. All animal experiments performed at the Leiden University Medical Center were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042, DEC 12043). The Dutch Experiments on Animal Act was established under European guidelines (EU directive no. 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes).

Accession number(s). Details of the DNA construct used in this study have been submitted to the database of genetically modified rodent malaria parasites (RMgmDB, http://www.pberghei.eu/; ID RMgm-152).

RESULTS

IN VITRO CULTURING OF *Pb* MOSQUITO STAGES

Pb mosquito stages were co-cultured with *Drosophila* S2 cells in FBS-supplemented Schneider's medium for 21 days, up to oocyst development and sporozoite production. A *Pb* transgenic line with a cassette expressing GFP and luciferase under the control of the *Pb*CSP promoter regions, was employed (*Pb*CSPGFP-Luc; **Fig. 2.1.1**), enabling the assessment of the *in vitro* development of the parasite's mosquito stages by bioluminescence.

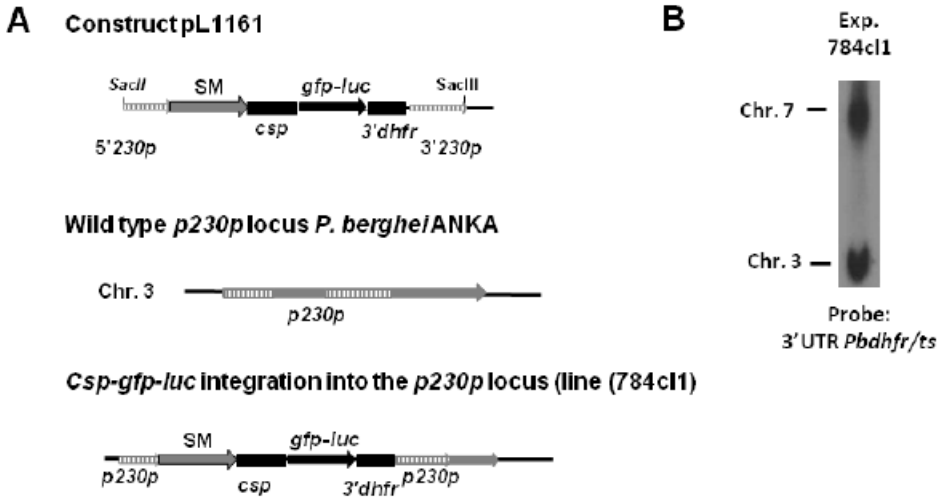

FIGURE 2.1.1 - Generation and genotyping of parasites expressing GFP-luciferase under control of the *csp* promoter (*Pb*CSPGFP-Luc; line 784cl1). (A) Schematic representation of the plasmid, the wild-type gene *230p* locus before and after incorporation of the construct pL1161 used to generate the transgenic line expressing GFP-luciferase under control of the *csp* promoter. The construct contains the *Toxoplasma gondii dhfr-ts* selectable marker cassette (SM: grey arrow) and the *csp-GFP-Luc* expression cassette (black arrow) and integrates by double cross-over homologous recombination into the *230p* locus. **(B)** Diagnostic Southern analysis of pulsed field gel-separated chromosomes confirms correct integration of the *csp-GFP-Luc* expression cassette in cloned line 784cl1. Separated chromosomes were hybridized with the 3'UTR of *Pb dhfr/ts* gene recognizing the endogenous *dhfr/ts* locus on chromosome 7 and the *csp-GFP-Luc* cassette on chromosome 3.

Blood was collected from *Pb*CSPGFP-Luc-infected mice and washed, and between 3×10^5 and 5×10^6 gametocytes were subsequently cultured as described in Materials and Methods. An ookinete formation rate of 25 to 33% of the total number of female gametocytes/gametes was estimated. Bioluminescence was measured at specific time points

thereafter and increased steadily over the initial 24 h of culture, corresponding to the parasite's progress from the gametocyte to the ookinete stage (**Fig. 2.1.2A**).

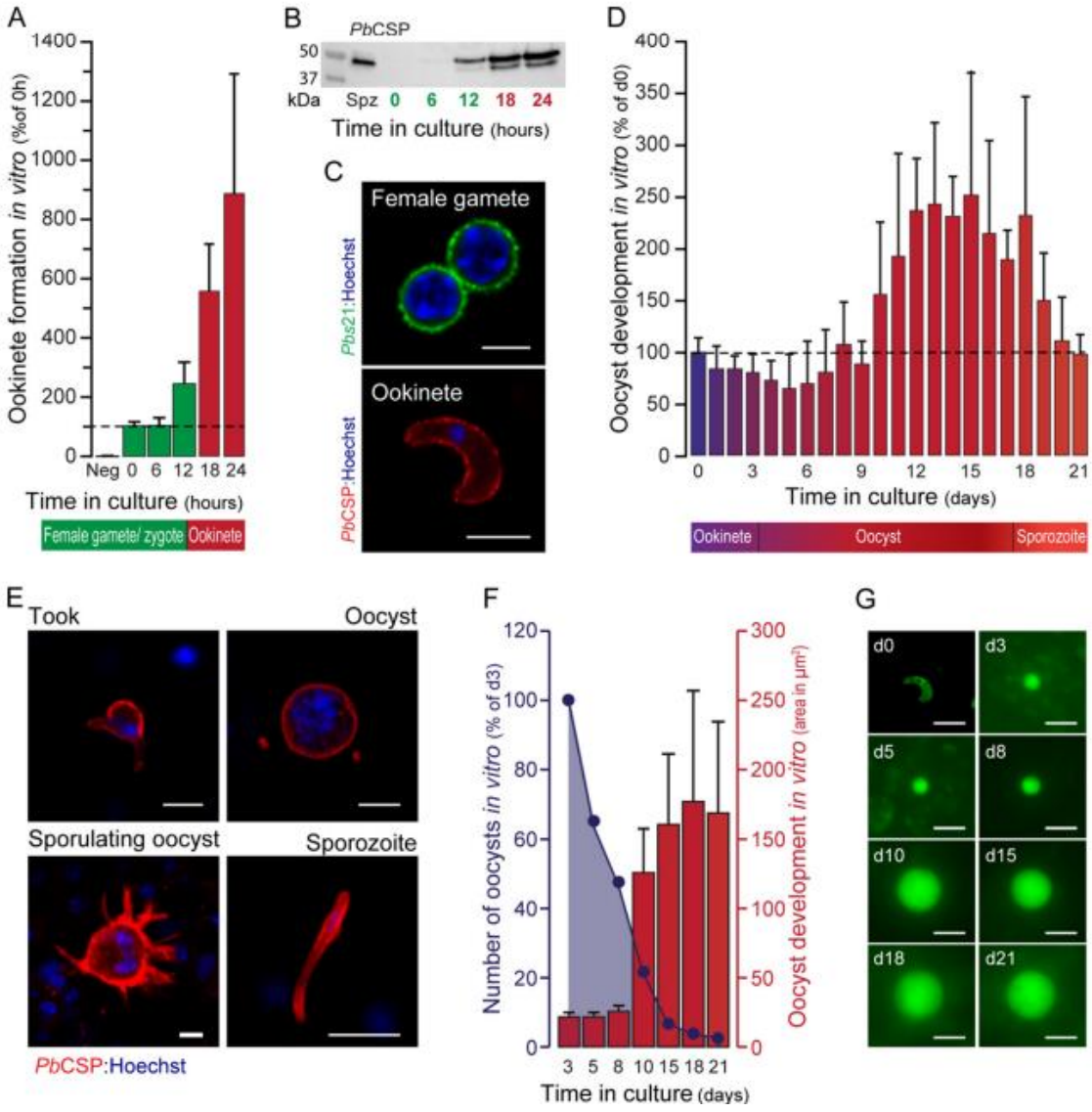

FIGURE 2.1.2 - A new system for culturing and detecting *Pb* mosquito stages. **(A)** Bioluminescence measured in relative luminescence units (RLU) at different time points throughout the formation and maturation of ookinetes, represented as a percentage of the RLU measured at the start of culture, 0 h. Neg corresponds to the uninfected blood sample used as a negative control. Results are expressed as the mean ± the standard deviation. **(B)** Western blot analysis of *Pb*CSP protein expression throughout the transformation of gametes into ookinetes *in vitro*. Salivary gland sporozoites were used as a positive control. **(C)** Immunofluorescence microscopy analysis of *Pb*CSPGFP-Luc gametes and ookinetes (green, *Pbs*21 protein; red, *Pb*CSP; blue, nuclei). Scale bars, 5 µm. **(D)** Bioluminescence measured in RLU throughout 21 days of oocyst culture, represented as a percentage of the RLU at time zero (d0) of culture. Results are expressed as the mean ± the standard deviation. **(E)** Representative

images of immunofluorescence staining of *Pb* tooks, oocysts, sporulating oocysts, and free sporozoites (red, *Pb*CSP; blue, nuclei). Scale bars, 5 µm. **(F)** Quantification of oocyst numbers and development by live fluorescence microscopy. Results are expressed as the mean ± the standard deviation (SD). d3, day 3. **(G)** Immunofluorescence staining of *Pb*CSPGFP-Luc ookinetes (time zero (d0)) and live imaging of *Pb*CSPGFP-Luc oocysts (day 3 (d3) to day 21 (d21)) cultured *in vitro*. Scale bars, 10 µm.

The intensity of the bioluminescence signal is in accordance with an unexpected pattern of expression of *Pb*CSP (**Fig. 2.1.2B**) on ookinetes and positively correlates with ookinete numbers (**Fig. 2.1.3**).

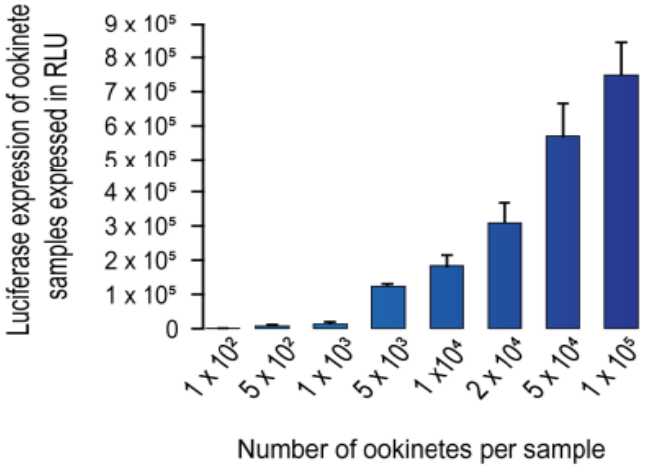

FIGURE 2.1.3 - Luciferase expression of ookinete samples. Bioluminescence of serial dilutions of samples of purified ookinetes produced *in vitro*. Data is expressed as a mean RLU +/- SD (triplicate wells).

Immunofluorescence microscopy analyses showed that expression of *Pb*CSP by ookinetes is a feature of the *in vitro* culturing process and is not an artifact of *Pb*CSPGFP-Luc parasites (**Fig. 2.1.2C**), as it is equally observed in cultures of *Pb*GFP-Luc$_{Con}$, a parasite whose constitutive luciferase expression is not driven by the *Pb*CSP promoter (**Fig. 2.1.4A**). Western blot analysis further confirmed that *Pb*CSP expression could be detected after 24 or 48 h of *in vitro* culturing of either *Pb*CSPGFP-Luc or *Pb*GFP-Luc$_{Con}$ (**Fig. 2.1.4B**), whereas the protein was not detected in mosquito midgut samples collected 24 and 48 h after mosquito infection with those parasites and analyzed by Western blotting or immunofluorescence microscopy (**Fig. 2.1.4B and C**).

Purified ookinetes (10 to 40% purity) were then cultured for an additional 21 days, with daily bioluminescence measurements (**Fig. 2.1.2D**). A detailed immunofluorescence

microscopy analysis of fixed samples further showed the development of transforming ookinete (took) forms, followed by oocysts containing multiple nuclei, with the presence of *Pb*CSP on the parasite's surface, as well as oocyst sporulation and free sporozoites from day 18 onward (**Fig. 2.1.2E**). Our data also showed that oocyst size increased, and sporulation started on day 10, reaching a maximum between days 13 and 18, while oocyst numbers decreased throughout the culturing process (**Fig. 2.1.2F**), from an initial ~5000 oocysts on day 3 to ~1500 on day 10 and ending with ~60 by day 21. Live fluorescence microscopy analyses at specific time points showed that ookinetes develop into growing oocysts and that the peak of signal intensity correlates with oocyst enlargement and sporulation **(Fig. 2.1.2G**).

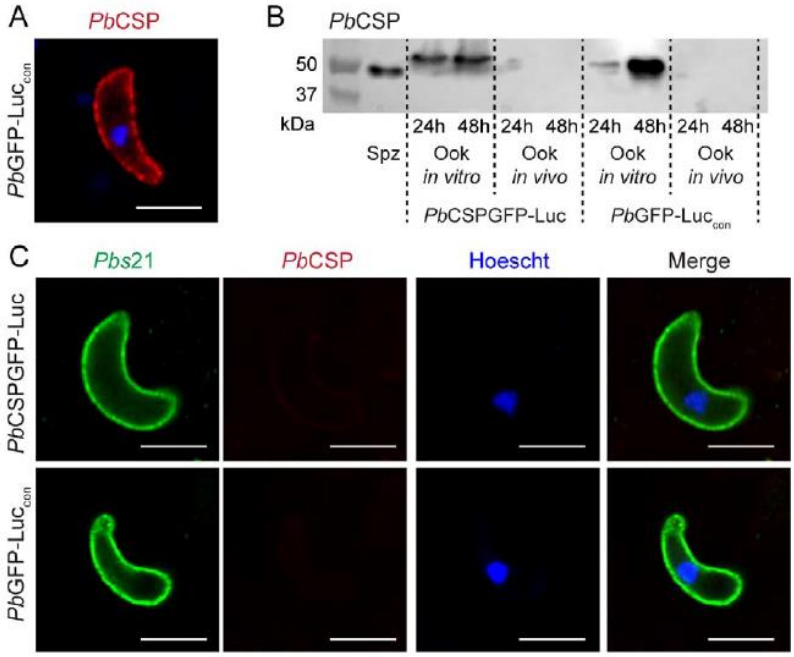

FIGURE 2.1.4 - CSP expression in ookinetes *in vitro* and *in vivo*. (A) Immunofluorescence staining of *Pb*GFP-LucCon ookinetes (red: *Pb*CSP, blue: nuclei). Scale bar: 5 µM. **(B)** Western blot analysis of *Pb*CSP in samples from *Pb*CSPGFP-Luc and *Pb*GFP-Luc$_{con}$ parasites, collected either *in vitro* or *in vivo* 24 and 48 hours after the start of the culture or of mosquito infection, respectively. **(C)** Immunofluorescence microscopy analysis of *Pb*CSPGFP-Luc and *Pb*GFP-Luc$_{con}$ parasites collected *in vivo* 24 hours after mosquito infection.

Collectively, our data reveal a culture system that effectively reproduces the mosquito stages of *Pb* parasites *in vitro*, from gametocytes to sporulating oocysts. Crucially, we also show that by employing a luciferase-expressing *Pb* line, the parasite's sporogonic development can be monitored by bioluminescence analysis.

EFFECTS OF ANTIPLASMODIAL COMPOUNDS ON *PLASMODIUM* MOSQUITO STAGES *IN VITRO*

The newly developed method described above can be employed to assess the activities of potential TB compounds *in vitro*. To prove the principle of this method, 10 well-established antimalarial compounds (Az, Ch, DA, Py, Lu, Ha, Po, At, Th, and Cy) belonging to different classes and with various expected effects on the development of *Plasmodium* mosquito stages were selected. The compounds' abilities to inhibit different phases of parasite development were assessed by bioluminescence analysis following compound addition at various stages of the culturing process (**Fig. 2.1.5A**).

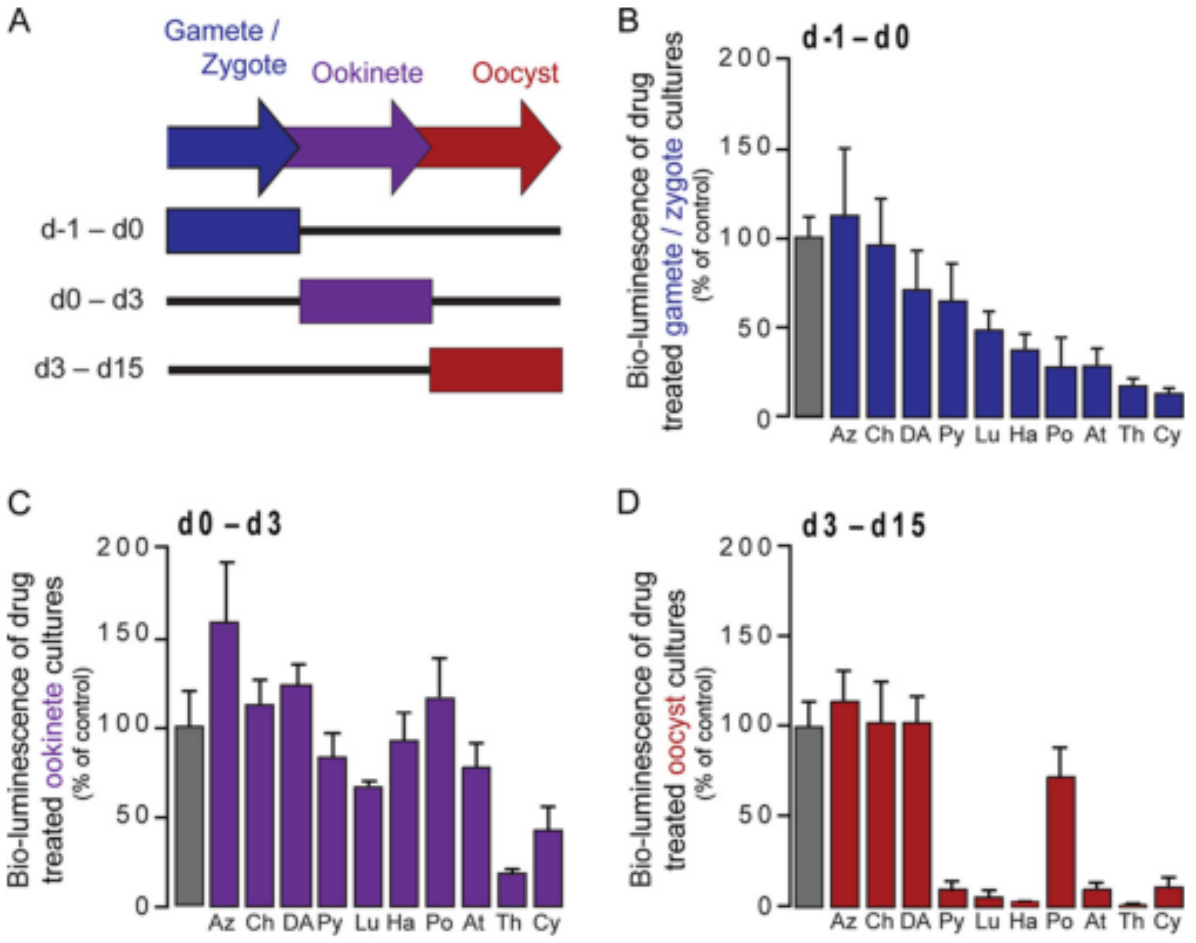

FIGURE 2.1.5 - Effects of selected compounds against *Plasmodium* mosquito stages *in vitro*. (A) Schematics of the progress of the parasite culturing process highlighting the different schedules of compound treatment employed. d, day. **(B)** Assessment of compound effects on ookinete formation and development, expressed as percentages of inhibition of *Pb* ookinete formation. **(C)** *In vitro* activities of selected compounds against early stages of oocyst development. **(D)** *In vitro* activities of selected compounds on young oocysts and subsequent

developmental stages. Ten compounds, Az, Ch, DA, Py, Lu, Ha, Po, At, Th, and Cy, were screened at a concentration of 10 µM. Bars correspond to RLU measurements represented as percentages of the RLU of the DMSO control. Results are expressed as the mean ± the standard error of the mean (SEM).

IDENTIFICATION OF COMPOUND EFFECTS ON OOKINETE FORMATION AND DEVELOPMENT

To assess the effects of the selected compounds on ookinete formation and subsequent development, compounds were added to gametes and zygotes (after 1 h of culture incubation). Bioluminescence was measured after 24 h of the culturing process and compared with that of control cultures to which only compound vehicle was added (**Fig. 2.1.5B**).

Our results show that At, Po, Th, and Cy have a strong effect on parasite development, whereas Ha and Py have a small effect on the zygote-to-ookinete transition stage and Az, Ch, DA, and Lu have no discernible effect on parasite development.

IDENTIFICATION OF COMPOUND EFFECTS ON OOCYSTS

To assess the compounds' activities on oocyst formation and early stages of development, the compounds were added to purified ookinetes and parasites were harvested after 3 days of culturing (**Fig. 2.1.5C**). Compound effects on late oocyst development were assessed by adding the compounds to already formed young oocysts (after 3 days of oocyst culturing) and harvesting the parasites after 15 days of culturing (**Fig. 2.1.5D**). The highest efficacy against early oocyst stages was obtained with Th and Cy, while the strongest effects against later stages of oocyst growth were observed with Th, Cy, Py, At, Ha, and Lu. The half-maximal inhibitory concentrations (IC_{50}s) calculated for Cy, Py, and Th upon addition of the compounds to young oocysts were 0.28 ± 0.32, 1.93 ± 1.72, and 1.16 ± 0.10 µM, respectively (**Fig. 2.1.6**).

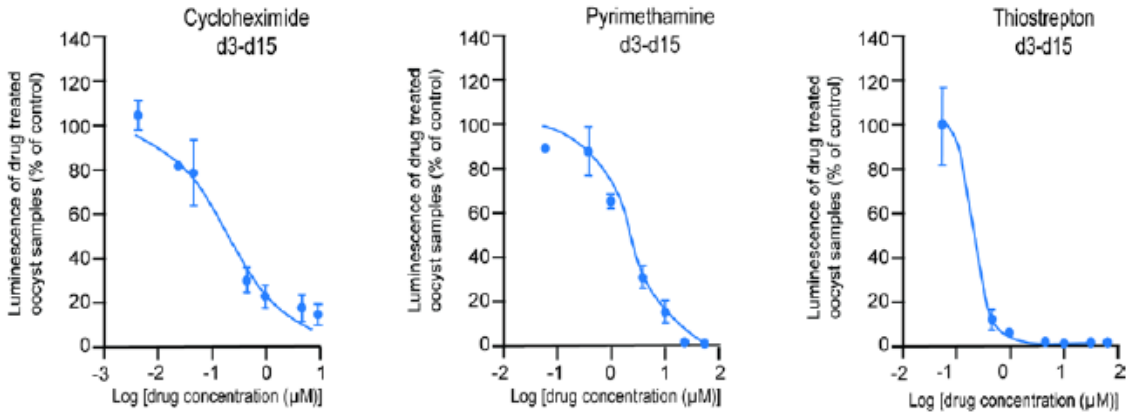

FIGURE 2.1.6 - IC50 determination for selected compounds active against oocyst maturation. IC50 values for Cy, Py and Th were 0.28 ± 0.32 µM, 1.93 ± 1.72 µM and 1.16 ± 0.10 µM, respectively.

Collectively, our results indicate that our bioluminescence-based method can be employed for the *in vitro* identification of compounds with activity against *Plasmodium* mosquito stages. The assay may also serve to distinguish between the different phases of parasite development where the compounds may be acting. Nonetheless, the different incubation times employed in the early- and late-stage assays do not allow exclusion of the possibility that apparent differences in the compounds' stage-specificity may also result from their faster or slower mode of action.

IN VIVO EFFECTS OF COMPOUNDS IDENTIFIED IN THE *IN VITRO* ASSAY

To test our newly developed screening method, the TB potential of At, Cy, and Th, which displayed potent activity against the parasite's mosquito stages *in vitro*, was assessed *in vivo*. DA, which displayed negligible activity in our *in vitro* screening, was employed as a negative control in these studies. *An. stephensi* mosquitoes infected 1 day earlier by feeding on *Pb*GFP-Luc$_{con}$-infected mice were starved for 2 days and subsequently allowed to feed on aqueous solutions containing the selected compounds at 50 µM (**Fig. 2.1.7A**). Ten days later, a fraction of the total number of mosquitoes in each group was dissected, their midguts were collected, and oocyst numbers (**Fig. 2.1.7B**) and development (**Fig. 2.1.7C**) were quantified by microscopy.

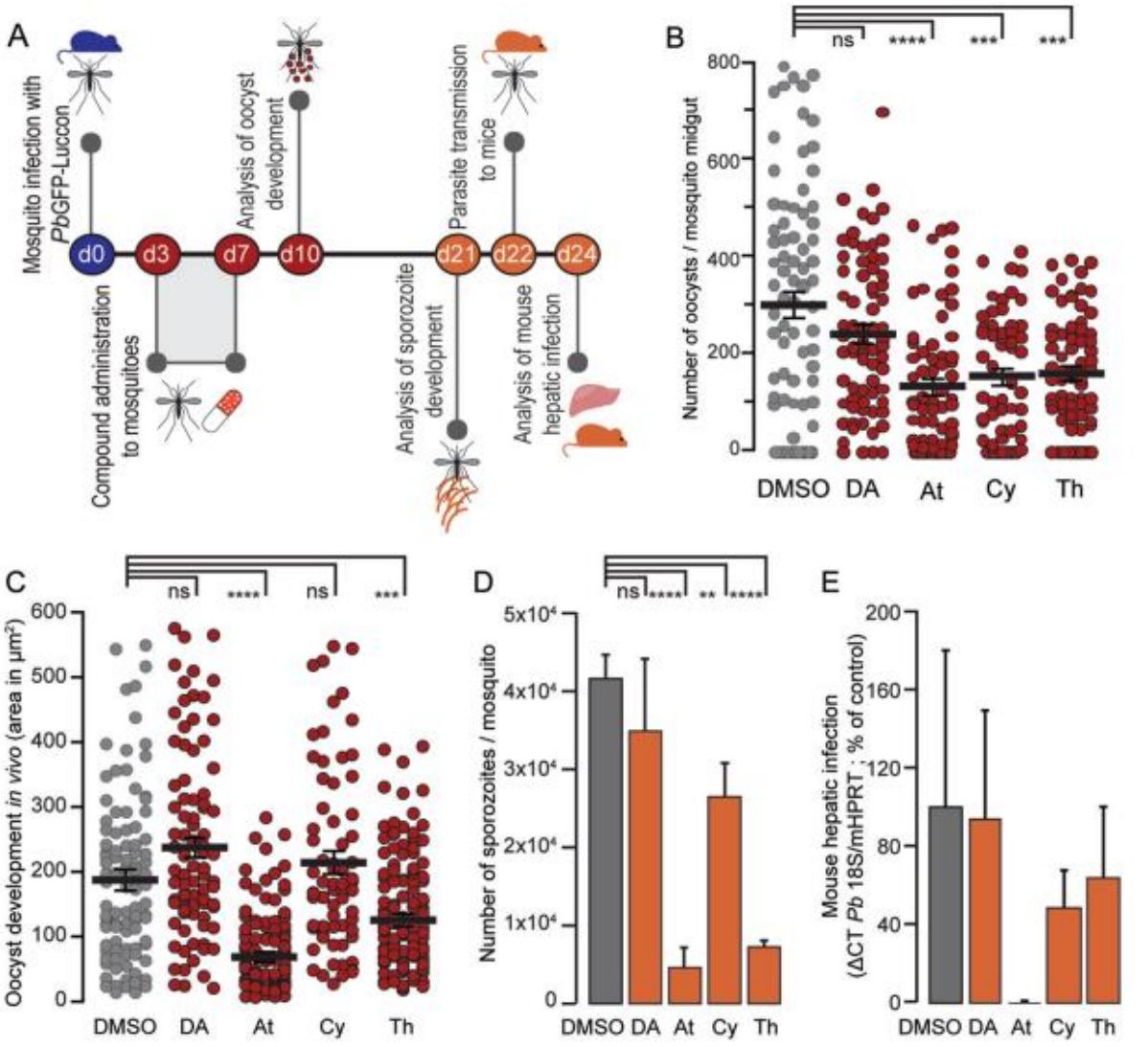

FIGURE 2.1.7 - Compound activity on *Pb* sporogonic development in infected mosquitoes and sporozoite infectivity of a mammalian host. (A) Schematics of assessment of *in vivo* compound activity on oocysts and sporozoites and liver parasite loads of mice infected by mosquitoes treated with selected compounds. d, day. **(B)** *In vivo* activities of selected compounds on the number of oocysts developing in mosquito midguts. Similar population sizes were analyzed, and results are expressed as the mean ± the SEM. **(C)** *In vivo* activities of selected compounds on oocyst development in mosquito midguts. Oocyst areas in square micrometers are presented for random samples of parasites observed in midguts of treated mosquitoes. Results are expressed as the mean ± the SEM. **(D)** *In vivo* activities of selected compounds on sporozoite formation. Results are expressed as the mean ± the SD. **(E)** Quantification of *in vivo* hepatic infection of mice infected by treated mosquitoes. Results are expressed as the mean ± the SEM. Compounds were screened at a concentration of 50 µM. **, $P < 0.0001$; ***, $P < 0.001$; ****, $P < 0.005$; ns, not significant.

Infection of the remaining mosquitoes in each group was allowed to proceed for an additional 11 days, after which either (i) mosquitoes were dissected and salivary gland sporozoites were counted (**Fig. 2.1.7D**) or (ii) mosquitoes were allowed to bite I mice and the parasite loads in the livers of these mice were determined 46 h later by quantitative real-time PCR (qRT-PCR) (**Fig. 2.1.7E**). Our results show that oocyst numbers in mosquitoes that fed on At-, Cy-, and Th-containing solutions were similar and significantly lower than those present in the midguts of vehicle- or DA-treated mosquitoes (**Fig. 2.1.7B**), whereas At and Th also significantly impacted oocyst development (**Fig. 2.1.7C**). Consequently, the number of sporozoites in the salivary glands of mosquitoes treated by any of these drugs is significantly decreased, and more markedly so in the case of treatment with At or Th (**Fig. 2.1.7D**). Importantly, our results also show that administration of the identified TB compounds to infected mosquitoes has a clear impact on an ensuing hepatic infection of mice bitten by these mosquitoes (**Fig. 2.1.7E**). Of note, these data further suggest that sporozoite viability is strongly impaired by At and is also impacted by Cy or Th.

Collectively, our results indicate that compound activity against sporogonic stages, as identified in our *in vitro* assay, is predictive of the activity of these compounds against parasite development *in vivo* in a previously infected mosquito vector, with a clear impact on transmission. Overall, these data show that our *in vitro* assay can identify compounds active against *Plasmodium* mosquito stages and inform their selection for further evaluation as potential TB compounds.

DISCUSSION

In the present study, we showed that the development of the parasite's mosquito stages can be monitored *in vitro* by bioluminescence measurements, enabling a new method to screen potential TB compounds. We employed a modified version of the *in vitro* culturing conditions described by Al-Olayan *et al.* (443) to obtain sporulating oocysts from gametocyte cultures of a transgenic *Pb* parasite line, *Pb*CSPGFP-Luc, which expresses GFP and luciferase at the mature ookinete, oocyst, and sporozoite stages of its life cycle. Xanthurenic acid was incorporated in the ookinete medium as a promoter of exflagellation, and we established an optimal pH for ookinete transformation of 7.6, in comparison with the pH of 8.4 established by Al-Olayan *et al*. In the culturing system described here, oocysts were successfully maintained and sporozoites were released by using a culture medium composed solely of Schneider's medium, which is a source of both glutamine and sodium bicarbonate, and FBS, which is composed of several growth factors that might be important for parasite maintenance *in vitro* (531). A pH of 6.6 to 6.8 was employed for oocyst development, whereas in the study of Al-Olayan *et a*l., the optimal oocyst yield was achieved at pH 6 (443). Finally, our system did not include a basal lamina substitute, and while S2 cells were found not to be essential for ookinete transformation, they proved necessary for oocyst maintenance *in vitro*, in agreement with what has been previously reported (532).

Luciferase-expressing *Pb* parasites have already been described as a valuable tool to assess hepatic infection *in vitro* and *in vivo* (78,533), as well as to measure blood parasitemia in rodent models of infection (534). Here, a firefly luciferase-expressing *Pb* parasite line was employed to monitor the parasite's sporogonic development *in vitro*, as well as a tool for the identification of compounds with TB activity. Our assay might potentially be improved in the future by the use of the recently reported *Pb* line expressing the novel luciferase enzyme NanoLuc (*Pb*NLuc), which has been shown to deliver a significantly enhanced luminescence signal, enabling single parasite detection in various stages of its life cycle, including in the mosquito vector (535). Another possible improvement of the assay might come from the use of a second reporter gene, placed under the control of a sporozoite-specific promoter, which would enable the specific monitoring of sporozoite formation and maturation.

Our *in vitro* study identified several sporontocidal compounds with potential TB activity. Unlike gametocytocidal compounds, sporontocidal drugs should ideally have a long half-life to interrupt transmission, as gametocytes can circulate for long periods of time before being ingested by a mosquito. However, sporontocidal agents may also present advantages over gametocytocidal compounds, as the former can interrupt transmission by mosquitoes taking a post infection blood meal. In fact, the *in vivo* activity of compounds identified in our *in vitro* assay was subsequently confirmed for Cy, Th, and At by adding these compounds to infected mosquito sugar feeds as previously described (536). Besides substantial activity against *Pf* blood stages (537), Cy has been shown to inhibit *Pf* exflagellation and *Pb* ookinete formation (463), appearing as one of the compounds with the most notable activity in a comparative study of potential TB compounds (463). Since Cy is a known inhibitor of translation (538), its inhibitory activity on exflagellation is consistent with the notion that protein synthesis is an important component of the morphological changes that occur upon activation of the exflagellation process (539). In our assay, Th displayed the broadest spectrum of activity and presented the strongest sporontocidal effect. Despite a very promising biological profile, Th has not been developed for clinical use, primarily because of its low aqueous solubility and formulation issues (540). However, given its dual activity on two independent targets, the parasite proteasome and the apicoplast, with the capacity to eliminate both intraerythrocytic asexual and transmission stages of the parasite, Th derivatives represent promising candidates for malaria therapy (541). At also demonstrated strong activity against ookinete formation and oocyst maturation in our study. Of note, hepatic infection of naive mice was completely abrogated following the administration of At to infected mosquitoes. Interestingly, At in serum collected from human volunteers was previously shown to block *Pb* development from ookinete to oocyst (542). This compound is frequently paired with other agents in patients with malaria. When administered alone, it is associated with unacceptable recrudescence rates and decreased parasite susceptibility following treatment (543). However, given its TB potential, an investment in new synergetic partners for At may be of interest.

One of the first steps toward finding TB compounds is to screen commercially available antimalarials with gametocytocidal activity (544). Although standard membrane feeding assays (SMFAs) are considered the gold standard for evaluating the infectivity of compound-treated gametocytes (545), this type of assay requires dissection and microscopic evaluation of individual mosquitos, which are very labor intensive. Assays measuring the viability or development of gametocytes without the need for mosquito dissection are comparatively inexpensive and highly scalable and will therefore continue to be required as a prioritization step in the screening pipeline for TB compounds (414).

Although the development of GFP-luciferase-expressing parasites has allowed for scalability of SMFAs (546), it is still important to establish filtering assays with higher throughput to prioritize compounds for subsequent evaluation in SMFAs. Even with *in vitro* culture, where protocols for *in vitro* generation of *Pf* ookinetes have been made available, the throughput of such assays remains a concern, with the possibility that the impact on ookinete viability and consequently oocyst formation being overlooked (547,548). In a recent comparison of SMFAs and gametocyte-based assays for identification of compounds with *Pf* transmission-reducing activity, a luciferase assay was shown to yield the strongest agreement with SMFAs (414). Thus, our assay appears as a valid tool for *in vitro* screening of TB compounds acting at various stages of the parasite's sporogonic development for subsequent *in vivo* evaluation. Furthermore, in facilities where the availability of *in vivo* models is sparse, this assay represents a relatively inexpensive alternative for compound screening. It establishes a fast method for screening a wide range of compounds in a relatively short time, allowing the specific assessment of compound activity against the various stages of *Plasmodium* sporogonic development.

2.2 INHIBITION OF *PLASMODIUM* SPOROGONIC STAGES BY IVERMECTIN AND OTHER AVERMECTINS

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AUTHOR CONTRIBUTIONS:

Azevedo R., Mendes A.M., and Prudêncio M. designed the experiments. Azevedo R. performed the experiments and wrote the paper. Mendes A.M. and Prudêncio M. supervised the work and revised the text.

INTRODUCTION

Wide-scale malaria interventions led to a decrease in malaria mortality and morbidity since 2000 (549). Although the number of malaria cases decreased from 2010, progress appears to have stalled, and a small increase in the global number of cases was actually observed in recent years (152). The most successful strategies to reduce the number of cases of malaria result from a combination of vector control strategies, and artemisinin combination therapies (ACT) that target the symptomatic blood stage of infection (62). However, the emergence of mosquito resistance to insecticides and of parasite resistance to antimalarial drugs severely threaten the efficacy of such measures (62,550). Therefore, the identification of new compounds that have a broad spectrum of action, long half-life and strong inhibitory activity remains a priority in the fight against malaria.

Avermectins are a class of macrocyclic lactones with insecticidal and antiparasitic properties. They are the most effective and well-developed class of endectocides, and are active against both endo- and ectoparasites (551). Ivermectin, the best studied semi-synthetic derivative of avermectins, has been considered one of the most successful discoveries in the fight against infections caused by roundworm parasites (552). Mass drug administration (MDA) of ivermectin in Africa and Latin America led to a reduction of onchocerciasis, as well as of lymphatic filariasis and scabies, which are also endemic in India and Southeast Asia (552–554). The impact of ivermectin on insect vectors (552,553), in particular its activity against *Anopheles* mosquitoes (174,555) prompted the investigation of its action against *Plasmodium* parasites, towards harnessing its potential as an integrated tool for malaria control (173,556). The host laboratory recently reported on ivermectin's activity against *Plasmodium* liver stages *in vivo* (530). However, discrepant results have emerged from several other studies aimed at assessing the Impact of ivermectin on the blood and mosquito stages of the *Plasmodium* life cycle (557–562). Whereas results obtained by Nasveld *et al.* (557) indicated that ivermectin displays very low activity against *P. falciparum* (*Pf*) blood stages *in vitro*, Panchal *et al.* (559) suggested that ivermectin inhibits the parasite's blood stage development by blocking nucleo-cytoplasmic shuttling of *Pf* signal recognition particle (SRP) components. This is in agreement with a recent study that demonstrated an impairment of sexual and asexual stages of *Pf* development by

ivermectin *in vitro* (562). On the other hand, ivermectin was reported to reduce oocyst prevalence and intensity in different mosquito species infected with *Pf* (558), contrary to reports by Kobylinski *et al.* (560) and Pinilla *et al.* (561), who did not observe a reduction on oocyst intensity, i.e. the number of oocysts per mosquito, but rather a decrease on oocyst prevalence, i.e., the proportion of mosquitoes harboring oocysts, for *P. vivax* (*Pv*) and *Pf*.

During the work described in this chapter, we aimed at clarifying the impact of ivermectin on *P. berghei* (*Pb*) blood stages *in vivo*, as well the impact of ivermectin and other avermectins on the parasite's mosquito stages *in vitro*.

MATERIALS AND METHODS

Experimental animals and *Pb* ANKA reference lines. Male BALB/cByJ mice (6–8 weeks-old) were purchased from Charles River Laboratories Inc. (Lyon, France). Work with laboratory animals was performed according to National and European regulations (Directive 2010/63/EU). Protocols were approved by the animal experimentation ethics committee (AWB_2015_09_MP_Malaria) of the Instituto de Medicina Molecular João Lobo Antunes and are in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines. Two parasite lines were employed in the experimental work, a transgenic parasite line termed *Pb* circumsporozoite-green fluorescent protein-luciferase (*Pb*CSGFP-Luc) (RMgm-152), expresses the fusion gene *gfp-luc* under the control of the circumsporozoite protein (*csp*) promotor (PBANKA_0403200) integrated into the silent *230p* gene locus (PBANKA_0306000) (415), and the transgenic parasite line *Pb* fluorescent female-red-male-green (Fluo-frmg) (RMgm-164), which expresses GFP under control of the 'male gametocyte-specific' promoter of PB000791.03.0 (dynein heavy chain, putative) and red fluorescent protein (RFP) under the control of the female gametocyte-specific promoter PB000504.02.0 (LCCL domain-containing protein CCP2). The *gfp* and *rfp* genes are integrated into the genome in the *230p* locus (PBANKA_0306000) (563).

Ookinete production. *Pb*CSGFP-Luc (RMgm-152) was maintained in *Anopheles stephensi* mosquitoes and BALB/cByJ mice. To keep gametocyte infectivity, up to six passages of parasites from infected to naïve mice were performed. Ookinete *in vitro* production was performed as previously described (415). Briefly, BALB/cByJ mice were treated with 0.1 ml phenylhydrazine (Sigma) (25 mg/ml) 3 days prior to infection with 10^7 *Pb*-infected red blood cells (iRBC) obtained from a donor mouse. On the third day after infection, gametocytemia was monitored by light microscopy for the presence of exflagellating gametocytes in the ookinete medium (1:4 dilution). Blood of 2 mice was collected by heart puncture and washed with Roswell Park Memorial Institute (RPMI) medium (Invitrogen) at 37 °C, followed by centrifugation at 1100× g for 10 min at 37 °C. After washing, 5 µl of blood containing exflagellating gametocytes was mixed with medium supplemented for ookinete formation

(RPMI1640 (Sigma), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (ThermoFisher Scinetific), 0.4 mM hypoxanthine (Merck), 100 mM xanthurenic acid (85570, Fluka), 10% foetal bovine serum (FBS) (Invitrogen) (pH 7.6)) in a final volume of 200 µl, and cultured in 96-well plates for 24 h at 19 °C. Additionally, blood containing exflagellating gametocytes was mixed with the ookinete medium in 1:20 ratio and cultured in T75 flasks for 22–24 h at 19 °C. Following incubation, ookinete enrichment was performed as previously described (415), with some modifications. Briefly, cultured blood was collected, and erythrocytes were lysed for 15 min on ice with 30 volumes of ice-cold 0.17 M ammonium chloride (VWR). Lysed erythrocytes were removed by washing with RPMI, and ookinetes were purified by centrifugation on a 63% Nycodenz (Axis-Shield) cushion at 650× g at 4 °C for 30 min. Following centrifugation, the ookinete-containing interface was collected, washed in ice-cold RPMI (Invitrogen) and resuspended in 0.5-1.0 ml of oocyst medium.

Oocyst cultures. Purified ookinetes were seeded with *Drosophila melanogaster* (*D. melanogaster*) S2 cells (*Drosophila* Genomics Resource Center, Bloomington, USA) in a 1:10 ratio (10^4 ookinetes and 10^5 S2 cells) in Schneider's medium (Sigma) supplemented with 15% FBS (Invitrogen), penicillin/streptomycin (50 U/ml, 50 µg/ml;ThermoFisherScientific) and gentamicin (50 µg/ml; ThermoFisher Scientific) to promote oocyst development. Oocysts were co-cultured with *D. melanogaster* S2 cells in flat bottom 96-well plates (Corning) for up to 15 days at 19 °C. One-quarter of the medium was changed 3 times per week (every 48 to 72 h), and 10^5 S2 cells were added once per week. In parallel, S2 cells were maintained at 27°C in Schneider's medium (Sigma) supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin (50 U/ml, 50 µg/ml; ThermoFisher Scientific).

Bioluminescence assay. A bioluminescence assay was used to assess the development of the mosquito stages of *Pb*CSGFP-Luc. In order to evaluate the effect of compounds on the development of the parasite's mosquito stages, samples were collected at 3 time points to determine the compounds' effect on ookinete and oocyst formation, and oocyst maturation, as previously described (415). The bioluminescence assay was performed using the firefly

luciferase assay kit (Biotium) according to the manufacturer's instructions, with some modifications. Briefly, the whole well contents were collected and spun down, washed with PBS (NZYTech), frozen in 50 µl of lysis buffer (1:5 ratio) and stored at -20 °C until further use. Collected samples were lysed and 30 µl of the resulting supernatant were transferred into white 96-well plates. Fifty µl of D-luciferin in Firefly luciferase assay buffer (1:50 ratio) were added to the samples and parasite load was determined by measuring luminescence intensity using a microplate reader (Tecan Infinite M200).

Evaluation of the *in vivo* activity of ivermectin. To assess the *in vivo* activity of ivermectin five BALB/cByJ (Charles River) mice per experimental group were infected by intraperitoneal injection of 10^7 *Pb*Fluo-frmg-infected red blood cells. Parasitaemia and gametocyteamia were measured every day by flow cytometry analysis of 4 µl of tail blood until parasitemia reached 3%. Blood was collected in 200 µl of PBS and 100 µl stored at 4 °C, while the remaining was transferred to 100 µl of PBS (NZYTech) containing 1.25 mM of the red fluorescent nucleic acid stain, Syto®61 (Thermo Fisher Scientific) and incubated for 20 min at room temperature in the dark. The samples were analyzed on a LSRFortessa X-20 flow cytometer (Becton, Dickinson and Company). Female and male gametocytes were gated based on the analysis of GFP and RFP fluorescence, and parasitemia was estimated based on the analysis of Syto®61 and forward scatter. The Syto®61-positive cell population was determined by comparison of infected blood samples with an uninfected blood sample. Results were then analyzed with the FlowJoTM Software (Version 10, FlowJoTM Software, Ashland, USA). Exflagellation was also monitored every day. To this end, 2.5 µl of tail blood were collected and mounted on a glass slide, and 8 min later the number of exflagellation events present in 4 independent fields of vision at 40× were determined. When parasitemia reached up to 3%, either dimethyl sulfoxide (DMSO; Sigma) or ivermectin were administered by oral gavage, at a concentration of 5 mg/kg, to five mice of each experimental group. Parasitemia, gametocytemia and exflagellation were monitored for 3 days after treatment, following which the mice were euthanized, and the experiment was terminated.

Evaluation of the *in vitro* activity of avermectin compounds. The effect of eprinomectin, abamectin, ivermectin, moxidectin, doramectin and emamectin on *Plasmodium* mosquito stages was evaluated as previously described (415). The compounds were dissolved in DMSO (Sigma) and the amount of DMSO (Sigma) equivalent to that present in the highest compound concentration was used as a control. The effect of 10 µM of each compound was assessed on ookinetes, as well as on oocyst development and maturation. Briefly, after 1 h of incubation, the compounds were added to 5 µl of infected blood cultures, and the bioluminescence intensity of the parasites on the ookinete cultures was assessed 24 h later. To assess the effect of compounds on oocyst development, they were mixed with the mature ookinetes, and cultures collected after 3 days. The effect on oocyst maturation was measured by adding compounds to the oocyst culture after 3 days, and parasites collected 15 days later.
Compound concentration resulting in 50% inhibition (IC_{50}) for oocyst growth and maturation were estimated for eprinomectin, abamectin, ivermectin, moxidectin, doramectin and emamectin (assayed at 0.05, 0.5, 1, 5, 10, 25 and 50 µM).

Evaluation of avermectin compounds' *in vitro* cytotoxicity. Compounds were screened for their *in vitro* cytotoxicity against *D. melanogaster* S2 cells, using the AlamarBlue® assay (Invitrogen). To assess the effect of the compounds on cell development, *D. melanogaster* S2 cells were seeded in a 1:10 ratio (10^5 S2 cells) in Schneider's medium (Sigma) supplemented with 15% FBS (Invitrogen), penicillin/streptomycin (50 U/ml, 50 µg/ml; ThermoFisher Scientific) and gentamicin (50 µg/ml; ThermoFisher Scientific). The compounds were added to the S2 cell cultures to a final concentration of 10 µM and the amount of DMSO equivalent to that present in the highest compound concentration was used as a control. Cultures were maintained for 7 days and one-quarter of the medium was changed 3 times per week every 48 to 72 h. Samples were collected every day by transferring 120 µl of medium to a 96 well flat bottom plate and adding 80 µl of AlamarBlue® (Invitrogen) previously diluted in Schneider's medium (1:10 dilution, Sigma) to each well. The suspension was incubated for one and a half hours at 37 °C. Fluorescence intensity was then measured using a microplate reader at 530 nm excitation and 590 nm emission wavelengths, respectively, to determine cell viability.

Statistical analysis. Data on the assessment of the compounds' effect *in vitro* were analyzed using the Kruskal-Wallis test. Data on the compounds' effect on parasitemia, gametocytemia and exflagellation *in vivo* were analyzed employing non-linear regression analysis. Results were considered significant for P-values < 0.05. Nonlinear regression analysis was employed to fit the normalized results of the dose-response curves for IC50 determination. All statistical tests were performed by GraphPad Prism (version 6.00, GraphPad Software, La Jolla, USA).

RESULTS

IN VIVO ACTIVITY OF IVERMECTIN AGAINST *Pb* BLOOD STAGES

We employed a mouse model of infection to evaluate the effect of ivermectin on *Pb* sexual and asexual blood stage forms *in vivo*, as described (**Fig. 2.2.1A**). Our results show no statistically significant differences between untreated controls and ivermectin-treated mice regarding parasitemia ($F(1, 26) = 0.074$, $P = 0.78$), percentage of the parasite's male and female gametocytemia ($F(1, 26) = 0.18$, $P = 0.67$ and $F(1, 26) = 0.079$, $P = 0.78$, respectively), and exflagellation events ($F(1, 26) = 0.33$, $P = 0.57$) up to 5 days after infection (**Figs. 2.2.1B-D**). These data suggest that, at the tested dosage, ivermectin does not appear to significantly inhibit *Plasmodium* asexual and sexual forms in the blood.

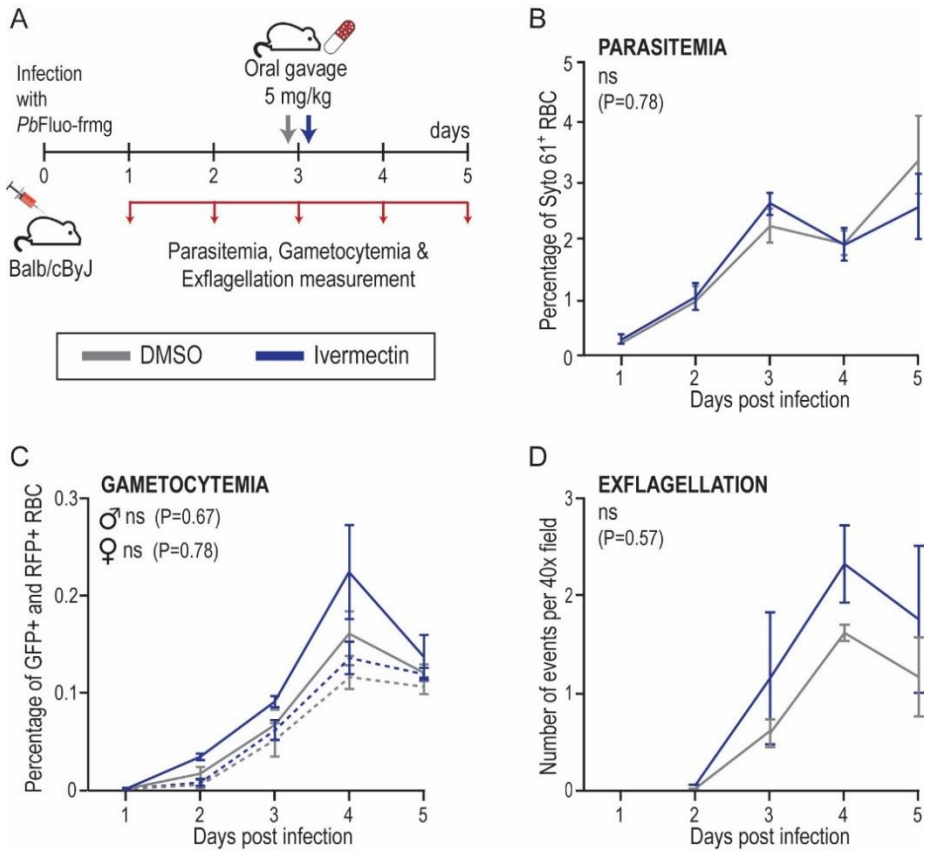

FIGURE 2.2.1 – Ivermectin activity against *Pb* asexual and sexual blood stages in the mammalian host. (A) Schematics of assessment of *in vivo* compound activity on exflagellation, parasitemia and gametocytemia of mice treated with ivermectin. **(B)** *In vivo* activity of ivermectin on parasitemia of mice treated by a single oral dosage of ivermectin. Results are a representation of Syto 61 positive events on flow cytometry analysis and are expressed as the mean of parasitemia values (percentage of infected red blood cells) ± standard deviation, SD.

(C) *In vivo* activity of ivermectin on female and male gametocytemia of mice treated by a single oral dosage of ivermectin. Female and male gametocytemia are represented by dashed line and solid lines, respectively. Female and male gametocytes were identified by flow cytometry analysis of RFP^+ or GFP^+ events, respectively. Results are expressed as the mean of gametocytemia values (percentage of gametocytes) ± SD. **(D)** *In vivo* activity of ivermectin on the number of exflagellation events per 40× microscopic field of mice treated by a single oral dosage of ivermectin. Results are expressed as the mean of observed exflagellation events ± SD. Abbreviation: ns, non-significant

IN VITRO ACTIVITY OF AVERMECTINS AGAINST *Pb* SPOROGONIC DEVELOPMENT

We then sought to clarify whether the proposed transmission-reducing activity of ivermectin would include a direct action of the drug on the parasite's transmission stages or if it would result solely from its effect on the mosquito. Given the marked structural similarities among avermectins, the effect of an additional 5 compounds of this family, doramectin, moxidectin, abamectin, emamectin, and eprinomectin on the parasite's sporogonic stages was also evaluated (**Fig. 2.2.2A**).

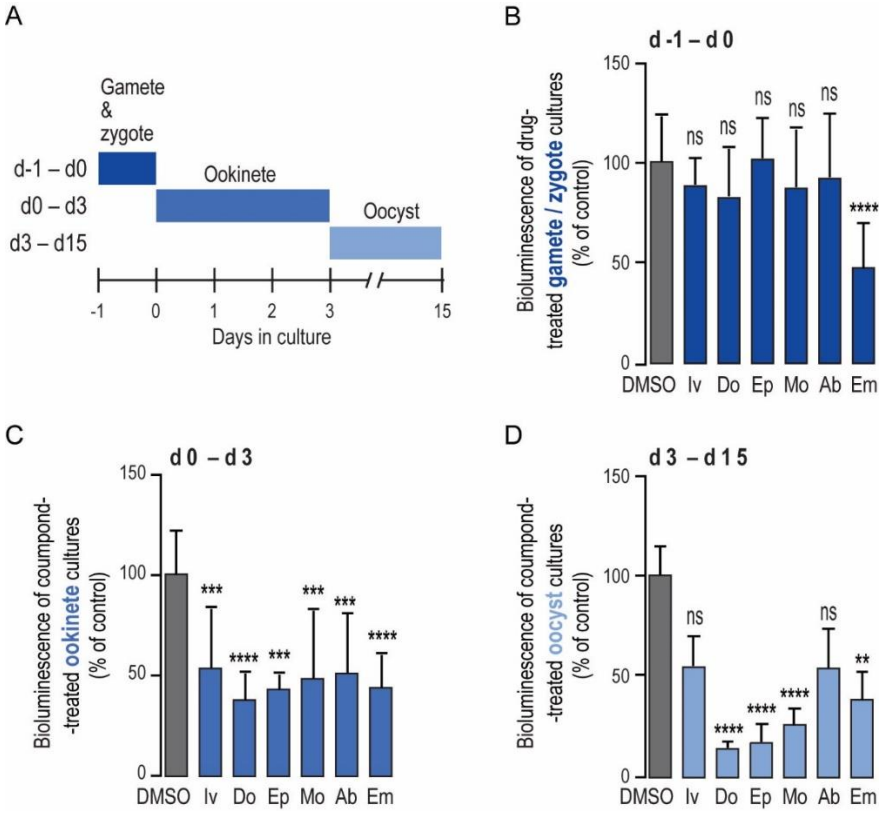

FIGURE 2.2.2 – Activity of avermectins against *Plasmodium* mosquito stages *in vitro*. (A) Schematics of the progress of the parasite culturing process, highlighting the different schedules of compound treatment employed.

(B) Assessment of compound effects on ookinete formation, expressed as the percentage of inhibition of *Pb* ookinete formation. **(C)** *In vitro* activity of selected compounds against oocyst formation. **(D)** *In vitro* activity of selected compounds on oocyst development. A total of 6 compounds were screened at a concentration of 10 µM: Iv; Do, Ep, Mo, Ab and Em. Bars correspond to RLU measurements, represented as the percentage of RLU of the DMSO control. Results are expressed as the mean + standard deviation, SD. ****P < 0.0001, ***P < 0.001, **P < 0.01, ns, non-significant. Abbreviations: Iv, ivermectin; Do, doramectin; Ep, eprinomectin; Mo, moxidectin; Ab, abamectin; Em, emamectin.

Our assessment of the compounds' effect on the transformation of gametes/zygote into ookinetes, showed that whereas emamectin inhibited parasite differentiation by approximately 47%, ivermectin and the remaining avermectins had a small and non-significant impact on the formation of ookinetes (**Fig. 2.2.2B**). The effect of avermectins on oocyst formation and growth was subsequently assessed. At 10 µM, both these processes are inhibited at least 50% by all compounds (**Figs. 2.2.2C, D**).

Half-maximal inhibitory concentrations (IC_{50}) of compounds on oocyst formation were then determined and found to range from ~5.7 µM to ~11.6 µM. Eprinomectin, doramectin and emamectin displayed the greatest potency against this early stage of parasite sporogony (**Table 2.2.1, Fig. 2.2.3A**). Their IC_{50} values for oocyst growth varied between ~4.3 µM and ~9.3 µM (**Table 2.2.1, Fig. 2.2.3B**), with eprinomectin, doramectin and moxidectin standing out as the three compounds that most potently inhibited oocyst development (**Table 2.2.1**).

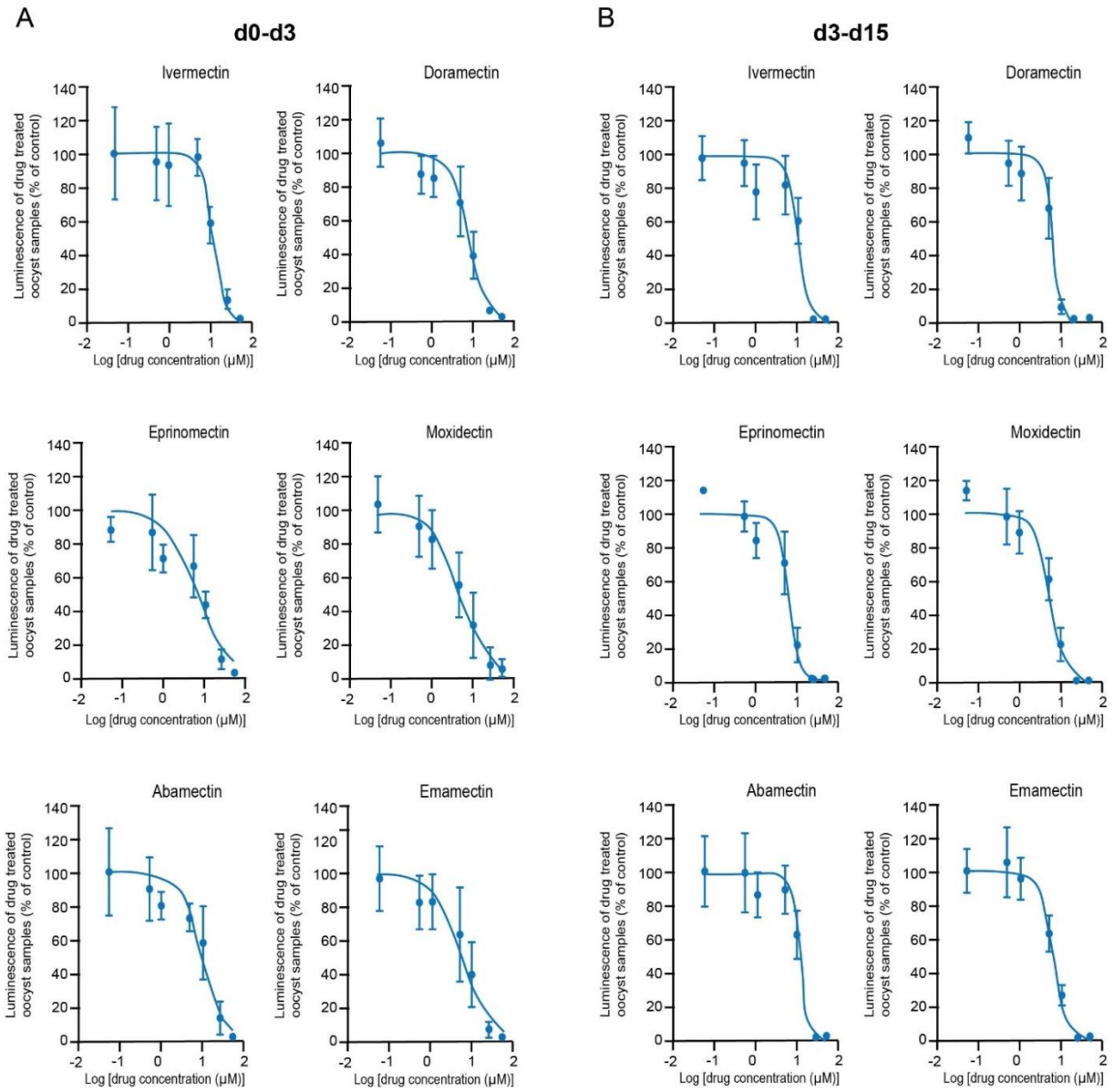

FIGURE 2.2.3 – Dose-response of avermectins against *Plasmodium* sporogonic stages. (A) Representative curves of avermectins effect resulting in 50% inhibition (IC_{50}) of oocyst formation. Curves are presented for eprinomectin, doramectin, emamectin, abamectin, moxidectin and ivermectin (assayed at 0.05, 0.5, 1, 5, 10, 25 and 50 µM). Results are expressed as the mean ± standard deviation, SD. **(B)** Representative curves of avermectin's effect resulting in 50% inhibition (IC_{50}) of oocyst maturation. Curves are presented for eprinomectin, doramectin, emamectin, abamectin, moxidectin and ivermectin (assayed at 0.05, 0.5, 1, 5, 10, 25 and 50 µM). Results are expressed as the mean ± SD.

TABLE 2.2.1 – IC_{50} of avermectins against oocyst formation and maturation *in vitro*. Compound concentration resulting in 50% inhibition (IC50) for oocyst growth and maturation were calculated for the compounds under evaluation (assayed at 0.05, 0.5, 1, 5, 10, 25 and 50 µM). Results are expressed as the mean ± standard deviation.

Compound	Day 0-Day 3 (µM)		Day 3-Day 15 (µM)	
	IC_{50}	SD	IC_{50}	SD
Eprinomectin	5.70	2.31	4.59	2.26
Doramectin	7.08	1.91	4.32	1.76
Emamectin	7.31	2.45	7.36	1.50
Abamectin	8.77	4.38	7.93	4.14
Moxidectin	10.85	8.56	5.49	1.00
Ivermectin	11.58	0.44	9.32	1.64

Since oocysts are co-cultured in the presence of *Drosophila* S2 cells, we further evaluated the latter's viability for up to 7 days in the presence of the compounds under study (**Fig. 2.2.4**). Of note, these results indicate that emamectin displays some cytotoxicity against S2 cells, which might suggest a moderate overestimation of this compound's activity against developing oocysts.

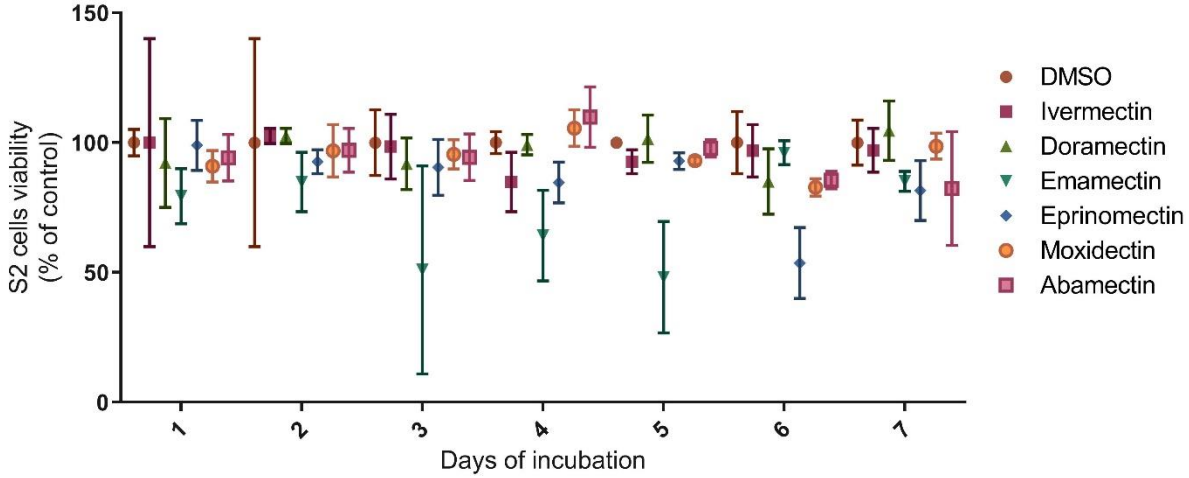

FIGURE 2.2.4 – Evaluation of avermectin's cytotoxicity on S2 cells. Determination of cell viability over a 7-day period in the presence of the test compounds, by the AlamarBlue® assay. Results are normalized to the DMSO control and expressed as the mean ± standard deviation, SD.

DISCUSSION

In the present study, we aimed to assess the impact of ivermectin on the blood stages of *Pb in vivo*, as well as the effect of several avermectins on the parasite's mosquito stages *in vitro*. Our results show that ivermectin is not active against *Pb* asexual and sexual blood forms in a mouse model. However, both ivermectin and other members of the avermectin family strongly inhibited parasite sporogony, at IC_{50} values consistent with those reported for the antibiotic thiostrepton and the antimalarial pyronaridine, whose impact on sporogony has been demonstrated (463).

In light of their versatility as antiparasitic and insecticidal compounds, avermectins, particularly ivermectin, have been considered as potential aids in the fight against malaria (173,174,530,558–560). Of note, the use of ivermectin in MDA to treat other tropical diseases which are co-endemic with malaria has led to the investigation of its potential to block malaria transmission (171,173,564). A recent study showed that a 3-weekly MDA of ivermectin in African villages reduced the incidence of uncomplicated malaria episodes among children, which the authors attributed to the drug's mosquitocidal effect (173). However, it should be noted that the results of this study have recently been questioned based on the statistical methods employed (565). Nevertheless, the issue of whether the impact of ivermectin stems solely from its impact on mosquitoes or, additionally, results from a combination of its insecticidal activity and of its ability to inhibit the parasite's blood and mosquito stages remains unresolved. In fact, while the inhibitory effect of ivermectin against the liver stages of *Plasmodium* parasites has been demonstrated (530), the evaluation of its impact against the blood (557,559,562) and sporogonic (555,560,561) stages of the parasite's life cycle has yielded contradictory results (555,557,559–562).

In invertebrates, ivermectin interacts with the glutamate-gated chloride channels in neuronal and neuromuscular tissues (566–568) and may also act on the γ-aminobutyric acid-gated chloride channels (569–571). However, neither of these molecular targets is present in *Pf*, which might explain the lack of ivermectin *in vitro* effect against *Pf* blood stages (557). Contradictorily, ivermectin was reported to lead to the arrest of the development of *Pf* blood stages by inhibiting the nuclear import of SRP polypeptides, thus arresting parasite growth

(559). The sporontocidal activity of ivermectin against *P. vivax* in *An. darlingi* led to a reduction of oocyst prevalence, but not of their intensity (561), similarly to what has been observed for *Pf* in *An. Gambiae* (174). This contradicts previous studies suggesting that ivermectin reduces oocyst prevalence and intensity in *An. dirus* and *An. minimus* (558). Although the mechanism of action of ivermectin against the sporogonic stages of *Plasmodium* parasites remains to be elucidated, these observations suggest that the compound may act on the mosquito midgut physiology, preventing parasite establishment (560). As such, the distinct results obtained experimentally in previous studies might result from differences in insect biology.

Our study sheds a new light on these controversial issues and helps clarify whether ivermectin exerts an impact on the blood and/or on the mosquito stages of *Plasmodium* parasites. Our *in vivo* investigation revealed that treatment of infected mice with 5 mg/kg of ivermectin had no impact on parasitemia, indicating an absence of activity of this compound against the parasite's asexual forms in the blood, in accordance with Nasveld *et al.* (557) for *Pf*. These results, supported by the *in vitro* screening of avermectins, suggest that the impact of ivermectin on *Plasmodium* transmission does not result from an inhibition of *Plasmodium* spp. transmissible forms in the mammalian host, and stems solely from its effect during the mosquito stage of infection. Our data show that the transition from gamete/zygote to ookinete is highly resistant to avermectins, and that the oocyst is the most vulnerable stage of the parasite's sporogonic cycle to treatment with avermectins. Further investigation on the mechanism of action of avermectins is required in order to fully clarify the exact targets of their activity against *Plasmodium* sporogony.

Collectively, these observations support the notion that, besides their mosquitocidal effect, avermectins may also directly target the parasite's sporogonic stages, which likely contributes to transmission-blocking activity. Our results lend further support to the use of avermectins for MDA as a tool for malaria control in endemic regions and suggest that the inclusion of members of the avermectin family of compounds besides ivermectin in these interventions should be considered.

2.3 THE IMPACT OF ANTIRETROVIRAL THERAPY ON MALARIA PARASITE TRANSMISSION

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AUTHOR CONTRIBUTIONS:

Azevedo R., Mendes A.M., and Prudêncio M. designed the experiments. Azevedo R. performed the experiments and wrote the paper. Mendes A.M. and Prudêncio M. supervised the work and revised the text.

INTRODUCTION

Human immunodeficiency virus (HIV) infects the immune system's CD4+ T cells, inducing chronic inflammation that may drive the progression into acquired immune deficiency syndrome (AIDS) (572). In 2021, 38.4 million people were reported to live with HIV, leading to an estimated 650 000 deaths in that year alone (573). In 2002, the World Health Organization (WHO) issued a set of guidelines to help determine the best usage of antiretroviral (ARV) compounds for the treatment of HIV-positive young adults and adolescents (574). Since then, these guidelines have been regularly updated and, from 2016, the recommended first-line antiretroviral therapies (ARTs) in adults, including pregnant women and adolescents, consists of two nucleoside reverse-transcriptase inhibitors (NRTIs) plus a non-nucleoside reverse transcriptase inhibitors (NNRTI) or an integrase strand transfer inhibitor (INSTI) (574). The recommendation for children between 3 and 10 years old is the combination of two NRTIs with the NNRTI efavirenz (EFV), while for children under 3 years of age is a combination of the NRTI backbone with the protease inhibitors (PIs) lopinavir/ritonavir (LPV/r) (574).

Plasmodium and HIV infections overlap geographically in tropical and subtropical regions, particularly in Sub-Saharan Africa, where 66 % of the world's HIV cases and 95% of the malaria cases are concentrated (326,549). Pregnant women, in whom *Plasmodium* infections are more severe, are at particular risk of co-infection (326,575). The outlook of either disease seems to be influenced by co-infection. On the one hand, the low CD4+ cell count of HIV carriers limits their immune system's ability to mount a response against a parasite infection (576), while, on the other hand, *Plasmodium* infection can cause T cell activation and cytokine release, which can stimulate HIV replication (576,577). Therefore, it is important to further understand the spectrum of activity of drugs used for treatment of either disease, and their possible impact on each other.

Numerous reports describe the effect of ART on the blood and liver stages of *Plasmodium* parasites (536,578–585). PIs have been systematically described as the most effective ARV compounds in inhibiting *Plasmodium* erythrocytic stages (578,579,582,584,586–588). Their ability to inhibit the growth of drug-susceptible and drug-resistant *P. falciparum* (*Pf*) parasite strains has also been documented (578,584). The PI lopinavir (LPV), has been

identified by several studies as the most potent ARV inhibiting *Pf* asexual stages *in vitro* (579,584,588). The PI indinavir (IDV) has also been reported to suppress *P. cynomolgi* growth and to delay pre-patency in monkeys infected with *P. knowlesi* (589). *P. vivax* was found to be more sensitive to the PIs ritonavir (RTV) and saquinavir (SQV) than *Pf* (587), whereas Peatey *et al.* showed that ARV PIs are more active against the trophozoite and schizont stages than against the ring stages of *Pf* asexual parasites in the blood (582). These authors also showed that the exposure of *Pf* gametocyte cultures to SQV, LPV, RTV, tipranavir and darunavir (DRV) (PIs) inhibited the formation of gametocytes – gametocytogenesis, but only tipranavir had the ability to kill gametocytes (582). Consistent with these results, Hobbs *et al.* showed that prolonged exposure to LPV/r, LPV, and SQV reduces early and late stage gametocyte viability, with the latter two drugs impacting parasite exflagellation (588). This impairment in parasite development was also reflected on oocyst infection in the mosquito, when mosquitoes fed on blood cultures previously treated with LPV and SQV (588). It was also shown in a high-malaria-transmission area, that ARV PI's LPV/r are associated with reduced gametocytemia in children (590).

The mechanism of action of PIs on *Plasmodium* is still unknown, but it has been theorized that PIs inhibit the development of malaria parasites by targeting plasmepsins (PMs) in their food vacuole (586,591), where they play an important role in haemoglobin degradation by *Pf* (592). More recently, it has been suggested these drugs might also target other non-vacuolar PMs (582,589,593,594). Another study suggested that treatment with PIs might affect positively the outcome of malaria infection due to an impairment of parasite sequestration by these drugs. This impairment could be explained by the deficiency in the CD36 receptor observed in some patients treated with ARV compounds (595). A recent investigation of the impact of ARV compounds on the liver stage of *Plasmodium* infection has shown that, consistent with what is observed for erythrocytic stages, the PJs LPV and RTV are potent inhibitors of the parasite's development in hepatic cells (580). A reduction of the *P. yoelii* (*Py*) liver burden *in vivo* by NNRTIs (581), and of the *P. berghei* (*Pb*) liver burden *in vivo* by etravirine (ETV) (585) have also been reported.

In this study, we employed a recently developed luminescence-based *in vitro* assay (415) to determine the ability of ARV compounds and current first-line ARTs to inhibit the development of *Plasmodium* mosquito stages *in vitro*. We further validated those results by assessing the *in vivo* inhibitory activity of the first-line ARTs and selected alternative drug combinations against the sexual stages of the parasite's life cycle, as well as their impact on oocyst infection. This study demonstrates that the current field treatments against HIV have an impact on the mosquito stages of *Plasmodium* and suggest the evaluation of the possible inclusion of both rilpivirine (RPV) and ETV in alternative ARTs.

MATERIALS AND METHODS

Animals and parasite lines. Six-to-eight weeks old male BALB/cByJ mice were purchased from Charles River Laboratories Inc. (France). Work with laboratory animals was performed according to National and European regulations (Directive 2010/63/EU). Protocols were approved by the animal experimentation ethics committee (AWB_2015_09_MP_Malaria) of the Instituto de Medicina Molecular João Lobo Antunes and are in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines. The *Pb* circumsporozoite-green fluorescence protein-luciferase (*Pb*CSGFP-Luc) (415) and *Pb* fluorescent-female red male green (*Pb*Fluo-frmg) (563) parasite lines were employed in the experimental work, which was carried out under biosafety level 1 (BSL1) or animal biosafety level 2 (ABSL2) conditions. The former parasite line expresses the fusion gene *gfp-luc* under the control of the circumsporozoite protein (*csp*) gene promotor (RMgm-152), and the latter expresses red fluorescence protein (RFP) and GFP under the control of stage-specific promotors for female and male gametocytes, respectively (RMgm-164). The genes were integrated by double recombination into the silent *230p* gene locus of the *Pb* genome.

Ookinete production and culturing. Ookinetes were generated as previously described (415). Briefly, two male BALB/cBbyJ mice were infected with 10^7 *Pb*CSGFP-Luc-infected red blood cells (iRBCs) three days post treatment with 0.1 ml phenylhydrazine (25 mg/ml). On the third day of infection, when 3-6 exflagellation events/field (1:4 dilution) were observed by light microscopy field (40x magnification), mice were sacrificed and approximately 2 ml of infected blood were collected by cardiac puncture and added to Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) 37 ºC. After washing with RPMI (Sigma), 5 µL of blood and 195 µL of ookinete culturing medium (RPMI-1640 (Sigma), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ThermoFisher Scientific), 0.4 mM hypoxanthine (Merck), 100 mM xanthurenic acid (Fluka, 85570), 10% fetal bovine serum (FBS, Invitrogen), pH 7.6) were added per well of a 96-well plate, and incubated for 24 h at 19 °C for ookinete formation. In parallel, a 1:20 dilution of blood in ookinete medium was cultured in T75 flasks for 22-24 h at 19 °C for production of ookinetes. Ookinetes were purified employing a

Nycodenz (Axis-Shield) gradient. The contents of the T75 flask were collected and the RBCs were lysed for 15 min on ice with 30 volumes of ice-cold 0.17M ammonium chloride (VWR). After removal of the lysed RBCs by washing with RPMI-1640 (Sigma), ookinetes were purified on a 69% Nycodenz (Axis-Shield) gradient by centrifugation at 650×g and 4 °C for 30 min. Following centrifugation, ookinetes were collected by aspiration of the dark brown ring formed, washed in RPMI-1640 (Sigma) and resuspended in 1 ml of oocyst medium (Schneider's medium (Sigma), 15% FBS (Invitrogen), penicillin/streptomycin (50 U/ml, 50 µg/ml; ThermoFisher Scientific)) and gentamicin (50 µg/ml; ThermoFisher Scientific)).

Oocyst cultures. Following purification, ookinetes were co-cultured with *Drosophila melanogaster* (*D. melanogaster*) S2 cells (*Drosophila* Genomics Resource Center, Bloomington, IN) in a 1:10 ratio (10^4 ookinetes and 10^5 S2 cells) in oocyst medium, as previously described (415). The cultures were maintained in 96-well plates for up to 15 days at 19 °C. One quarter of the medium volume was replaced by fresh medium three times a week and 10^5 S2 cells were added to the medium once per week.

Evaluation of the activity of ARV compounds against *Plasmodium* mosquito stages *in vitro.* The activity of 10 µM of each ARV compound was assessed against ookinetes and oocysts. This concentration was selected based on the standards established by previous experimental work by Delves *et al.* and Azevedo *et al.* on the *Plasmodium* transmission-blocking effect of compounds, and after a preliminary screen of the compounds under evaluation at 50, 10 and 1 µM (415,463). Eighteen compounds belonging to four different classes of ARV compounds were evaluated: 1) PI – amprenavir (APV), atazanavir (ATV), DRV, IDV, LPV, nelfinavir (NFV), RTV and SQV; 2) integrase strand transfer inhibitors (INSTIs) – raltegravir (RAL); 3) NRTIs – abacavir (ABC), tenofovir (TDF), emtricitabine (FTC), zidovudine (AZT) and lamivudine (3TC); 4) NNRTIs – ETV, nevirapine (NVP), EFV and RPV. ARV compounds were obtained from the NIH AIDS and Reference Reagent Program. Ten mM stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO, Sigma) and serially diluted to 10 µM of each compound for *in vitro* activity assessments. A concentration of DMSO (Sigma) equivalent to that present

in the highest compound concentration was also used as a control in all activity assays. The compounds' effect on gametocyte to ookinete transition was determined by adding them to 1 h old gametocyte cultures. After 24 h, the parasite load was assessed by bioluminescence employing the Firefly Luciferase Assay Kit (Biotium) according to the manufacturer's instructions, with some modifications. Briefly, the well contents were collected, washed with PBS (NZYTech), spun down and frozen in 50 µL of 1:5 lysis buffer. Thirty µL of the lysed supernatant were transferred into each well of a white 96-well plate. Fifty µL of luciferin Firefly luciferase assay buffer (1:50 ratio) were added to the samples and the parasite load was determined by measuring luminescence intensity using a microplate reader (Tecan Infinite M200). To assess their effect on oocyst formation, compounds were mixed with ookinetes and cultured with *D. melanogaster* S2 cells for 3 days, following which the cultures were collected and lysed, and parasite load was determined by luminescence measurement, as described above. The effect of the compounds on oocyst development was assessed by adding them to 3-day old oocyst cultures, lysing the cultures 12 days later, and determining the parasite load by bioluminescence, as described above.

Evaluation of the activity of ARV compounds against *Plasmodium* blood and mosquito stages *in vivo.* To evaluate the *in vivo* antiplasmodial activity of first-line ARV regimens and proposed modifications, three male BALB/cByJ mice per experimental group were infected with 10^7 iRBCs of the parasite line *Pb*Fluo-frmg from a donor previously infected from a parasite stock vial. After 24 h, and during the following 4 days, parasitemia and gametocytemia were assessed by the collection of 4 µl of tail blood in 200 µL of PBS. One hundred µl of the solution were further diluted in PBS (NZYTech) at a 1:1 ratio and stored at 4 °C, while the remainder was diluted in a 1:1 ratio of PBS containing 1.25 mM of red fluorescent nucleic acid stain Syto® 61 (ThermoFisher Scientific) and incubated for 20 min at room temperature in the dark. The samples were analysed on an LSR Fortessa X-20 flow cytometer (Becton, Dickinson and Company). Forty-eight h post-infection, a suspension of the compounds in sunflower oil was administered by oral gavage. Compounds were administered at an allometry-scaled dose and, in accordance with the administration regimen recommended for humans. The compounds

were administered on a 24-hour schedule except for ETV, which was administered every 12 hours (**Table 2.3.1**). DMSO in a dosage equivalent to the highest amount of this solvent present in any compound combination was used as a control.

TABLE 2.3.1 – Schedules and allometry-scaled doses of antiretroviral drugs employed *in vivo*. Abbreviations: TDF, tenofovir; 3TC, lamivudine; EFV, efavirenz; ETV, etravirine; RPV, rilpivirine; AZT, zidovudine; LPV/r, lopinavir/ritonavir

Drug combination	Dose in humans (w)	Dose in mice (w/W)	Schedule (h)
TDF+3TC+EFV	300 mg TDF	31.2 mg/kg TDF	24/24
	300 mg 3TC	31.2 mg/kg 3TC	24/24
	600 mg EFV	62.4 mg/kg EFV	24/24
TDF+3TC+ETV	300 mg TDF	31.2 mg/kg TDF	24/24
	300 mg 3TC	31.2 mg/kg 3TC	24/24
	200 mg ETV	20.8 mg/kg ETV	12/12
TDF+3TC+RPV	300 mg TDF	31.2 mg/kg TDF	24/24
	300 mg 3TC	31.2 mg/kg 3TC	24/24
	25 mg RPV	2.6 mg/kg RPV	24/24
AZT+3TC+LPV/r	90 mg AZT	9.9 mg/kg AZT	12/12
	45 mg 3TC	4.9 mg/kg 3TC	12/12
	120 mg LPV	13.1 mg/kg LPV	12/12
	20 mg RTV	2.2 mg/kg RTV	12/12

On the fifth day of infection, approximately 50 previously starved *Anopheles stephensi* mosquitoes per experimental condition fed for approximately 30 min on anesthetized, infected, drug-treated mice. Mosquitoes were kept in standard dietary conditions, at 20 °C with 80% humidity under a 12 h light/dark cycle. Ten days after infection, mosquito midguts were dissected and stained with a solution of 0.025% mercurochrome in order to quantify oocyst density by microscopy analysis. Images were acquired on a Leica DM2500 microscope and analyzed with the FIJI software (528).

Statistical analysis. Data regarding the compounds' *in vitro* effect and mosquito infection were analyzed using the Kruskal-Wallis test. A chi-squared test was used to compare mosquito infection prevalence. Data on the compounds' effect on parasitemia and gametocytemia *in vivo* were analyzed by non-linear regression analysis. Results were considered significant for P values < 0.05. All statistical tests were performed using the GraphPad Prism software (version 6.00, GraphPad Software, La Jolla California USA).

RESULTS

IN VITRO ACTIVITY OF ARV COMPOUNDS AGAINST OOKINETE FORMATION

The *in vitro* activity of 10 µM of 18 ARV compounds from 4 different drug classes – PI: APV, ATV, DRV, IDV, LPV, NFV, RTV and SQV; integrase strand transfer inhibitors (INSTIs): RAL; NRTIs: ABC, FTC, 3TC, TDF and AZT; and NNRTIs: EFV, ETV, NVP and RPV – was evaluated (**Fig. 2.3.1A**). Our results showed that the PIs LPV and RTV led to approximately 50% reduction in ookinete formation relative to the controls, whereas the NNRTI ETV inhibited parasite development by ~40% (**Fig.2.3.1B**). Conversely, neither of the NRTI and INSTI compounds under evaluation displayed an inhibitory activity against this stage of the parasite's sporogonic development at the concentration used in this assay (**Fig. 2.3.1B**).

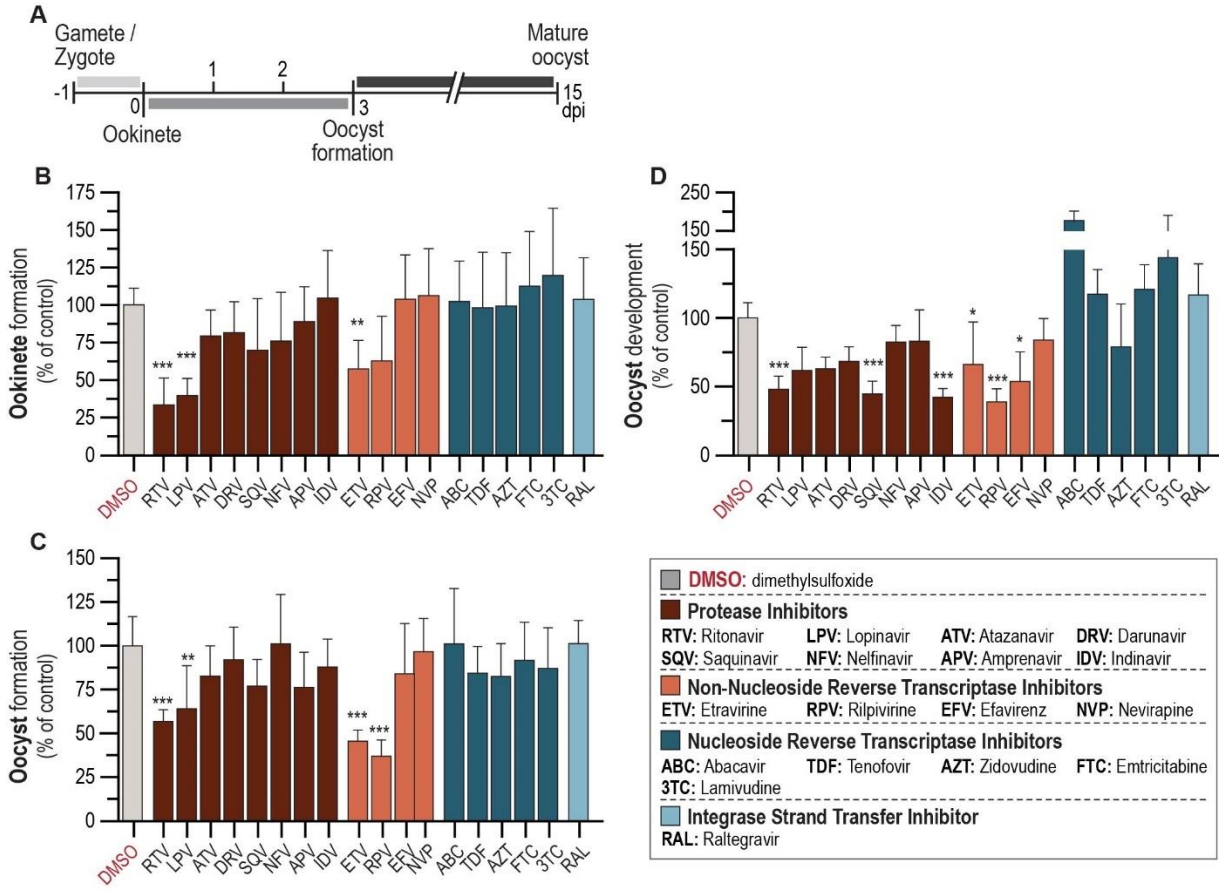

FIGURE 2.3.1 – *In vitro* activity of ARV compounds on *Pb* sporogonic stages. (A) Timeline of *Pb* sporogonic development and drug incubation periods. **(B)** Activity of ARV compounds on the conversion of zygotes/gametes into ookinetes. **(C)** Activity of ARV compounds on oocyst formation. **(D)** Activity of ARV compounds on oocyst development. All compounds were employed at 10 µM. Bars correspond to RLU measurements represented as

the percentage of RLU of the DMSO control. Results are expressed as the mean +/- SD. Statistically significant differences between control and treated conditions were analyzed using the Kruskal-Wallis test. N = 3-6. ***, P < 0.001; **, P < 0.01; *, P < 0.05.

IN VITRO EFFECT OF ARV COMPOUNDS ON OOCYST FORMATION AND DEVELOPMENT

We subsequently assessed the activity of the 18 compounds listed above on oocyst formation and development. Our results showed that 10 µM concentrations of the NNRTIs ETV and RPV, and of the PIs RTV and LPV, inhibited oocyst formation by approximately 50%, whereas the remaining compounds under evaluation did not display activity against this stage of the parasite's life cycle (**Fig.2.3.1C**). The PIs IDV, RTV and SQV, and the NNRTI RPV led to ∼50% inhibition of oocyst development (**Fig. 2.3.1D**). A milder ∼30% inhibition was observed for the NNRTIs ETV and EFV (**Fig. 2.3.1D**). Interestingly, treatment with the NRTIs ABC and 3TC consistently led to increased parasite loads relative to vehicle-treated controls (**Fig. 2.3.1D**).

IN VITRO ACTIVITY OF FIRST LINE ARTs AGAINST *Pb* SPOROGONIC STAGES

According to WHO recommendations, ARV compounds should be administered as an integral part of well-established ART regimens. The preferred backbone for first-line treatment against HIV in adults and adolescents is composed of two NRTIs and an NNRTI or INSTI, while for treatment of children less than 3 years old, WHO's suggested drug combination is AZT + 3TC + LPV/r (574). We assessed the activity of the first-line ARV regimen for adults and adolescents, TDF (NRTI) + 3TC (NRTI) + EFV (NNRTI), and children, AZT (NNRTI) + 3TC (NNRTI) + LPV/r (PIs), against the parasite's sporogonic development (**Figs. 2.3.2A, B, C**). In parallel, informed by our results regarding the most active ARV compound on each parasite developmental stage, we evaluated alternative drug combinations for adults and adolescents where EFV was replaced by either of the NNRTIs ETV or RPV, and alternative drug combinations for children where LPV/r was replaced by either of the best performing PIs in the individual screen, SQV or IDV (**Figs. 2.3.2A, B, C**). Our results showed that a combination of 10 µM of the drugs AZT + 3TC + LPV/r displayed a ~50% inhibitory activity against gametocyte to ookinete transition, whereas the drug combinations TDF + 3TC + ETV and RPV inhibited this process by ∼30% (**Fig. 2.3.2A**). The combinations AZT + 3TC + LPV/r and TDF + 3TC + RPV markedly

inhibited oocyst formation (~90% and 80% inhibition, respectively) and development (~50% and ~40% inhibition, respectively), whereas TDF + 3TC + ETV inhibited oocyst formation by 70% but was ineffective on oocyst development. Finally, the combination of TDF+ 3TC + EFV also resulted in a ~50% reduction of oocyst development but did not impact oocyst formation (**Figs. 2.3.2B, C**).

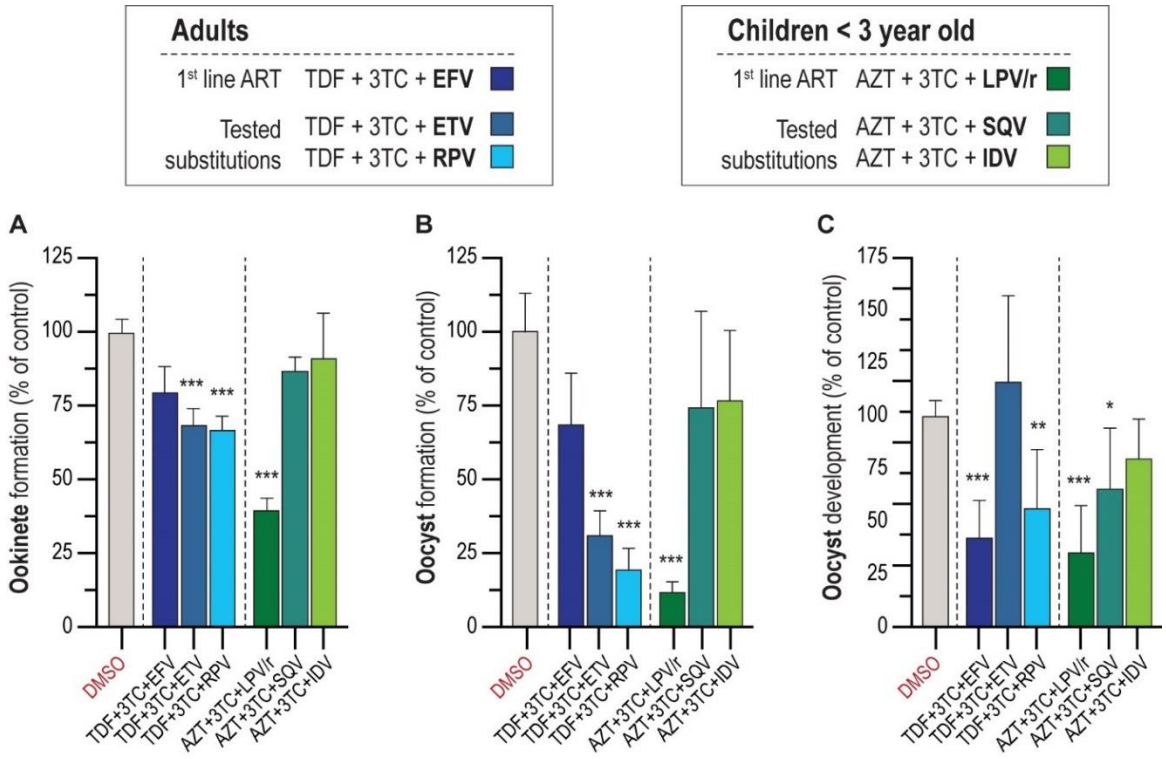

FIGURE 2.3.2 – *In vitro* activity of ARTs on the *Pb* sporogonic stages. (A) Effect of first-line ARTs employed for adults and adolescents, and for children under 3 years old and suggested substitutions on the conversion of zygotes/gametes into ookinetes. **(B)** Activity of first-line ARTs and suggested substitutions on oocyst formation. **(C)** Activity of first-line ARTs and suggested substitutions on oocyst development. All compounds were screened at 10 µM. RLU measurements represented as the percentage of RLU of the DMSO control. Statistically significant differences between control and treated conditions were analyzed using the Kruskal-Wallis test. Results are expressed as the mean +/- SD. N = 3-4. ***, P < 0.001; **, P < 0.01. Abbreviations: ART, antiretroviral therapies; TDF, tenofovir; 3TC, lamivudine; EFV, efavirenz; ETV, etravirine; RPV, rilpivirine; AZT, zidovudine; LPV/r, lopinavir/ritonavir; SQV, saquinavir; IDV, indinavir

EVALUATION OF ART EFFECT ON *Pb* SPOROGONIC STAGES *IN VIVO*

To validate our *in vitro* results, the antiplasmodial effect of the first line drug combinations TDF + 3TC + EFV and AZT + 3TC + LPV/r, were evaluated in an *in vivo* setting (**Fig.**

2.3.3A). Informed by our *in vitro* data, ETV and RPV were also screened in combination with TDF + 3TC. Our results showed that neither of the drug treatments employed had an impact on *Pb* parasitemia and gametocytemia, when compared with vehicle-treated mice (**Figs. 2.3.3B, C, D**).

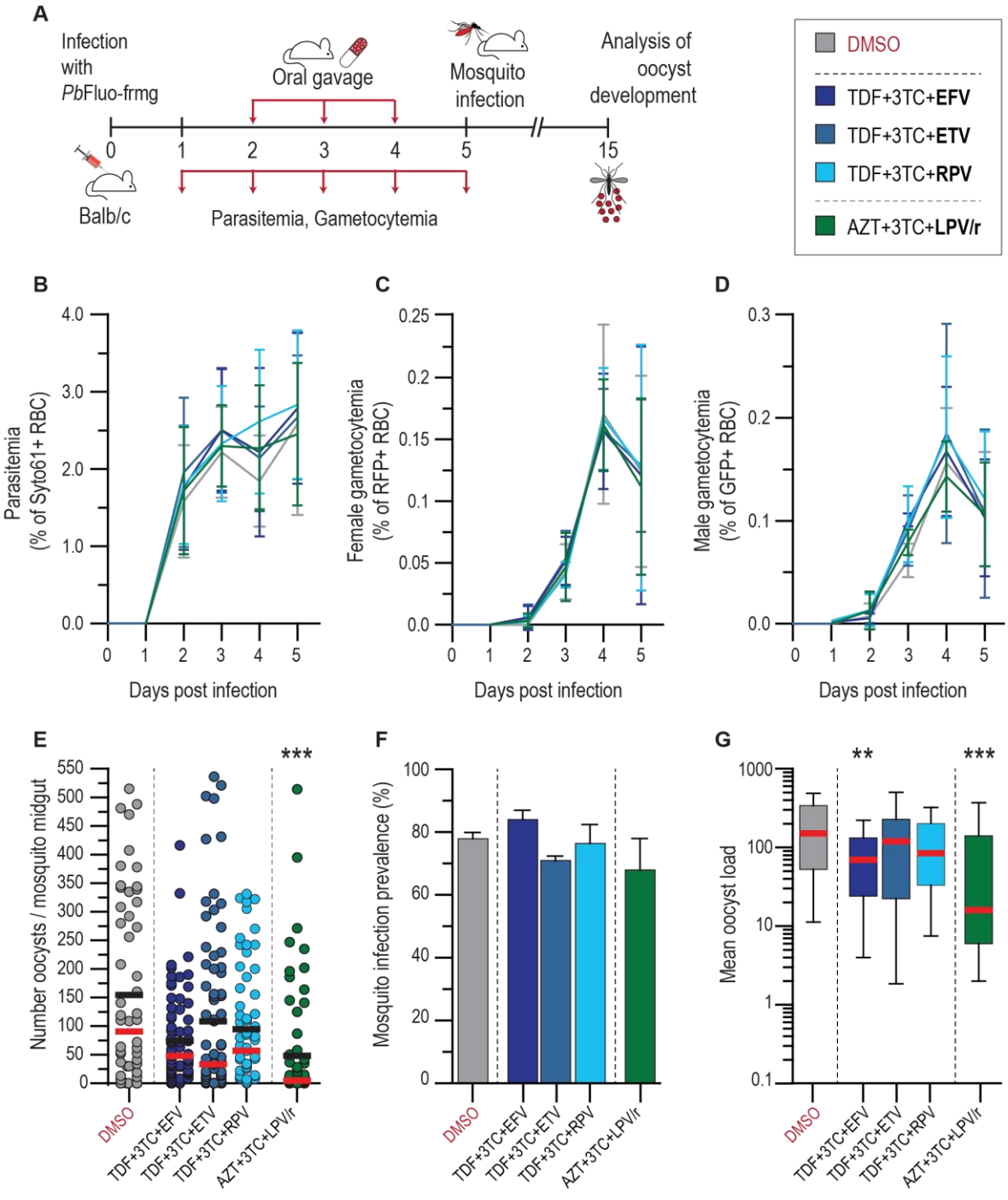

FIGURE 2.3.3 – *In vivo* activity of ART on blood, and transmission stages of *Pb*. (A) Schematics of drug administration and sample collection schedules. **(B, C, D)** Activity of ART and suggested alternative drug

combinations on *Pb* parasitemia **(B)**, female **(C)**, and male **(D)** gametocytemia in mice. Results are expressed as the mean percentage of Syto 61-positive events +/- SD for parasitemia, percentage of RFP^+ events for female gametocytemia and percentage of GFP^+ for male gametocytemia. **(E)** Impact of ART and suggested alternatives on *Pb* mosquito infection measured as oocyst intensity per mosquito. Results are represented individually by number of parasites per mosquito midgut. Horizontal red and black lines represent median and mean respectively. **(F)** Prevalence of oocyst infection in mosquitoes infected with *Pb* expressed as the mean +/- SD. **(G)** Average *Pb* oocyst infection intensity upon ART and suggested alternatives in infected mosquitoes. Box plot represent the median and 25^{th} and 75^{th} percentile. N=2. ***, $P < 0.001$; **, $P < 0.01$. In B, C and D, statistically significant differences between control and treated conditions for blood stage *Pb* development were analyzed using a non-linear regression analysis. In E, Kruskal-Wallis test was used to calculate p-values and determine the significance of parasite numbers. A chi-squared test was used to compare infection prevalence values in F. The detailed statistical analysis is presented in Table 3.2. Abbreviations: ART, antiretroviral therapies; TDF, tenofovir; 3TC, lamivudine; EFV, efavirenz; ETV, etravirine; RPV, rilpivirine; AZT, zidovudine; LPV/r, lopinavir/ritonavir.

Our data further showed that the drug combination AZT + 3TC + LPV/r displayed a strong ~90% impact on median oocyst infection in the mosquitoes, whereas the remaining drug combinations with EFV, ETV and RPV led to smaller reductions on the intensity of infection (**Fig. 2.3.3E and Table 2.3.2**). This reduction does not result from an increase in the number of non-infected mosquitoes, but rather from a reduction in the oocyst load on infected mosquitoes, which is also significant upon treatment with the TDF + 3TC + EFV combination (**Figs. 2.3.3F, G and Table 2.3.2**).

TABLE 2.3.2 – Evaluation of ARV compounds' activity against *Pb* mosquito stages *in vivo*. Additional statistical data of *Pb* mosquito infection results presented in Fig.3E-G.

	DMSO	TDF+3TC+EFV	TDF+3TC+ETV	TDF+3TC+RPV	AZT+3TC+LPV/r
n (with zeros)	54	69	79	61	68
Range (with zeros)	0-515	0-416	0-536	0-331	0-514
Median (with zeros)	90.5	48.0	33.0	57.0	5.0
Percentile (25%-75%)	9.7-310.3	14.0-115.5	0.0-170.0	8.0-160.5	0.0-36.5
% decreased median		47.0%	63.5%	37.0%	94.5%

Mean (with zeros)	155.5	75.0	108.2	95.4	49.5
Std. Deviation	162.6	82.4	145.3	103.5	99.4
% decreased mean		51.8%	30.4%	38.7%	68.2%
Kruskal-Wallis test p-value		ns	ns	ns	<0.0001 (***)
Prevalence	77.9%	84.3%	71.4%	76.4%	68.0%
Std. Deviation	2.0%	3.1%	1.4%	6.85%	10.3%
Chi-square test p-value		ns	ns	ns	ns
n (without zeros)	42	59	56	49	43
Range (without zeros)	6-515	1-416	1-536	3-331	1-514
Median (without zeros)	150.5	70.0	120.0	85.0	16.0
Percentile (25%-75%)	52.7-339.8	24.0-132.0	22.2-227.5	33.0-202.0	6.0-141.0
% decreased median		53.5%	20.3%	43.5%	89.4%
mean (without zeros)	199.9	87.7	152.6	118.7	78.3
Std. Deviation	158.4	82.6	151.8	102.8	116.0
% decreased mean		56.1%	23.7%	40.6%	60.8%
Kruskal-Wallis test p-value		<0.01 (**)	ns	ns	<0.0001 (***)

DISCUSSION

HIV and *Plasmodium* co-infections raise serious concerns in the regions where both organisms overlap geographically (326,575). It has been hypothesized that the interaction between HIV and *Plasmodium* is both synergistic and bidirectional. Thus, infection with HIV might increase the severity of *Plasmodium* infection, while the HIV viral load has been shown to increase during a *Plasmodium* infection (326,596). Numerous studies report the effect of HIV ARV compounds on the different stages of the *Plasmodium* life cycle (579–582,584–589). However, little is known about how ARTs may impair the transmission and mosquito stages of *Plasmodium* parasites.

The results presented here show that several ARV compounds impair various stages of *Plasmodium* sporogonic development *in vitro*, and that the WHO-recommended first-line ARTs employed against HIV have a significant impact on *Plasmodium* infection in the mosquito vector (**Figs. 2.3.3E, G**). However, neither of the current first-line ART, nor the alternative combinations evaluated in this work, inhibited *Pb* asexual and gametocyte stages at clinically relevant concentrations *in vivo* (**Figs. 2.3.3B, C and D**). It has been shown that HIV infection leads to an increase in the production of proinflammatory cytokines tumor necrosis factor (TNF), interleukin (IL)-1β and IL-6, which can be partially reversed by ART (597,598), suggesting a possible indirect effect of ART on *Plasmodium* infection. However, the lack of an impact of ARV compounds on the blood stages of *Plasmodium in vivo* suggests that a different mechanism may be responsible for the effects observed on the parasite's sporogony. Our results suggest that PIs might either affect the parasite's fusing process by impairing exflagellation *in vitro*, as previously suggested for *Pf* (588), or act further downstream of the fertilization process.

Similar to what has previously been shown for the blood stages of *Pf*, our results indicate that PIs display the strongest *in vitro* inhibitory activity against *Pb* transition from gametocytes to ookinetes (**Fig. 2.3.1B**). It has been suggested that PIs act on PMs, a class of *Plasmodium*' aspartic proteases (579,582,593,599). Although HIV aspartic proteases are structurally different from *Plasmodium* PMs, several of the latter have been described in the sexual stages of *Pf*, *Pb* and *Py* (247,600). Our results show that PIs strongly inhibit the formation

and development of oocysts *in vitro* (**Figs. 2.3.1C, D**). Accordingly, the first-line ART containing the PIs LPV/r had the strongest impact on oocyst intensity *in vivo* (**Figs. 2.3.3E, G**). Although, to the best of our knowledge, the effect of ARV compounds on ookinetes has not been previously reported, it is known that PMs IV, VII and X (601,602) are expressed by this parasite stage, thus providing a possible explanation for the effect of PIs on the transformation of ookinetes into oocysts. Moreover, PM VI, whose role is yet undefined, seems crucial for the early oocyst stages of sporogonic development (602). The observed inhibition of oocyst formation and development *in vitro* by PIs (**Figs. 2.3.1C, D**) may suggest that PM VI could be a target of drugs belonging to this class.

We further observed that LPV and RTV had a stronger inhibitory activity on sporogony when tested in combination than individually (**Figs. 3.1B, C, D and 3.2A, B, C**). RTV is currently administered exclusively as a pharmacokinetic enhancer of other PIs due to its effect on cytochrome P450 3A4 isoenzyme (603). However, since this enzyme is absent from the *in vitro* system employed here, the results obtained may be explained by an additive effect of LPV and RTV. We also observed a moderate inhibition of the sporogonic stages of *Pb in vitro* by several NNRTIs (**Figs. 2.3.1A, B, C**). A reverse transcriptase telomerase has been previously identified and characterized in *Pf* (604), and although it differs from HIV's reverse transcriptase, it might contribute to explaining the observed effect of these drugs on the early and late oocyst stage of the parasite's life cycle (**Figs.2.3.1C, D**).

Both the first-line ART and the alternative ARV combinations tested here had similar impacts on oocyst infection in the mosquito (**Figs. 2.3.3E, G**). Our results show that the current first-line ART for children under 3 years old AZT+3TC+LPV/r is the drug combination that most effectively inhibits the sporogonic stages of *Pb in vivo*. Interestingly, previous studies showed that LPV/r inhibits oocyst infection in *Pf* (588) and reduces parasite *Py* liver burden (580) . The impact of current first-line ART for adults and adolescents observed on mosquito infection, which includes EFV, was similar to that of the suggested alternatives employing ETV and RPV. Interestingly, a previous study by Machado *et al.* identified ETV as a stronger inhibitor of the hepatic stages of *Pb* than the current recommended ART with EFV (585).

Our results suggest that both ETV and RPV, as well as other ARV compounds that may have an impact on *Plasmodium* transmission, should be contemplated when considering alternative ARTs in malaria-endemic regions. However, in order to fully ascertain the possible impact of these findings in such settings, additional work is required to assess the impact of these compounds on transmission of *Pf* sporozoites. Furthermore, given the importance of the mosquito microbiota on infection by *Plasmodium*, it would be interesting to replicate the results obtained *in vivo* in mosquitoes depleted of microbiota (605,606). Finally, a further understanding of the mechanism of action of ARV compounds against *Plasmodium* parasites is essential for developing new drugs that might have both ARV and antiplasmodial activity. By identifying the target of HIV PIs on *Plasmodium* parasites, new drugs could be developed that have a stronger impact on *Plasmodium* infection.

3 PROGRESS ON TRANSMISSION-BLOCKING VACCINES

3.1 DEVELOPMENT OF A MULTI-STAGE MALARIA VACCINE

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AUTHOR CONTRIBUTIONS:

Azevedo R., Mendes A.M., and Prudêncio M. designed the experiments. Azevedo R., Carvalho M. and Franke-Fayard B. performed the experiments. Azevedo R. wrote the paper. Mendes A.M. and Prudêncio M. supervised the work and Prudêncio M. revised the text.

INTRODUCTION

In recent years the interest in malaria elimination and eradication has received renewed interest with the World Health Organization (WHO) urging for the acceleration on the progress of reduction of malaria-related morbidity and mortality worldwide (143). Achieving these goals will depend on combining different strategies, among which the development of a vaccine against malaria is fundamental (143). Malaria vaccines can be designed to target different stages of the parasite's life cycle: the liver, blood and the sexual and mosquito stages, with several vaccine candidates against *Plasmodium falciparum* (*Pf*) and *P. vivax* (*Pv*) currently under development [reviewed in (189,214,317)]. So far, results of clinical trials employing such candidates have stayed far from the goal of 75% protective efficacy set by WHO (189,214,317). Nevertheless, RTS, S, a pre-erythrocytic circumsporozoite protein (CSP) based subunit vaccine, despite conferring limited and short lived protection (205,206,607,608), has been recently endorsed by WHO for administration to children living in regions of moderate to high transmission of *Pf* (609). Breakthroughs in the development of a highly effective malaria vaccine have been stalled by the complexity of the *Plasmodium* parasite, which has evolved to evade the host's immune response, varying vaccine target antigens or by modulating the host's immune response (610). Moreover, most vaccine candidates target a single stage of the parasite's life cycle. However, immunity against one life cycle stage, as conferred by RTS,S for the pre-erythrocytic stage of infection, does not confer immunity to other life cycle stages, such as the blood stage. Consequently, if in a following infection of an individual vaccinated with one such vaccine, even a single parasite escapes the host's stage-specific immunity responses, it can then proceed to multiplying into thousands of parasites (317,610).

The most broadly studied and most effective vaccines candidates target the pre-erythrocytic stages of the *Plasmodium* parasite, with the CSP, the most abundant antigen on the surface of sporozoites and crucial in hepatocyte invasion (85,199,202,611), being the most explored in malaria vaccine research (203). Even though the CSP has previously been shown to be a dominant protective antigen (612), sterile immunity could be achieved by immunization of transgenic mice T cell tolerant to the CSP with irradiated sporozoites (612). These results suggest that protection is achieved as a response to the full array of the parasite antigens being

presented to the host's immune system, pointing towards the benefits of immunization with the whole parasite (526).

Whole-sporozoite vaccines (WSV) rely on using live-attenuated human malaria parasites inducing anti-sporozoite or anti-liver stage immunity to prevent the onset of a blood stage *Plasmodium* infection (223), and are the only ones that have been consistently shown to induce sterile immunity in humans (197). They include three different vaccination strategies, employing *Pf* sporozoites: radiation-attenuated sporozoites (RAS), genetically attenuated sporozoites (GAP, early and late- arresting) and chemoprophylaxis with sporozoites (CPS). In GAP immunization approaches essential genes for parasite development are modified or deleted so that the parasite arrests at different time points during the liver stage of infection. Immunization with late-arresting pre-erythrocytic parasites was shown to induce higher protection than with early arresting parasites, exposing the host's immune system to a more diverse cohort of antigens (253,254). Mendes *et al.* have envisaged employing an alternative WSV, using genetically modified rodent *Plasmodium* parasites to present *Pf*CSP, eliciting cross-species immune responses while inducing specific immunity against the inserted target antigen (275). Immunization with this vaccine candidate, termed *Pb*Vac, was shown to induce anti-*Pf*CSP immune responses capable of inhibiting *Pf* hepatocyte infection of liver-humanized mice (275). In a phase I/IIa clinical trial with *Pb*Vac a delay in patency of approximately 2 days was observed as well as an estimated 95% reduction in parasite density (286). However, sterile immunity could not be achieved, warranting for the optimization of *Pb*Vac's efficacy. Considering *Plasmodium*'s antigen variability and the stage-specificity of immune responses elicited by most vaccine candidates, improvements of *Pb*Vac may include the incorporation of highly conserved antigens covering multiple stages of the human parasite's life cycle (317).

*Pf*Rh5 is an erythrocytic stage antigen involved in the process of the parasite red blood cell invasion (302). It is highly conserved across different *Pf* strains and capable of inducing protective immune responses in immunized primates challenged with heterologous *Pf* parasites (307–310). However, no post-immunization boosting was observed upon natural infection, which could be indicative of short-lived protection (310). Nonetheless, due to its degree of conservation, somewhat rare in blood stage antigens, and the possibility of inducing

a synergistic effect when combined with other blood stage antigens (306), the inclusion of *Pf*Rh5 in a multistage vaccine candidate warrants further study.

For many years research was mostly focused on liver and blood stage vaccines, often overlooking the transmission and mosquito stages (181,189,195,214,317,466,613,614), which became a fundamental piece of malaria elimination programs, and will continue to become more relevant with the threat of global warming expecting to increase malaria burden worldwide, and urbanization and deforestation affecting transmission dynamics (143). Transmission-blocking vaccines (TBVs) target surface antigens of the mosquito and sexual stages of *Plasmodium*, preventing parasite transmission from the mosquito to the human host (188,189). When combined with vaccines targeting other life cycle antigens, they have the potential of interrupting the parasite's life cycle and preventing the spread of the disease in the community (466). *Pfs*48/45 is a gametocyte surface antigen involved in male gametocyte fertility (499–501), with low polymorphism across different *Pf* strains (355,508,615–617). Previous studies have shown the potential of antibodies against *Pfs*48/45 to inhibit parasite development in the mosquito vector (510,513). Nevertheless, attempts to produce a *Pfs*48/45 based vaccine have been hindered by the challenges in producing the full length antigen (505). Nevertheless, a vaccine candidate that could incorporate both the highly conserved *Pf*Rh5 and the low-polymorphic *Pfs*48/45 antigens could potentially result in an increase in the protective efficacy of *Pb*Vac.

Therefore, building on the previous knowledge generated by *Pb*Vac, we propose to produce a multi-stage transgenic *Plasmodium* vaccine, using a rodent malaria parasite platform to express *Pf*CSP, a pre-erythrocytic stage antigen, *Pf*Rh5, an erythrocytic stage candidate antigen, and *Pfs*48/45, a sexual stage antigen. Here, we will focus on the characterization of *Pf*CSP and *Pfs*48/45 expression on a *P. berghei* (*Pb*) delivery platform.

MATERIALS AND METHODS

Experimental animals. Male C57BL/6 and BALB/cByJ, 6 to 8 weeks old, from Charles River were housed in the rodent facility of Instituto de Medicina Molecular-João Lobo Antunes (IMM-JLA, Lisboa).

***Plasmodium berghei* (*Pb*) reference lines.** The following *Pb* transgenic lines, generated at Leiden University Medical Center (LUMC) were employed throughout the experimental work:

***Pb*Vac**

*Pb*Vac is a transgenic *Pb* parasite line, in which a *Pfcsp* expression cassette was inserted in the non-essential *230p* locus of the 1596cl1 mother line using the 'gene insertion/marker out' (GIMO) technology. The *Pfcsp* cassette was inserted under the control of the *Pbuis4* promoter by double crossover recombination (275).

676m1cl1

Also termed *Pb*GFP-Luc_{con} (RMgm-29 in www.pberghei.eu), this transgenic parasite line expresses a fusion of GFP (gfp-mu3) and Luciferase (LucIAV) inserted in the non-essential *230p* gene locus of *Pb* by double cross-over recombination. The reporter gene was inserted under the eef1a promoter (278).

3265, 3267, 3279 and 3281

The transgenic lines 3265, 3267, 3279 and 3281 were generated using the GIMO technology to insert the *Pfs48/45* and *Pfrh5* genes in the *Pb*Vac genome in non-essential locus *230p* (*Pfcsp*), sporozoite expressed gene 1 (*S1*) (*Pfrh5*) and silent intergenic locus on chromosome 6 (*SIL6*) (*Pfs48/45*) by double cross-over recombination. Parasite line 3265 expresses the *Pfcsp, Pfs48/45* and *Pfrh5* cassette under the control of the *Pbuis4* promoter. Line 3267 expresses the *Pfcsp* cassette under the control of the *Pbuis4* promoter, the *Pfs48/45* gene under the control of *Pbanka_1349300* promoter and *Pfrh5* under the *Pbuis4* promoter. Line 3279 expresses the *Pfcsp* and *Pfs48/45* genes under the control of the *Pbuis4* promoter the *Pfrh5*

gene under the *Pbanka_1349300* (here on mentioned as *Pb1349300*) promoter. Line 3281 expresses the *Pfcsp* cassette under the control of the *Pbuis4* promoter, and the *Pfs48/45* and *Pfrh5* genes under the *Pbanka_1349300* promoter. Data referencing the different promoters of target genes is summarized in the table below:

TABLE 3.1.1 – Promoters of the different transgenic lines generated at LUMC.

Gene / Line	*Pfcsp*	*Pfs48/45*	*Pfrh5*
3265	*Pbuis4*	*Pbuis4*	*Pbuis4*
3267	*Pbuis4*	*Pbanka_1349300*	*Pbuis4*
3279	*Pbuis4*	*Pbuis4*	*Pbanka_1349300*
3281	*Pbuis4*	*Pbanka_1349300*	*Pbanka_1349300*

Parasite fitness in the mosquito. *Anopheles stephensi* mosquitoes reared in the insectary facility of IMM-JLA, kept at 20 °C and 80% humidity on a 12 h light/dark cycle and fed on 100 mg/ml of glucose (Sigma) and 2mg/ml of para-aminobenzoic acid (PABA, Sigma) solution, were allowed to feed from previously infected BALB/cByJ mice by intraperitoneal injection with 10^7 infected red blood cells (iRBC) of parasite lines *Pb*Vac, 3265, 3267, 3279 and 3281 on day 4 to 5 post-infection. Ten to 12 days later, approximately 20 mosquitoes per transgenic parasite line were dissected and the oocyst density assessed by staining the midguts with 0.025% mercurochrome-H_2O and counting on a Leica DM2500 microscope at 100×. On the 21st day post mosquito infection, sporozoite yield for each of the transgenic lines was assessed by dissecting approximately 50 mosquitoes' salivary glands and counting sporozoites in each of the groups on a Neubauer chamber at 400× magnification.

Parasite gliding motility. Coverslips were placed in 24-well plates (Corning) and coated overnight with mouse anti-*Pf*CSP primary monoclonal antibody 2A10 (1.8 µg/ml) at 4 °C. All antibodies were diluted in 1% bovine serum albumin (BSA, VWR). On the following day, coverslips were washed with phosphate buffered saline (PBS, NZYtech), and 10 000 sporozoites of each transgenic line *Pb*Vac, 3265, 3267, 3279 and 3281 were pipetted per well with 400 µl

of Dulbecco's modified eagle medium (DMEM, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific), 2mM L-glutamine (ThermoFisher Scientific) 100 U/ml penicillin and 100 µg/ml streptomycin (P/S, ThermoFisher Scientific). After centrifugation at 3000 rpm for 5 min, plates were maintained at 37 °C, 5% CO_2 for 30 min to allow sporozoites to glide, followed by medium removal, washing with PBS and overnight fixing with 4% paraformaldehyde (PFA, tebu-bio). Samples were then blocked for 1 h at room temperature (RT) with 1% BSA (VWR)-PBS (NZYTech), incubated for 1 h at RT with mouse anti-*Pf*CSP primary monoclonal antibody 2A10 (9 µg/ml), followed by washing 3× with PBS (NZYTech) and staining with Alexa Fluor 488-conjugated donkey anti-mouse IgG secondary antibody (Abcam, 6.66 µg/ml) for 1 h, at RT. Coverslips were mounted in microscope slides using fluoromount-G (SouthernBiotech), images acquired in Zeiss Axio Observer Z1 fluorescence microscope at 400× magnification and analyzed using the FIJI software (528).

Parasite fitness in the liver – hepatocyte invasion. Fifty thousand sporozoites from *Pb*Vac, 3265, 3267, 3279 and 3281 lines were collected from dissected salivary glands of previously infected *An. Stephensi* mosquitoes, and incubated with an hepatoblastoma derived cell line, HepG2, seeded to a 50 000 cells/well confluency in the previous 24 h in DMEM (ThermoFisher Scientific) complete as described above, supplemented with 50 µg/ml gentamicin (ThermoFisher Scientific) and 0.8 µg/ml fungizone (ThermoFisher Scientific). Samples were centrifuged at 3000 rpm for 5 min, and incubated at 37 °C, 5% CO_2 for 2 to 3 h. Afterwards, samples were washed 3× with PBS and fixed with 4% PFA (tebu-bio) overnight at 4 °C. Hepatocyte invasion was determined according with the protocol by Sinnis *et al.* (618). Briefly, after fixation and washing with PBS (NZYTech), samples were blocked with 1% BSA (VWR)-PBS (NZYTech) for 30 min at RT and subsequently incubated with mouse anti-*Pf*CSP primary monoclonal antibody 2A10 (7.2 µg/ml) and mouse anti-*Pb*CSP primary monoclonal antibody 3D11 (13.8 µg/ml) for 1 h, at RT. All antibodies were diluted in 1% BSA (VWR)-PBS (NZYTech). Samples were then washed 3× with PBS (NZYTech) and incubated with Alexa Fluor 568-conjugated donkey anti-mouse IgG (ThermoFisher Scientific, 6.66 µg/ml) for 1 h, at RT. Samples were again washed 3× with PBS, incubated for 15 min in 100% cold methanol and washed again

with PBS 3×, after which, coverslips were blocked with 1% BSA (VWR)-PBS (NZYTech) for 30 min at RT. Finally, samples were re-incubated with the above-mentioned primary antibodies for 1h at RT, followed by washing and incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG secondary antibody (Abcam, 6.66 µg/ml) for 1 hour at RT. After washing 3× with PBS (NZYTech), samples were mounted using fluoromount-G (Southern Biotech), images acquired using Zeiss Axio Observer Z1 fluorescence microscope at 200× magnification and samples analyzed using the FIJI software (528).

Parasite fitness in the liver – *in vitro* hepatic infectivity and development. Previously seeded HepG2 cells in 24 well plates (Corning), at a confluency of 50 000 cells/well, were infected with 50 000 sporozoites of *Pb*Vac, 3265, 3267, 3279 and 3281 lines 24 h post-seeding in DMEM (ThermoFisher Scientific) complete as described above. After centrifugation at 3000 rpm for 5 min, plates were incubated for 48 h at 37 °C, 5% CO_2, followed by medium removal, washing coverslips with PBS (NZYtech) and incubation with 4% PFA (tebu-bio) overnight at 4 °C. In the following day, PFA was removed, samples washed 3× with PBS (NZYTech), blocked and permeabilized with 0.1% Triton-X100 (Roth)-1% BSA (VWR)-PBS (NZYTech) for 1 h, at RT. Coverslips were stained with mouse anti-*Plasmodium* heat shock protein 70 (HSP70) monoclonal primary antibody 2E6 (60 µg/ml) and goat anti *Pb* upregulated in sporozoite 4 (UIS4, 5 µg/ml, SICGEN) overnight at 4 °C, followed by washing 3× with PBS (NZYTech) and incubation for 1 h at RT with secondary antibodies Alexa Fluor 488-conjugated donkey anti-mouse IgG (6.66 µg/ml, Abcam), Alexa Fluor 568-conjugated donkey anti-goat IgG (6.66 µg/ml ,ThermoFisher Scientific) and of Hoechst-33342 (ThermoFisher Scientific, 5 µg/ml). Coverslips were mounted using fluoromount-G in microscope slides, images acquired in Zeiss Axio Observer Z1 fluorescence microscope at 200× magnification and samples analyzed using the FIJI software (528).

Parasite fitness in the liver – *in vivo* hepatic infectivity and development. Hepatic infectivity and development were determined *in vivo*, by infecting C57BL/6 male mice 6 to 8 weeks-old with 10 000 sporozoites of *Pb*Vac, 3265, 3267, 3279 and 3281 parasite lines by intravenous (iv)

retroorbital injection. Forty-eight hours post-infection (hpi), mice were euthanized, livers excised, and lobes maintained overnight in 4% PFA (tebu-bio) at 4 °C. The following day, 50 µm liver lobe sections were prepared in the Leica VT 1200S vibratome and permeabilized and blocked in 0.1% Triton-X100 (Roth)-3% BSA (VWR)-PBS (NZYTech), for 1 h, at RT. Then, 3 to 4 liver slices per well in a 24 well plate were incubated with goat anti *Pb*UIS4 antibody (5 µg/ml, SICGEN) overnight at 4 °C. Afterwards, liver slices were washed 3× with PBS (NZYTech) and incubated with Alexa Fluor 568-conjugated donkey anti-goat IgG (6.66 µg/ml, ThermoFisher Scientific) for 1 h at RT and Hoechst-33342 (5 µg/ml, ThermoFisher Scientific). Liver slices were mounted in microscope slides using fluoromount-G (Southern Biotech), images acquired in Zeiss Axio Observer Z1 fluorescence microscope at 200× magnification and samples analyzed using the FIJI software (528).

Target antigens expression – mRNA expression *in vitro* and *in vivo*. To detect mRNA expression of the *Pfcsp* and *Pfs48/45* genes, sporozoites, infected HepG2 cells samples at 48 hpi, and infected mouse livers at 48 hpi with transgenic parasite lines *Pb*Vac, 3256, 3267, 3279 and 3281 were collected. Pooled samples of 400 000 sporozoites, 100 000 *in vitro* infected hepatic cells and livers infected with 10 000 sporozoites by iv retroorbital injection were flash frozen in liquid nitrogen, and the two prior ones lysed with TripleXtractor from the directRNA kit (Grisp) and mouse liver lobes homogenized in 3 ml of denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7), 0,5% N-lauroylsarcosine in diethylpyrocarbonate (DEPC)-treated water), supplemented with 0,1M β-mercaptoethanol and lysed in a MiniBeadBeater (BioSpec Products). Gametocyte culture samples were lysed with TripleXtractor and used as positive controls for *Pfs*48/45 mRNA expression. Samples' RNA was quantified in the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and used at 15 to 70 ng/µl for sporozoites and infected hepatocytes *in vitro* and at 200 ng/µl for infected mouse livers. Samples were treated with turbo DNase (ThermoFisher Scientific) according with the manufacturer guidelines. cDNA was synthesized from 1 µg of RNA for infected livers, and from 197 ng to 920 ng for sporozoites and infected hepatic cells, using the NZYTech cDNA synthesis kit and according with the manufacturer's recommendations, employing the

following thermocycler parameters: 25 °C for 10 min, 55 °C for 30 min, and 85 °C for 5 min. The qPCR reaction was performed in a final volume of 10 or 20 µl , depending if reactions were run in 96 or 386 well plates (Applied Biosystems), employing the SYBR Green PCR master mix (NZYTech) and following manufacturer's instructions with the following thermocycling parameters: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, melting stage was done at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 30 s. Primer pairs employed to amplify the target gene transcripts are listed below (Table 3.2). Expression of *Pb18s* rRNA was normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase *(HPRT*) using the comparative Ct method (ΔΔCt) and the target genes *Pfcsp* and *Pfs48/45* expression was normalized to *Pb18s* rRNA. Expression of *Pfs48/45* in gametocyte control samples were normalized to *Pfcsp*. Confirmation of amplicon size of sporozoite qPCR products was performed by gel electrophoresis. Briefly, a 1% agarose (NZYTech) gel pre-stained with gelRed nuclei acid stain (0.02 µl/ml, Biotium) was prepared and the resultant qPCR reaction samples mixed with gel loading dye purple (New England BioLabs) according with manufacturers guidelines. Seven microliters per sample plus the quick-load purple 1 kb plus DNA ladder (New England Bio Labs) were loaded in each gel well and the reaction run at 100 volts for approximately 30 min.

TABLE 3.1.2 – Primer pair list employed in the qPCR analysis.

Gene	Primer sequence (5' – 3')
Pfs48/45	**FW** TCTGCGAAAAAGGCTCAAGTTGC **RV** ACCTACGTTCACGCATATCTGGC
Pfcsp	**FW** GGAAGTTCGTCAAACACAAGG **RV** TGCATTTGGATCAGGATTACC
Pf18s	**FW** GAGCTTGCGGCTTAATTTGA **RV** CATGCATCACCATCCAAGAA
Pb18s	**FW** AAGCATTAAATAAAGCGAATACATCCTTAC **RV** GGAGATTGGTTTTGACGTTTATGTG
HPRT	**FW** TTTGCTGACCTGCTGGATTAC **RV** CAAGACATTCTTTCCAGTTAAAGTTG

Target antigens expression – detection of protein expression by immunofluorescence microscopy. Infected HepG2 cells with sporozoites from each parasite line, as described above, were fixed at 48 hpi with 4% PFA (Sigma) overnight at 4 °C, following which, samples were washed 3× with PBS (NZYTech), permeabilized with 0.1% Triton-X100 (Roth)-PBS (NZYTech) for 10 min and blocked with 1% BSA (VWR)-PBS (NZYTech) for 1 h, at RT. Samples were stained with goat anti *Pb*UIS4 monoclonal primary antibody (5 µg/ml, SICGEN) and with either mouse anti-*Pf*CSP primary monoclonal antibody 2A10 (12 µg/ml) or with rat anti-*Pfs*48/45 primary monoclonal antibody 85RF45.3 (3.33 µg/ml, kindly provided by LUMC) overnight at 4 °C. The next day, samples were washed with PBS (NZYTech) and incubated with secondary antibodies Alexa Fluor 568 (ThermoFisher Scientific)-conjugated donkey anti-goat IgG (6.66 µg/ml), Alexa Fluor 488 (ThermoFisher Scientific)-conjugated donkey anti-rat IgG (6.66 µg/ml) or Alexa Fluor 488 (ThermoFisher Scientific)-conjugated donkey anti-mouse IgG (6.66 µg/ml, Abcam) and Hoechst-33342 (5 µg/ml, ThermoFisher Scientific) for 2 h at RT. Samples were mounted in microscope slides using fluoromount-G (Southern Biotech), images acquired on a Zeiss LSM 880 confocal microscope at 600× magnification and samples analyzed using the FIJI software (528).

Target antigens expression – detection of protein expression by mass spectrometry. Liquid nitrogen-frozen samples of 400 000 sporozoites of lines 3265 and *Pb*Vac, as well as samples of blood stage cultures of *Pf*, were lysed with a solution of 4% (SDS)-0.5 M Tris-HCl-H_2O, pH 7.4 in a 1:1 proportion to the pellet and remaining washing solution. Samples were incubated at 95 °C, 700 rpm for 5 min, cooled at RT and further diluted in 50 mM Tris-HCl to a final concentration of SDS of 1%. Benzonase stock solution (benzonase 19%, 0.024M $MgCl_2$, 39.3 mM Tris-HCl, pH 7.4) was added to the samples at 1:24 dilution followed by incubation for 30 min at 37 °C and 850 rpm. Half of the original amount of benzonase stock solution was added to the samples followed by incubation for 45 min at 37 °C and 850 rpm. Cell lysates were centrifuged at 20 000g for 20 min at RT, the supernatant collected and snap frozen in liquid nitrogen for mass spectrometry analysis. Data regarding detection of samples' target proteins peptide sequence was generated at Cellzome GmbH.

Immunogenicity of target antigens. Humoral responses elicited by target antigens inserted in the transgenic parasite lines was assessed by immunizing groups of 2 to 3 C57BL/6 mice aged 6 to 8 weeks-old with 100 000 sporozoites of all the transgenic parasite lines, in a three-dose immunization regimen on day 0, 7, and 14 post first immunization. Forty-eight h post immunization, administration of a daily dose of 0,7 mg/mouse of chloroquine was initiated, interrupted on the day of immunization and the following day, and terminated on the seventh day post last immunization. On the fifteenth day after the last immunization, blood was collected from immunized mice by heart puncture and serum separated by centrifuging samples at 13000 rpm for 8 min at 4 °C. Nunc MaxiSorp ELISA plates (BioLegend) were coated individually with *Pf*CSP synthetic peptide (ThermoFisher Scientific) consisting of the repeat region of the amino acid sequence (NANP)4NVDPC at 2.5 μg/ml and *Pfs*48/45 6C region recombinant protein at 2.5 μg/ml (kindly provided by Teun Bousema) overnight at 4 °C. On the following day, plates were washed 3× with PBS (NZYTech) and blocked with 100 μl of 5% milk-0.05% Tween 20 (Promega)-PBS (PBST, NZYTech) for 1 h, at RT. Plates were washed with 0.1% PBST and the standard curve initial dilution of each target antibody prepared in 1% milk-0.05% PBST with anti-*Pf*CSP primary monoclonal antibody 2A10 (36.08 μg/ml) or serum from immunized mice with R0.6C, a chimera of the C-terminal 6-Cys domain of *Pfs*48/45 (6C) and the Ro region of the asexual stage Glutamate Rich Protein expressed in *Lactococcus lactis*. Samples were pipetted in 2-fold dilutions from 1:50 to 1:25600. In parallel, serum from immunized mice with the transgenic parasite lines was pipetted in 4 subsequent 2-fold serial dilutions starting at 1:70 for anti-*Pf*CSP and 1:50 for anti-*Pfs*48/45 antibody detection, and incubated for 3 h at RT. Afterwards, plates were washed 3× with 0.01% PBST followed by incubation with 50 μl per well of secondary antibody goat anti-mouse IgG horseradish peroxidase (0.08 μg/ml, Santa Cruz Biotech) for 30 min. Plates were again washed 3× with 0.1% PBST, followed by washing 3× with PBS. Tetra-methyl-benzidine (TMB) solution was prepared according to manufacturer's instructions and 100 μl per well added for 10 min at RT, after which the reaction was stopped with 50 μl per well of H_2SO_4 (2N). Fluorescence was measured at 450 nm using the Microplate Reader TECAN Infinite M200.

Statistical analysis. Statistically significant differences between control and new transgenic lines were analyzed with the Kruskal-Wallis test. Assessment of statistically significant differences between gliding motility groups in the control and transgenic lines were analyzed with the mixed effects model. Differences were considered not to be significant at a *P* value of >0.05. Under this value, all differences were considered to be statistically significant. All statistical tests were performed by GraphPad Prism (version 8.00; GraphPad Software, La Jolla, USA).

Ethics statement. All work with laboratory animals was performed in accordance with national and European regulations. All procedures followed the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and protocols were approved by the animal experimentation ethics committee of the IMM-JLA (AWB_2021_07_MP_Strategy4Malaria). All animal experiments performed at the Leiden University Medical Center were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042, DEC 12043). The Dutch Experiments on Animal Act was established under European guidelines (EU directive no. 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes).

RESULTS

FITNESS OF CHIMERIC PARASITE LINES

*Pb*Vac, the precursor of the newly generated transgenic parasite lines, has been previously characterized by Mendes *et. al*, who showed that the genetic modifications necessary for the vaccine candidate construction had no overall impact on the parasite's fitness, relative to a wild-type *Pb* control (275). However, further incorporation of new antigens could still impair parasite development in the vector and/or mammalian host. Thus, we began by evaluating the sporogonic development of transgenic lines 3265, 3267, 3279 and 3281, compared to that of the parental line *Pb*Vac, followed by the assessment of the parasites' ability to infect, and develop inside hepatic cells *in vitro* and *in vivo*.

No significant impact of the genetic modifications was observed on the production of oocysts and sporozoites by infected mosquitoes with lines 3265, 3267, 3279 and 3281, when compared to the *Pb*Vac control (**Fig. 3.1.1A, B**).

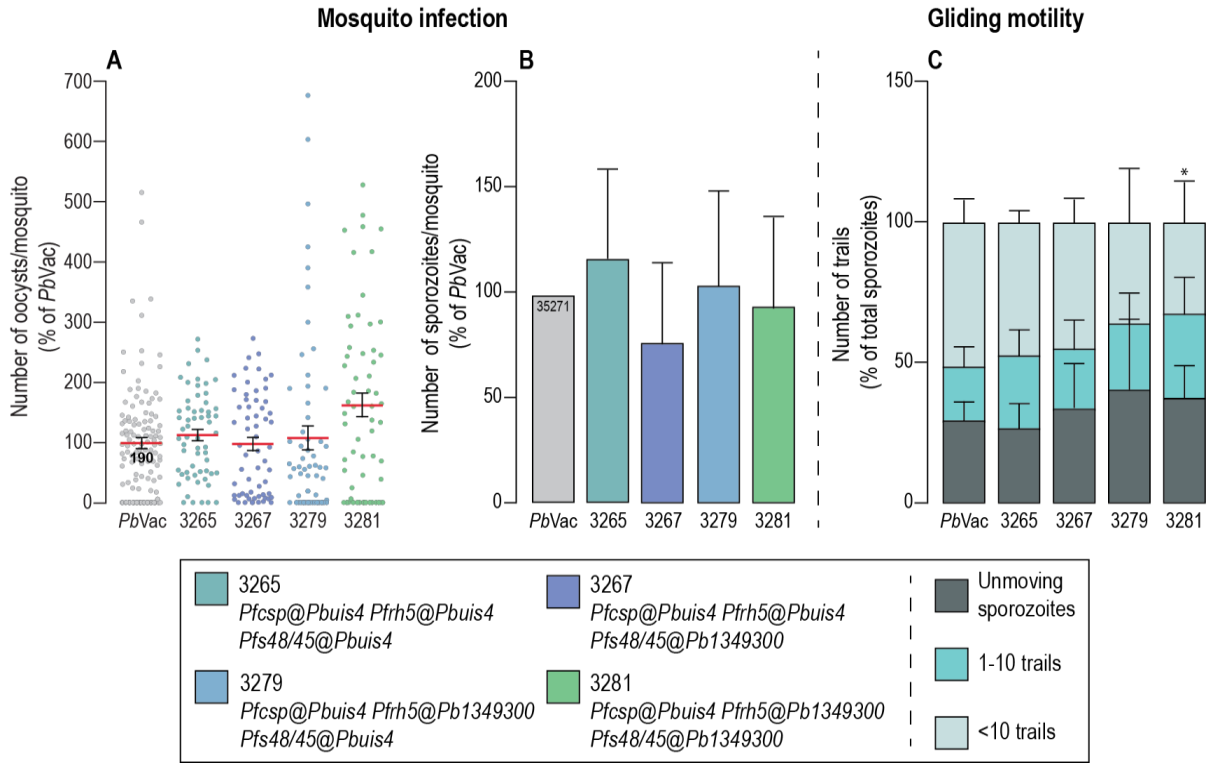

FIGURE 3.1.1 – Chimeric parasite lines fitness: oocysts and sporozoites. (A) Oocyst density in mosquitoes infected with lines *Pb*Vac, 3265, 3267, 3279 and 3281. Absolute average oocyst production of *Pb*Vac-infected mosquitoes' is presented as an indicative of infection performance (190 oocysts per mosquito). Results are

presented as the mean ± the standard error of the mean (SEM). N=3-6, n=60-120 **(B)** Salivary glands sporozoites' production in infected mosquitoes with chimeric lines *Pb*Vac, 3265, 3267, 3279 and 3281. Absolute average oocyst production of *Pb*Vac-infected mosquitoes' is presented as an indicative of infection performance (35271 sporozoites per mosquito). Results are presented as the mean ± the standard deviation, SD. N=7, n=20-70 **(C)** Gliding motility of sporozoites of each transgenic line is categorized according with the number of gliding trails: unmoving sporozoites, 1 to 10 trails and more than 10 trails. Results are presented as the mean ± the SD.*p< 0.05 N=3-6, n=180-360

Upon reaching the bloodstream, sporozoites use their gliding motility to migrate through cells and invade their final target cell - the hepatocyte. Thus, we characterized the transgenic sporozoites' gliding motility, showing that parasite line 3281, expressing *Pfcsp* under the *Pbuis4* promoter, and *Pfrh5* and *Pfs48/45* under the *Pb1349300* promoter, glides at a lower frequency than the *Pb*Vac control (**Fig. 3.1.1C**). The gliding motility of the remaining transgenic lines 3265, 3267 and 3279 was not significantly different from that of *Pb*Vac (**Fig. 3.1.1C**).

We then sought to characterize the parasite's fitness throughout the infection in the liver, beginning by determining their ability to invade HepG2 cells *in vitro*. We showed that the number of sporozoites found inside HepG2-infected cells with lines 3265 and 3267 is significantly lower than that found in *Pb*Vac-infected cells (**Fig. 3.1.2A**). The parasites that successfully invade hepatic cells will develop into exoerythrocytic forms (EEFs). Thus, we evaluated the size and infection rate of HepG2 cells and hepatocytes of previously infected mice at 48 hpi, showing that, aside from transgenic line 3265, in which a significant reduction was observed, no impairment was observed in parasite size *in vitro* (**Fig. 3.1.2B**). Contrarily, a significant reduction in size could be registered *in vivo*, in mice infected with all new transgenic lines when compared to *Pb*Vac (**Fig. 3.1.2D**). Regarding the hepatic infectivity of transgenic lines *in vitro*, no impairment was observed, while an impairment in *in vivo* infectivity was observed for lines 3265, 3267, 3279 and 3281 (**Fig. 3.1.2C, E**).

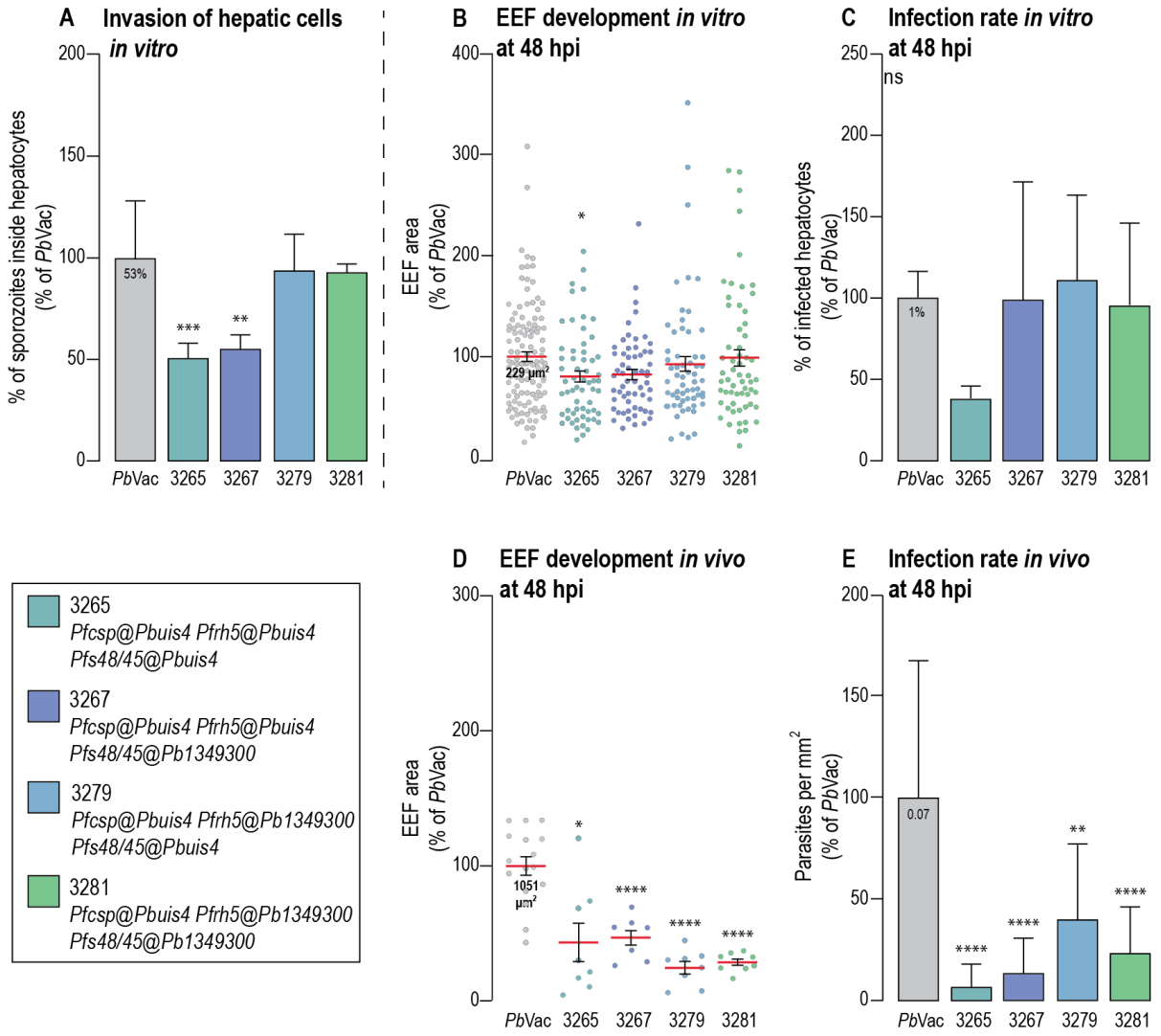

FIGURE 3.1.2 – Fitness of chimeric parasite lines in hepatic cells. (A) Invasion of hepatic cells *in vitro* with lines 3265, 3267, 3279 and 3281. Average invasion of HepG2 cells *in vitro* with *Pb*Vac is presented without normalization (53%). Results are presented as the mean ± the SD.**p≤ 0.01, ***p≤ 0.001 N=2-6, n=60 **(B)** Parasite hepatic development *in vitro* at 48 hours post-infection (hpi) with chimeric lines. Average absolute parasite size is shown for *Pb*Vac (229 µm^2). Results are presented as the mean ± the SEM. N=3-6, n=60 **(C)** Infectivity of transgenic lines *in vitro* at 48 hpi in HepG2 cells. Average percentage of infected hepatocytes is shown for *Pb*Vac (1%). Results are presented as the mean ± the SD.*p<0.05, N=3-6, n=180-360 **(D)** Parasite hepatic development in infected mice 48 hpi with all chimeric lines. Absolute average parasite size of *Pb*Vac (1051 µm^2) is presented. Results are presented as the mean ± the SEM. ** p≤ 0.01, *** p≤ 0.001 N=2-4, n=8-16 **(E)** Infectivity of transgenic lines *in vivo* at 48 hpi in mouse hepatocytes. Absolute average ratio of parasites per mm^2 of *Pb*Vac (0.07) is presented. Results are presented as the mean ± the SD.* p<0.05, *** p≤ 0.001, **** p≤ 0.0001. N=2-4, n=16-32

We can conclude that the overall fitness of the new chimeric lines in the mosquito is not impaired, except for the gliding motility of parasite line 3281. However, the *in vivo* hepatic infectivity with the new transgenic lines is reduced when compared to that of *Pb*Vac.

TARGET ANTIGENS mRNA EXPRESSION

We then proceeded to evaluate the target antigens' mRNA expression *in vitro* and *in vivo*. Although these transgenic lines incorporate three different antigens - *Pfcsp*, *Pfs48/45* and *Pfrh5* - only the expression of the two former ones will be addressed throughout this work. We analyzed the expression of the *Pfs48/45* and *Pfcsp* genes at the mRNA level in sporozoites, infected HepG2 cells and in infected mouse hepatic cells at 48 hpi. In sporozoites, *Pfs48/45* and *Pfcsp* expression was identified in the transgenic lines 3265, 3267, 3279 and 3281. The highest mRNA expression of the target genes was observed for lines 3267 and 3281, in which *Pfs48/45* was inserted under the *PB1349300* promoter (**Fig. 3.1.3A, B**). We confirmed the correct size of the *Pfs48/45* qPCR products of all the new chimeric lines sporozoites by gel electrophoresis, running them in parallel with the qPCR product of *Pf* gametocytes (**Fig. 3.1.3C**).

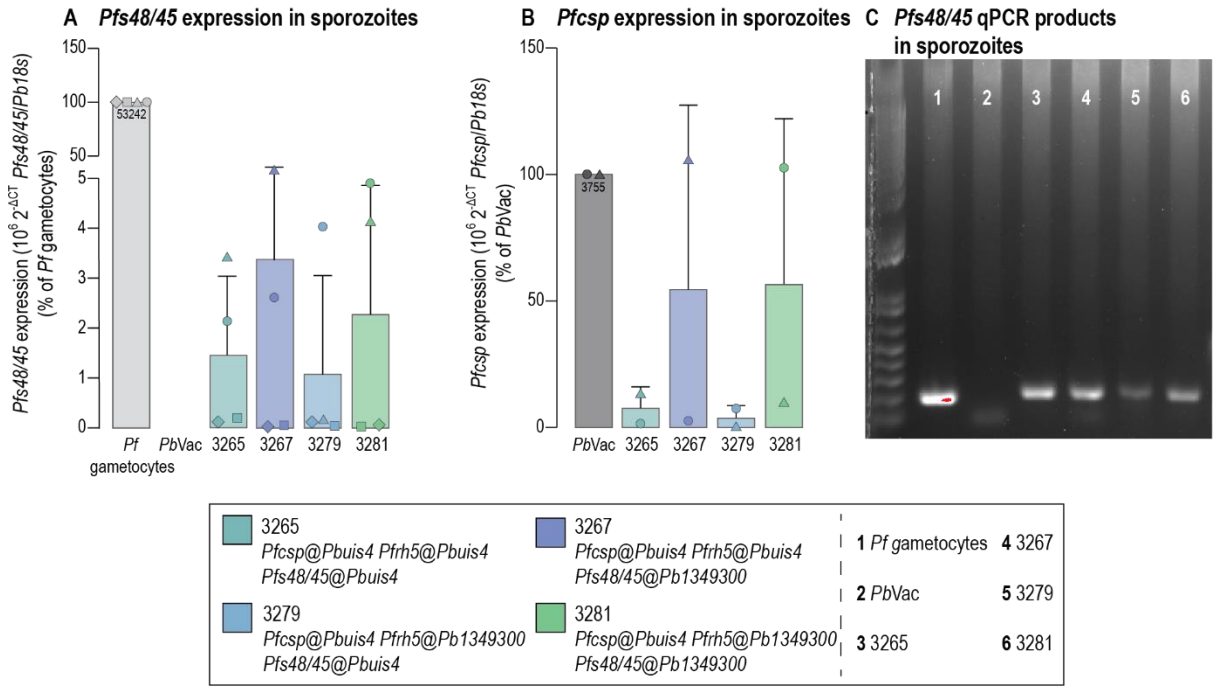

FIGURE 3.1.3 – *Pfs48/45* and *Pfcsp* mRNA expression in sporozoites. (A) *Pfs48/45* mRNA expression in sporozoites of all chimeric lines. Absolute average level of *Pfs48/45* expression is presented as $10^6\ 2^{-\Delta CT}$

Pfs48/45/Pb18s for *Pf* gametocytes sample (53242). Results are presented as the mean ± the SD. N=4, n=4 **(B)** *Pfcsp* mRNA expression in sporozoites of all chimeric lines. Absolute average *Pfcsp* expression is presented as 10^6 $2^{-\Delta CT}$ *Pfcsp/Pb18s* for *Pb*Vac sporozoites sample (3755). Results are presented as the mean ± the SD. N=2,n=2 **(C)** *Pfs48/45* qPCR amplicons of sporozoites of all new chimeric lines and *Pf* gametocytes run by gel electrophoresis.

Both target genes could be identified in HepG2-infected cells, with lines 3265 presenting the highest mRNA expression of both the *Pfs48/45* and *Pfcsp* genes (**Fig. 3.1.4A, B**). Lines 3267 and 3279 had the lowest *Pfs*48/45 and *Pfcsp* mRNA expression, respectively (**Fig. 3.1.4A, B**). Regarding infection, the highest *Pb18s* expression was detected in cells infected with line 3279 (**Fig. 3.1.4, C**).

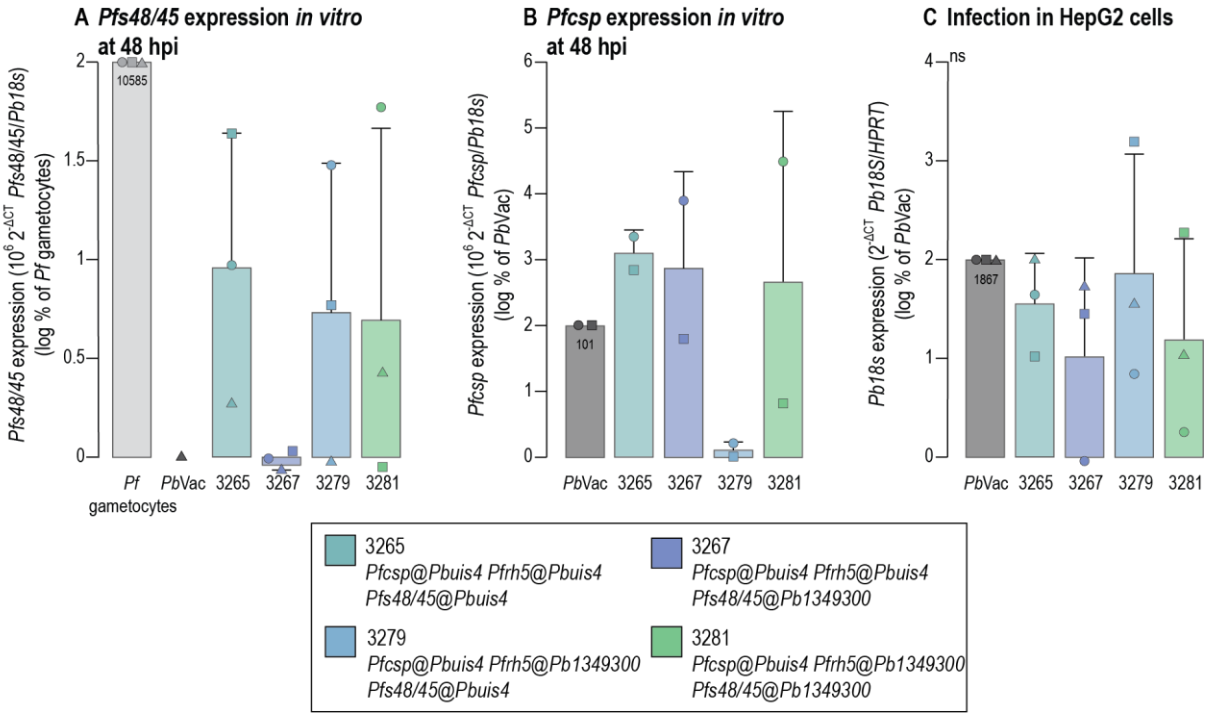

FIGURE 3.1.4 - *Pfs48/45*, *Pfcsp* and *Pb18S* mRNA/rRNA expression in infected HepG2 cells at 48 hpi. (A) *Pfs48/45* mRNA expression in infected cells with all chimeric lines. Absolute average *Pfs48/45* expression is presented as 10^6 $2^{-\Delta CT}$ *Pfs48/45/Pb18s* for *Pf* gametocytes sample (10585). Results are presented as the mean ± the SD. N=3,n=3 **(B)** *Pfcsp* mRNA expression in infected cells with all chimeric lines. Absolute average *Pfcsp* expression is presented as 10^6 $2^{-\Delta CT}$ *Pfcsp/Pb18s* for *Pb*Vac infected cells sample (101). Results are presented as the mean ± the SD. N=2, n=2 **(C)** *Pb18S* rRNA expression in infected HepG2 cells with all transgenic lines. Absolute average *Pb18S* expression is presented for reference purposes as $2^{-\Delta CT}$ *Pb18s/HPRT* for *Pb*Vac. Results are presented as the mean ± the SD. N=3, n=3.

After assessing mRNA expression of the target genes *in vitro*, we sought to understand mRNA/rRNA expression of *Pfs48/45*, *Pfcsp* and *Pb18s in vivo*. To this end, livers of infected mice were collected at 48 hpi, and analyzed by qRT-PCR. This analysis revealed expression of *Pfs48/45* mRNA, albeit at a relatively low level in transgenic line 3279 (**Fig. 3.1.5A**). *Pfcsp* expression could only be detected in lines 3279 and 3281 and in the *Pb*Vac control (**Fig. 3.1.5B**).

We confirmed mRNA *in vitro* expression of the target genes, both in sporozoites and infected HepG2 cells at 48 hpi, while *in vivo, Pfcsp* expression could only be detected in lines 3279 and 3281.

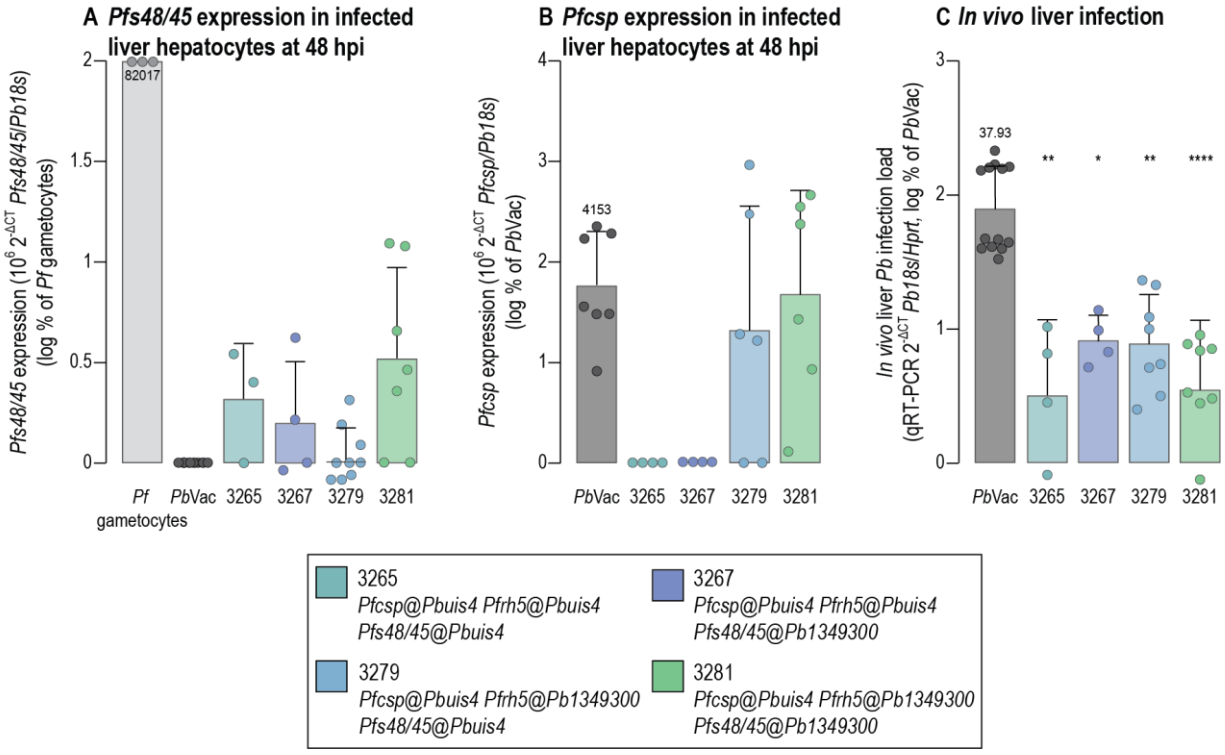

FIGURE 3.1.5 – *Pfs48/45*, *Pfcsp* and *Pb18s* mRNA/rRNA expression in infected hepatocytes *in vivo* at 48 hpi. (A) *Pfs48/45* mRNA expression in infected hepatocytes *in vivo* with all chimeric lines. Absolute average *Pfs48/45* expression is presented as $10^6\ 2^{-\Delta CT}$ *Pfs48/45/Pb18s* for *Pf* gametocytes sample (82017). Results are presented as the mean ± SD. N=2-4,n=3-13 **(B)** *Pfcsp* mRNA expression in infected hepatocytes *in vivo* with all chimeric lines. Absolute average *Pfcsp* expression is presented as $10^6\ 2^{-\Delta CT}$ *Pfcsp/Pb18s* for *Pb*Vac infected cells sample. Results are presented as the mean ± SD. N=2-4, n=4-13 **(C)** *Pb18s* rRNA expression in infected hepatocytes *in vivo* with all transgenic lines. Absolute average *Pb18s* expression is presented for reference purposes as $2^{-\Delta CT}$ *Pb18s/Hprt* for *Pb*Vac. Results are presented as the mean ± SD. N=2-4, n=4-13.

TARGET ANTIGENS PROTEIN EXPRESSION

To understand if the mRNA expression of the *Pfs48/45* and *Pfcsp* genes translated into protein expression, HepG2 cells infected with all the above-mentioned transgenic lines cells, plus a *Pb*GFPLuc$_{con}$ negative control of *Pf*CSP expression were analyzed by immunofluorescence microscopy at 48 hpi. *Pfs*48/45 protein expression was observed in lines 3265, 3267, 3279 and 3281, while, as expected, being absent from the *Pb*Vac negative control (**Fig. 3.1.6**).

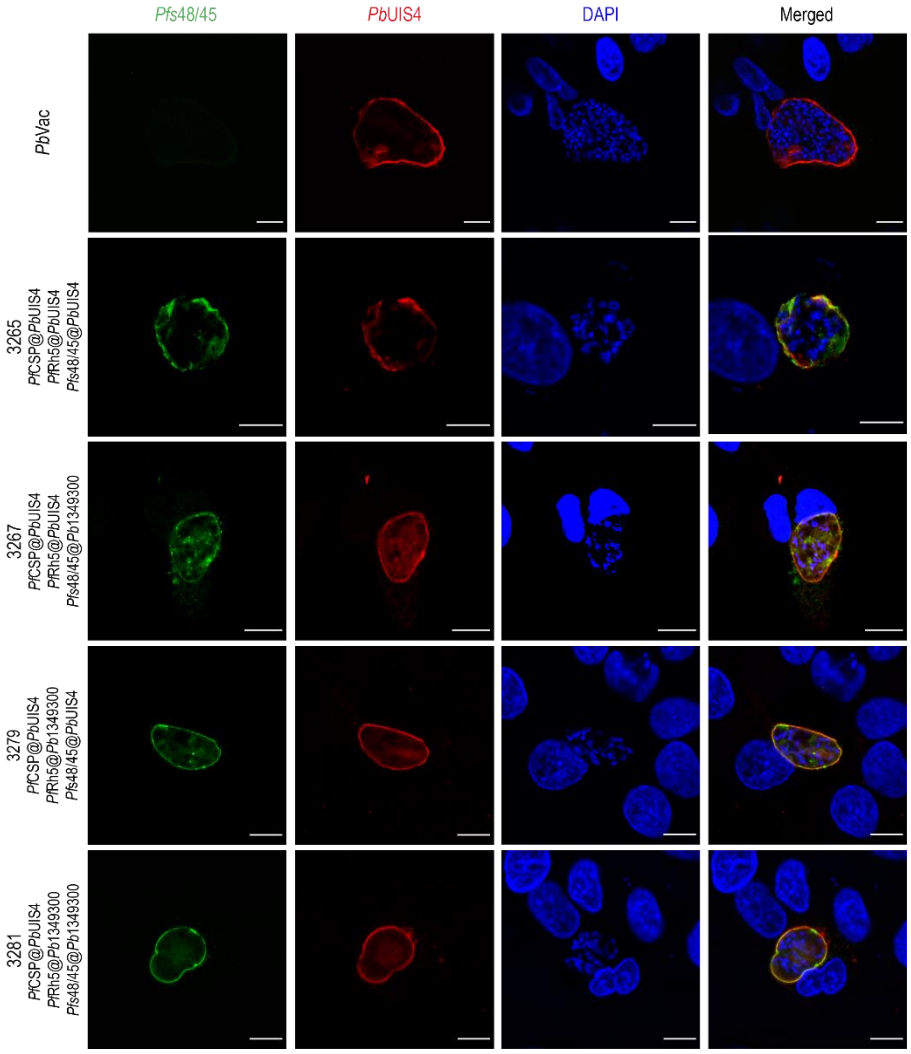

FIGURE 3.1.6 – *Pfs*48/45 protein expression in infected HepG2 cells at 48 hpi with lines *Pb*Vac, 3265, 3267, 3279 and 3281. (Green, *Pfs*48/45; red, *Pb*UIS4; blue, DAPI). Scale bar, 10 µm.

*Pf*CSP expression was observed in lines 3265, 3267, 3279 and 3281, as well as in the *Pb*Vac positive control (**Fig. 3.1.7**). No *Pf*CSP expression was observed in the *Pb*GFP-Luc$_{con}$-infected cells used as negative control (**Fig. 3.1.7**).

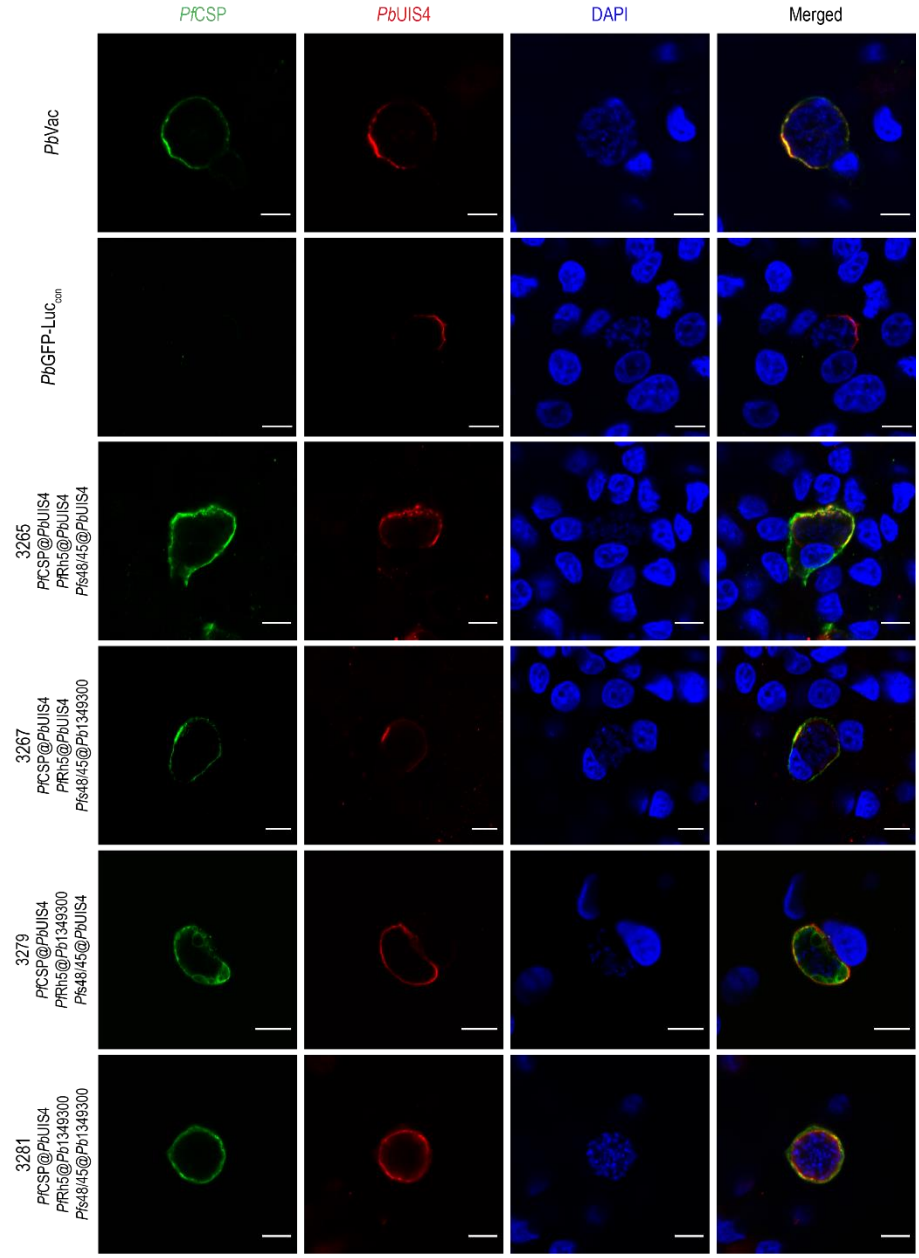

FIGURE 3.1.7 – *Pf*CSP protein expression in infected HepG2 cells at 48 hpi with lines *Pb*Vac, *Pb*GFP-Luc$_{con}$, 3265, 3267, 3279 and 3281. (Green, *Pf*CSP; red, *Pb*UIS4; blue, DAPI). Scale bar, 10 µm.

In parallel, samples of lysed sporozoites of transgenic line 3265 and controls were analyzed by mass spectrometry at Cellzome GmbH to identify peptide sequences of the target proteins. This analysis showed that *Pf*CSP and *Pfs*48/45 could be identified not only in the control samples of *Pb*Vac sporozoites and *Pf* blood stage cultures, respectively, but also in the sporozoite samples of parasite line 3265 (**Table 3.1.3**).

TABLE 3.1.3 – Identification of peptide sequences of *Pf*CSP and *Pfs*48/45 in sporozoite samples by mass spectrometry analysis.

Sample	Proteins identified	Total PSM	MS1 intensity
3265 sporozoites	*Pf*CSP, *Pfs*48/45	1, 1	6.6, 5.6
3265 sporozoites	*Pf*CSP, *Pfs*48/45	2, 1	6.7, 5.8
3265 activated sporozoites	*Pf*CSP, *Pfs*48/45	1, 1	6.7, 5.9
*Pb*Vac sporozoites	*Pf*CSP	2	6.7
Pf blood stage cultures	*Pfs*48/45	1	6.2

TARGET ANTIGENS IMMUNOGENECITY

Finally, to understand if the new transgenic parasite lines could induce an immune response against the target antigens *Pf*CSP and *Pfs*48/45, mice were immunized with a three-dose regimen of 100 000 sporozoites of lines 3265, 3267, 3279, 3281, as well as with the *Pb*Vac and *Pb*GFP-Luc_{con} negative controls for antibody production against *Pfs*48/45, and the *Pb*GFP-Luc_{con} negative control for antibody production against *Pf*CSP. Our results show that immunization with the new chimeric lines can elicit an immune response against *Pf*CSP to an extent similar to *Pb*Vac, with no differences observed in the anti-*Pf*CSP antibody titers in the serum of immunized animals (**Fig. 3.1.8A, B**). Total anti-*Pfs*48/45 IgGs in the serum of immunized mice could not be quantified due to unknown anti-*Pfs*48/45 antibody titers on anti-R0.6C serum employed in the standard curve dilutions. Therefore, results are presented as absolute absorbance values and were analyzed based on the comparison to the negative controls, showing that immunization with the different transgenic lines elicited different immune responses (**Fig. 3.1.8**). In serum from mice immunized with transgenic lines 3265 and 3267 no anti-*Pfs*48/45 antibodies could be detected when compared to the negative controls **(Fig. 3.1.8C**). However, immunization with lines 3279 and 3281 elicited an immune response against *Pfs*48/45, albeit low, when compared to anti-*Pfs*48/45 background levels detected in the serum of negative control immunized animals (**Fig. 3.1.8D**). Even so, immune responses against *Pfs*48/45 were not homogenous in the serum of 3279- and 3281-immunized animals,

with the absorbance values of some samples being similar to the background levels observed in the negative controls (**Fig. 3.1.8D**).

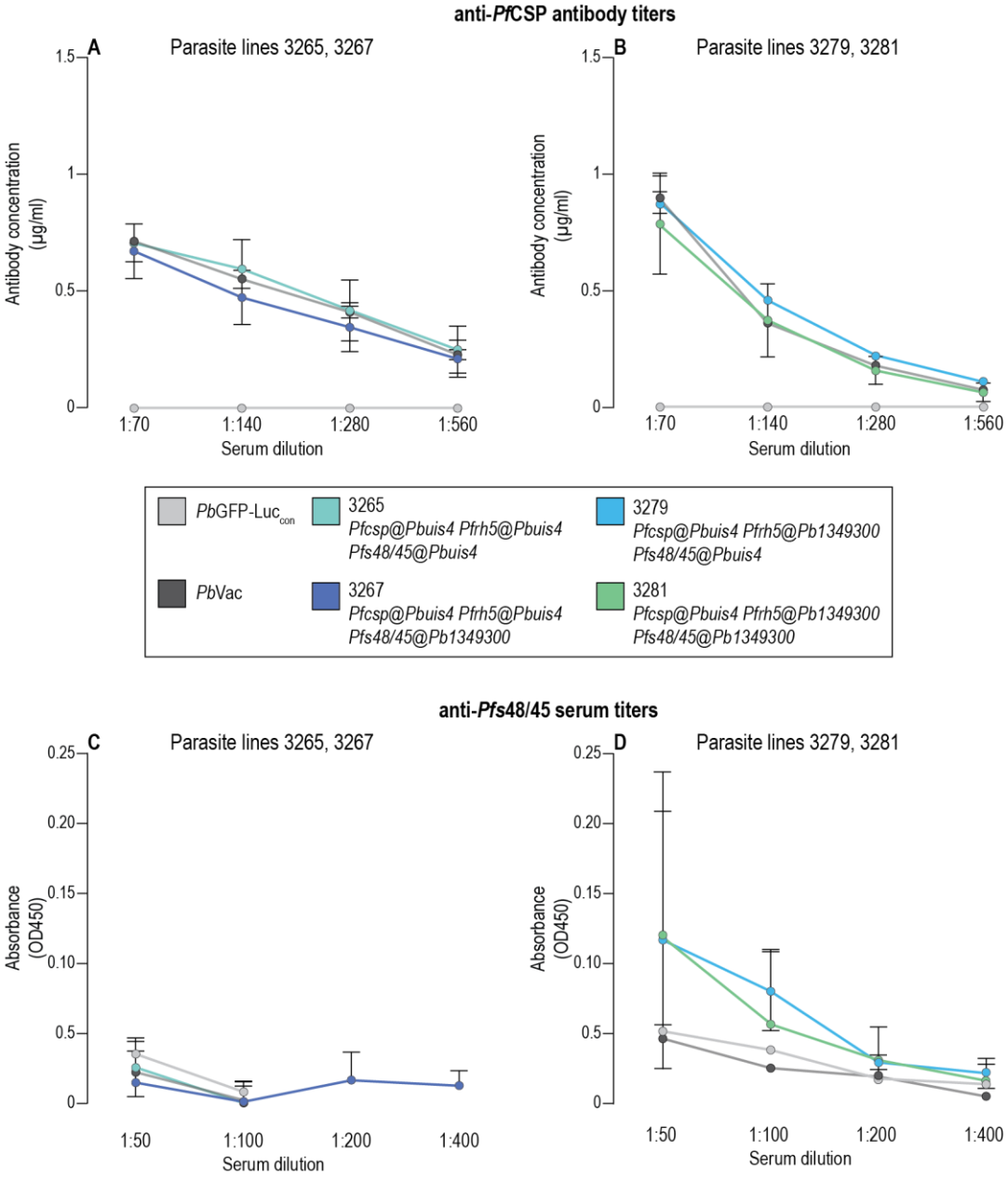

Figure 3.1.8 – Immune responses in mice immunized with transgenic parasite lines. (A) Anti-*Pf*CSP antibody titers in serum of mice immunized with transgenic lines *Pb*Vac, PbGFP-Luc$_{con}$, 3265 and 3267. **(B)** Anti-*Pf*CSP antibody titers in serum of mice immunized with transgenic lines *Pb*Vac, PbGFP-Luc$_{con}$, 3279 and 3281. **(C)** Anti-*Pfs*48/45 antibody titers in serum of mice immunized with transgenic lines *Pb*Vac, PbGFP-Luc$_{con}$, 3265 and 3267. **(D)** Anti-*Pfs*48/45 antibody titers in serum of mice immunized with transgenic lines *Pb*Vac, PbGFP-Luc$_{con}$, 3279 and 3281. All results are presented as the mean ± the SD. N=1, n=2-3

Collectively, our results show that multi-stage chimeric *Pb* parasite lines display partial impairment of overall parasite fitness, which can influence the degree of immune responses generated. Even so, this *Pb* platform can successfully allow the integration of multiple genes from different stages of the *Pf* life cycle, as well as the expression of these heterologous antigens.

DISCUSSION

The development of malaria vaccines has been limited by the intrinsic biological complexity of the *Plasmodium* parasite (610), which undergoes several morphological changes and expresses stage-specific antigens during its life cycle, thereby eliciting stage-specific immunity upon host infection (619). In addition, *Plasmodium* parasites have evolved to escape the host's immunity, varying their antigens or modulating the host's immune responses (610). Therefore, a multi-stage malaria vaccine would likely be more efficacious than a single stage malaria vaccine, which has struggled to achieve the goal of 75% protective efficacy defined by WHO, due to the induction of multiple layers of immunity (143,610). Currently, the most advanced malaria vaccine, RTS,S, which has limited efficacy (approximately 30%) (205), focuses on one single pre-erythrocytic stage antigen, the CSP (204).

Although *Pb*Vac is not the first whole-sporozoite vaccine to be developed, nor the first transgenic rodent malaria parasite line expressing human-infective *Plasmodium* genes, it has been the first chimeric vaccine employing *Pb* as a delivery platform of *Pf* antigens to elicit a protective immune response against malaria (275,620–622). Like other WSV, *Pb*Vac has the advantage of expressing full length target proteins and induce a wider range of antibody responses than sub-unit vaccines, also due to the ability of the *Pb* backbone to elicit cross-species immune responses against *Pf* (275). While promising, the results from a clinical trial showed that sterile immunity could not be achieved by immunization of humans with a suboptimal dose of *Pb*Vac (286). Nevertheless, vaccination with *Pb*Vac led to a delay in patency and an estimated 95% reduction in *Pf* liver burden (286). Therefore, we aimed to enhance the immunogenic potential of *Pb*Vac by adding other life cycle stage antigens besides the pre-erythrocytic stage CSP. To this end, we addressed the potential of incorporating a TB stage protein, *Pfs*48/45, in the construction a multi-stage malaria vaccine capable of inducing immune responses that eliminate the parasite at the pre-erythrocytic stage but also prevent the transmission of the parasite to the host by eliminating its sexual stages from the mosquito.

We began by evaluating sporogonic development of the newly generated chimeric lines followed by their *in vitro* and *in vivo* hepatic development. Although the development of the different parasite lines in the mosquito was not impaired, mixed results were obtained

regarding their hepatic infection. We showed that the ability of transgenic lines 3265 and 3267 to invade hepatic cells *in vitro* was compromised, although this did not seem to affect both the *in vitro* hepatic infectivity and parasite size of line 3267. However, a two-dimensional HepG2 culture model does not completely mimic an *in vivo Plasmodium* hepatic infection. The liver is a complex organ with a three-dimensional architecture composed of several interacting cell types, including hepatocytes, which cannot be completely replicated in an HepG2 monolayer culture system (623). Moreover, although HepG2 cells retain some features of differentiated hepatocytes, they lack crucial metabolic enzymes of mature hepatocytes, which makes them poorly suited to fully understand the dynamics of an *in vivo Plasmodium* infection (624,625). Therefore, we replicated this analysis *in vivo*, showing that infectivity and parasite size of all new transgenic lines was reduced when compared to that of *Pb*Vac. These differences may be explained by the small sample size in the *in vivo* experimental groups, which decreases the power of the statistical analysis (626), but could also be justified by the low reproducibility between the monolayer 2D model employed here and a complex 3D model such as the liver.

After determining the fitness of the new chimeric lines in mosquitoes and mammalian host's livers, it was crucial to assess the mRNA expression of the *Pfcsp* and *Pfs48/45* genes in sporozoites and infected hepatic cells. Although mRNA of the target genes could be identified in sporozoite and infected HepG2 samples, the level of expression of each target genes on both sample types was not similar. *In vivo*, in liver samples infected with lines 3265 and 3267 *Pfcsp* could not be detected. Since we previously established that *Pfcsp* mRNA was expressed in HepG2 cells infected with these parasite lines, our results suggest that the combination of low hepatic parasite size and infectivity *in vivo* may affect the detection of *Pfcsp* mRNA expression, which may be below the qPCR detection limit. The addition of a pre-amplification step before the qPCR reaction might increase the number of copies of *Pfcsp* mRNA, facilitating its detection (627). Alternatively, new primers for the *Pfcsp* gene could be designed to increase detection sensitivity. Since different housekeeping genes had to be employed in the detection of *Pfs*48/45 in control and test samples, differences in the target genes expression are not comparable to those of the control. Therefore, primers for homologous sequences between the *Pb*18s and *Pf1*8s housekeeping genes regions between control and test samples should be

considered. In addition, due to the low level of expression of the target genes, when compared to those of the housekeeping gene *Pb18s,* differences of expression of the target genes between test samples are difficult to interpret, suggesting that other housekeeping parasite genes, such as PBANKA_061540 and PBANKA_011140 could be added to the qPCR analysis, as previously shown by Mancio *et al.* (628).

Collectively, our results regarding parasite fitness contrast with what was previously reported for *Pb*Vac, for which no impairment of sporogonic and hepatic development was observed (275). Our data now show that the genetic modification of *Pb* with the insertion of multiple *Pf* antigens may impair parasite hepatic infectivity and development. Even though *Pb*Vac was the first transgenic parasite line to be employed as a vaccination agent, other transgenic rodent malaria parasites have previously been generated [Reviewed in (629,630)]. For some of these parasites, an impairment of parasite fitness throughout their development in the mosquito has been reported (621,622,631). We can postulate that the simultaneous expression of multiple exogenous antigens from different life cycle stages in a *Pb* platform or the insertion of a single exogenous antigen impair parasite development in the liver, either due to expression of the exogenous proteins in life cycle stages other than the one they are endogenously expressed in, or due to the interaction between exogenous proteins that can lead to inhibition of parasite growth or parasite death. To better understand the dynamics of protein expression in these new chimeric lines, we plan to generate and characterize a *Pb* transgenic parasite line to which only the *Pfs48/45* has been added.

Finally, the evaluation of antibody titers in the serum of immunized mice revealed that immunization with transgenic lines 3279 and 3281 elicited, although at a relatively low level, the production of antibodies against *Pfs*48/45, which could not be identified in serum of mice immunized with lines 3265 and 3267. Considering the low *in vivo* liver infectivity of lines 3265 and 3267, when compared to the *Pb*Vac control, it is possible that the low abundance of the *Pfs*48/45 protein might be responsible for the absence of a detectable antibody response against this protein in the serum of immunized mice. However, anti-*Pf*CSP antibody titers in the serum of these mice were similar between those infected with *Pb*Vac and the new transgenic lines, suggestive of *Pf*CSP high immunogenicity. Therefore, differences in infectivity

between control and new transgenic parasite lines might not affect the titers of antibodies against *Pf*CSP to the same extent as they affect the titers of anti-*Pfs*48/45 antibodies. Although the production of antibodies against *Pf*CSP in animals immunized with the new transgenic lines are in agreement with what was previously observed for *Pb*Vac, further studies regarding the functional capacity of the anti-*Pf*CSP antibodies induced by immunization with these chimeric parasite lines, as previously described for *Pb*Vac, are required, in order to assess their ability to inhibit hepatic infection by *Pf* (275).

The low immunogenicity of *Pfs*48/45 raises a crucial question regarding the correct expression of the full-length protein using the *Pb* antigen delivery platform. Previously, the development of new vaccine candidates targeting the *Pfs*48/45 antigen has struggled with the expression of the correctly folded full-length protein, which is known to have conformation-dependent target epitopes, crucial for the induction of TB immunogens (499,509,632). Although our immunofluorescence microscopy results show expression of the *Pfs*48/45 protein, the 85RF45.3 antibody employed in these studies recognizes conformational epitope III in the C-terminal region of *Pfs*48/45, and does not bind to conformational epitopes I and IIb in the C-terminal region and epitope V in the N-terminal module (632). Therefore, improper folding of *Pfs*48/45, which may lead to degradation and removal of the protein through the ubiquitin pathway (633,634), cannot be overruled. Perhaps more plausibly, the absence of another antigen with which *Pfs*48/45 forms a stable complex, *Pfs*230, could lead to fast protein degradation (482). Another hypothesis would be that glycosylation might be inducing post-translational modifications of *Pfs*48/45, consequently affecting its immunogenicity, as previously shown by Milek *et al.* in an attempt to express *Pfs*48/45 in a vaccinia virus (635–639). Concluding, further experiments are required to clarify these hypotheses, which may include Western blot analysis to assess protein size and possible glycosylation, or immunofluorescence microscopy analysis of the ability of antibodies in the serum of immunized mice to bind to *Pf* gametocytes, where *Pfs*48/45 is present in its native conformation. In addition, parasite staining with different anti-*Pfs*48/45 antibodies that recognize different epitopes of this protein, as shown by Othman *et al.,* could lend further confirmation of correct folding (640).

Collectively, the data presented here show that it is possible to genetically modify *Pb* to serve as a delivery platform for multistage *Pf* antigens. Even though a compromise on parasite fitness was observed, further experiments are required to understand the dynamics of the expression of multiple exogenous proteins in such a system. Importantly, the incorporation of a fusion of proteins *Pfs*48/45 and *Pfs*230 should be considered, due to their ability to elicit increased TB activity in standard membrane feeding assays when compared to each protein alone (513). Ultimately, the addition of efficient TB antigens to *Pb*Vac would be a pivotal step towards enhancing the scope of immunogenicity of this vaccine candidate, in an attempt to contribute to the goal of malaria eradication.

4 GENERAL DISCUSSION

Malaria is one of the most concerning public health problems worldwide, and a leading cause of death and disease in many developing countries, where young children and pregnant women are particularly vulnerable (152). The WHO's Global Technical Strategy for Malaria 2016-2030 defined a set of strategic measures to reduce malaria mortality and incidence in 90%, eliminate malaria from 35 countries by 2030 and prevent the re-establishment of malaria in all malaria-free countries (143). These goals rely on the implementation of a multifaceted strategy in which different tools like prevention, diagnostics, treatment and surveillance are combined towards achieving malaria elimination (143). Although the use of antimalarial tools has reduced the malaria burden from 262 million in 2000 to 214 million in 2015, since then the progress in decrease of malaria cases and deaths has stalled, with the current COVID-19 pandemic disrupting initiatives for malaria prevention, diagnosis and treatment (152). This stagnation is the result of several combined challenges, including the lack of funding, especially in low-income countries (641), and the decrease in responsiveness of mosquitoes to vector control measures, which leads to residual transmission (143,642,643). Until 2015, ITNs alone were responsible for an estimated 68% decrease in malaria cases in endemic African countries (154), illustrating the importance of interventions that target malaria transmission through the mosquito vector. Although insecticide resistance poses a threat to this type of malaria control intervention, strategies combining improvements in current malaria control tools and innovative methods that are cost-effective and long-lasting should be considered (644,645).

TBS have the advantage of reducing parasite prevalence in an endemic population by blocking its transmission through the mosquito, while potentially stopping the spread of drug-resistant parasite strains and insecticide-resistant vectors (351). Additionally, they could address parasite transmission by asymptomatic carriers, a persistent issue in endemic regions (646). The transmission stages of the parasite's life cycle, which include the blood stage gametocyte and mosquito stage parasite forms, are attractive targets of TB interventions, due to limited parasite polymorphisms of gametes, ookinetes and oocysts, which are not subjected to the host's immune pressure (355), the bottlenecks the parasite goes through inside the mosquito (647–650), and the timeframe during which the parasites remains extracellular (approximately 24 h compared to ~1 min it takes for the merozoite to invade the RBC [reviewed

in (189,438)]) (see also Introduction, section 1.8). Moreover, different modeling studies on the dynamics of parasite transmission, the within-human parasite dynamics, and the impact of malaria control measures clearly show the potential role of TBS in malaria elimination (154,651–654).

TBS that target directly the parasite can be broadly divided in TBDs and TBVs, two strategies explored throughout this thesis. Most antimalarials currently employed for treatment in field settings fail to eliminate the transmission stages of the parasite's life cycle, or to impact its development in the mosquito (415,422,463). Yet, three antimalarials are well known for their transmission-blocking efficacy – atovaquone (423,655,656), methylene blue (463,657,658) and primaquine (658–661). Primaquine is known to cause hemolytic anemia in G6PD-deficient individuals, – a genetic trait that is widespread across sub-Saharan Africa, restricting for many years its use in this region (662). As a response, the WHO recommended administering a low dose of primaquine, which toxicity is unlikely at a single dose of 0.25 mg/kg, for reduction of *Pf* transmissibility in malaria low transmission settings (160,412). Additionally, questions regarding methylene blue's toxicity have arisen, and atovaquone targets a limited range of parasite forms (it does not act on mature gametocytes), making the incorporation of these drugs in first-line treatments against the disease, as transmission-blocking compounds, restrictive (160,426,536,663,664).

The search for compounds that target the transmission stages of the parasite has commonly been a byproduct of screening campaigns prioritizing the identification of new compounds with blood stage activity, perceived as an additional benefit in dual-active antimalarials. Additionally, the logistic demands and the elevated costs of adding complexity to the already existing first-line drug combinations has led to the deployment of interventions that incorporate compounds with a large spectrum of activity, eliminating both asexual and sexual or mosquito stage parasites. However, such compounds usually act on a common parasite target expressed through different life cycle stages, which can lead to dissemination of resistant alleles in a population (665). Moreover, *in vitro* data suggests that most transmission-blocking molecules require higher drug concentrations than those employed for asexual parasite elimination in order to be effective (433,461,666), implying that dosing in the

field could be sub-effective. Consequently, mutated parasites could be pressure-selected and passed on, and the scientific/political investment could cease due to underperformance of compounds in blocking parasite transmission (438,667).

With the growing emergence of drug-resistant parasites, and the financial load inherent to the drug discovery process, alternatives in other therapeutic fields could be considered, opening the door to drug repurposing. Anticancer drugs, immunomodulators, antibiotics, or even antiretrovirals, as shown in the work developed during this thesis, could represent a cost-effective transmission-blocking alternative to be administered in combination with drugs targeting the asexual parasite stages (668–671). Nevertheless, how compounds that are not already administered in malaria endemic regions with the purpose of treating other endemic diseases (which is the case of antiretroviral therapy) could be used to block transmission remains to be explored by the scientific community.

Alternatively, new compounds in the drug discovery pipeline with specific transmission or mosquito stage activity could be employed in combination with schizontocidal drugs. In this scenario, asexual parasites would be eliminated with minimal probability of selection of resistant parasites by the TBD (smaller target population) and protecting the lifespan of blood stage drugs (resistant gametocytes will not spread mutated alleles) (438,672).

Still, questions remain regarding the implementation of TBDs in the field. Asymptomatic sub-microscopic gametocyte carriers are frequently found in malaria-endemic regions (673–677), with no currently available tools being both fast, cost-effective and sensitive enough to detect such cases (678). Therefore, a test-and-treat approach to MDA interventions with compounds aiming to decrease transmission in the community seems unfeasible. However, this gametocyte reservoir cannot go untreated. A solution would be to deploy MDA with TBDs across a community regardless of their health status. This poses additional questions regarding the regulatory and ethical, and political aspects of treating patients who might be considered “healthy”. From a regulatory perspective, control and monitoring of the administration of drugs could prove difficult, with the added constraint of patients possibly refusing treatment, which would then have to be legislated to become mandatory, consequently raising ethical issues (679). Moreover, without further clinical trials

that clearly highlight the benefits of TBDs, it might seem counterintuitive for governments to willingly invest in such initiative (666).

Another approach that could possible address many of these questions would be MDA with ivermectin. Its potential for blocking transmission through the elimination of the mosquito vector has been previously demonstrated in field clinical trials (171,173). Thus, considering the co-endemicity of malaria with other parasitic diseases, such as onchocerciasis and lymphatic filariasis, which have undergone chemoprevention by MDA regimens incorporating ivermectin for many years (680–685), this option could be effective in preventing infection with different parasitic worms while eliminating the vector of *Plasmodium* transmission in malaria-endemic regions (552–554). Whether ivermectin will be administered alone in seasonal MDA interventions or in parallel with other TBS remains to be explored.

TBVs, another TBS mentioned above, face similar challenges regarding ethic and regulatory approval as TBDs, as well as the issue of financial endorsement of a vaccine that does not envision prevention of disease in the vaccinated individual. TBVs can be designed to target parasites at two different stages of development: the sexual stages, i.e., gametocytes, against which immunity can be boosted by natural infection (508,686); and mosquito stage parasites, i.e., gametes, ookinetes and oocysts, which cannot be boosted by natural infection, but in turn, since they never interact with the host's immune system, do not undergo the same selective pressure as the others [reviewed in (188,189)]. Which of these strategies would be the most beneficial for malaria control remains to be determined. Most TBV research has focused on four antigens: the gamete proteins P48/45 and P230, and the zygote/ookinete surface proteins P25 and P28. Although others will enter the clinic in the future, such as HAP2, P47 and CelTOS (see Introduction, section 1.8.6), the number of TBV candidate antigens is minimal when compared to that of the pre-erythrocytic and erythrocytic antigens currently under study [reviewed in (189,214)]. Consequently, the development of novel TBV candidates, which largely will depend on the identification of new transmission stage immunogens, has progressed slowly.

The TBV development pipeline still faces numerous hurdles. It is unclear what degree of efficacy is required of a TBV candidate in pre-clinical studies to warrant its advance into

clinical trials. It is also unknown how effective such a vaccine must be in order to reduce transmission in the field. Although initially a reduction of 80% in oocyst intensity was proposed for vaccine candidates to move forward in clinical development (468,687), a population transmission model has shown that this threshold might be too restrictive (352). Moreover, results from SMFA are frequently inconsistent between different laboratories and not always correlate with results obtained by DMFA (688,689). Therefore, a growing effort to optimize assays that explore the potential of TBVs and accelerate their approval by regulatory authorities has been made (689,690). The development of CHMI models to evaluate different TBS represented a significant improvement on the development pipeline of both TBDs and TBVs (691,692), but the design of a field clinical trial to assess the efficacy of a TBV candidate should be approached carefully. How would a company/institute obtain regulatory approval to test a new vaccine candidate that does not aim to prevent infection of an individual, but instead protects the community? A clinical trial in which different arms receive the vaccine candidate and others do not (cluster randomized clinical trial), thus comparing the number of infections between them, might produce misleading results, since malaria transmission patterns and parasite densities could differ between clusters. Not only would several clusters be necessary to account for variability between them, but researchers would have to ensure that mosquitoes would not travel between clusters, which can be extremely challenging (679). Alternatively, uncontrolled clinical trials in which the entire community receives the TBV candidate, comparing the number of infections before and after vaccine administration (during the same season), could be envisaged. However, a certain degree of bias is to be expected when compared to a controlled clinical trial, due to cofounding factors such as the possibility of patients requiring treatment for other diseases throughout the duration of the study (693).

Concluding, TBS have often been overlooked when it came to invest in innovative tools for malaria elimination. However, a growing effort to develop new TBDs and TBVs that could be incorporated in malaria control interventions have recently been pushed forward. Although numerous issues remain to be answered from a regulatory perspective, solutions can be explored between researchers and government authorities to accelerate TBS development pipeline.

In the future, new tools should continue to be explored to be integrated in malaria control initiatives in endemic regions.

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CURRICULUM VITAE AND PUBLICATIONS

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Education:

- **Post graduate degree**, Monitoring and research in clinical trials, 2022
- **MSc**, Biomedical Sciences, Instituto de Higiene e medicina tropical, 2016
- **BSc**, Biology, Evolutionary biology, Faculdade de Ciências da Universidade de Lisboa, 2014

Work Experience:

April 2018-Present | PhD Student Biomedical Sciences, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa

- Full time researcher at MPrudêncio Laboratory, focusing on *Plasmodium* infection & anti-malarial interventions; experienced in designing experimental plans, data collection, critically analyzing generated data and communicating experimental results. Also regularly teaching experimental techniques to new lab members;
- Master student supervisor, mostly focused on supervising the design and implementation of experimental plans, teaching experimental techniques, and providing guidance in interpretation of experimental results;
- Participating in national and international conferences and meetings, synthesizing results to address multidisciplinary audiences.

Jan 2017-Mar 2018 | Research Technician, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa

- Working within a multidisciplinary team mostly focused on designing experimental protocols for optimization of *in vitro* culture systems and interpreting and communicating experimental results.

Jun 2015-Oct 2016 | Master Thesis Student, Biomedical Sciences, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa

- Working on the assessment of antimalarial compounds transmission blocking activity *in vitro*;
- Scheduling multiple experiments within a short time frame, maximizing efficiency of the experimental process;
- Learning experimental techniques relevant for the thesis project;
- Discussing and critically think about experimental generated data, with a multidisciplinary team.

Jan 2015-Jun 2015 | Internship, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal, Group of Immune Parasitology and Vaccine Development

- Learning experimental techniques in molecular and cellular biology within the research scope of the laboratory.

Complementary education:

IATA, Dangerous Goods Training, Mayo Clinic – **January 2022**

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Design and Interpretation of Clinical Trials, Johns Hopkins University online via coursera – **March 21**

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Writing in the sciences, Stanford online via coursera – **May 2020**

Graphical design: creating figures for publications – **May 2019**

1st FLxFlow Course: Principles and Applications of Flow Cytometry – **October 2017**

'Laboratory Animal Science Course', Felasa category B, Lisbon, **May 2016**

Cambridge Advanced English certificate – grade B, **2014**

Additional experience:

- Co-organizer of CAML / IMM PhD Student's Meeting, 2019-2021 (contacting possible sponsors to raise funds for the meeting; contacting national and international renowned speakers; managing the program including schedule of oral and poster presentations; managing logistics and coordinate event on-site)

Publications

- **AZEVEDO, R.,** Mendes, A. M., and Prudêncio, M. (2020). The Impact of Antiretroviral Therapy on Malaria Parasite Transmission. *Front. Microbiol.* 10, 3048. doi:10.3389/fmicb.2019.03048.
- **AZEVEDO, R**., Mendes, A. M., and Prudêncio, M. (2019). Inhibition of *Plasmodium* sporogonic stages by ivermectin and other avermectins. *Parasites & Vectors*. 12, 549. doi:10.1186/s13071-019-3805-0
- **AZEVEDO, R**. *et al.* (2017). A bioluminescence method for *in vitro* screening of *Plasmodium* transmission-blocking compounds. *Antimicrob. Agents Chemother.* AAC.02699-16 doi:10.1128/AAC.02699-16
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Oral Communications

- 5º conferência anual da RedeSaúde: 30 November 2021, Lisbon, **AZEVEDO R.** *et al*. 'Development of a multi-stage malaria vaccine'
- Parasitology series, Jan 2022, Instituto de Medicina Molecular, Lisboa, **AZEVEDO R.** *et al*. 'Development of a multi-stage malaria vaccine'
- Parasitology series, Dec 2021, Instituto de Medicina Molecular, Lisboa, **AZEVEDO R.** *et al.* 'Development of a multi-stage malaria vaccine'
- IMM PhD Students' Meeting, 5-7 May 2021, virtual format, **AZEVEDO R.** *et al*. 'Development of a multi-stage malaria vaccine'
- Parasitology series, Mar 2021, Instituto de Medicina Molecular, Lisboa, **AZEVEDO R.** *et al.* 'Development of a multistage malaria vaccine'
- Pizza Seminar, Oct 2020, Instituto de Medicina Molecular, Lisboa**, AZEVEDO R.** *et al.* 'Plasmodium transmission blocking strategies'
- Parasitology series, Apr 2019, Instituto de Medicina Molecular, Lisboa, **AZEVEDO R.** *et al.* 'HIV treatment has Plasmodium transmission blocking activity'
- Parasitology series, May 2018, Instituto de Medicina Molecular, Lisboa, **AZEVEDO R.** *et al*. 'The role of avermectins and antiretrovirals as transmission-blocking compounds'
- Rodrigues A, Santos-Mateus D, Alexandre-Pires G, Valério-Bolas A, Rafael Fernandes M, Pereira M, Jesus J, **ALVES-AZEVEDO R**, *et al*. Kupffer cells and blood derived macrophages respond differently to *L. infantum* parasites. 6th world congress on leishmaniasis. Toledo Conference Centre, Toledo, Spain, 16-20 May 2017
- Rodrigues A, Santos-Mateus D, Alexandre-Pires G, Valério-Bolas A, Rafael Fernandes M, **ALVES- AZEVEDO R** *et al*. G. *Leishmania infantum* modulates the metabolism of dog

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Poster communications

- BioMalPar XVII: biology and pathology of the malaria parasite, 25-27 May 2021, virtual format, **AZEVEDO R.** *et al*. 'Drug repurposing for malaria control: impact on mosquito and transmission stages'
- IMM PhD Students' Meeting, 15-17 May 2019, Lisbon, **AZEVEDO R.** *et al*. 'A Novel Bioluminescence Method to Evaluate the Transmission Blocking Activity of Avermectins'
- TwinnToInfect Spring School in Infection and Immunity, 19-22 March 2018, Sesimbra. **AZEVEDO R.** *et al*. 'Development of a new *in vitro* assay for screening malaria transmission blocking compounds'