**UNIVERSIDADE DE LISBOA**
**FACULDADE DE CIÊNCIAS**
**DEPARTAMENTO DE BIOLOGIA ANIMAL**


# Characterization of a new malaria vaccine candidate against *Plasmodium vivax* using genetically modified rodent *Plasmodium* parasites

**Miguel Filipe Duarte**

Dissertação de Mestrado

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

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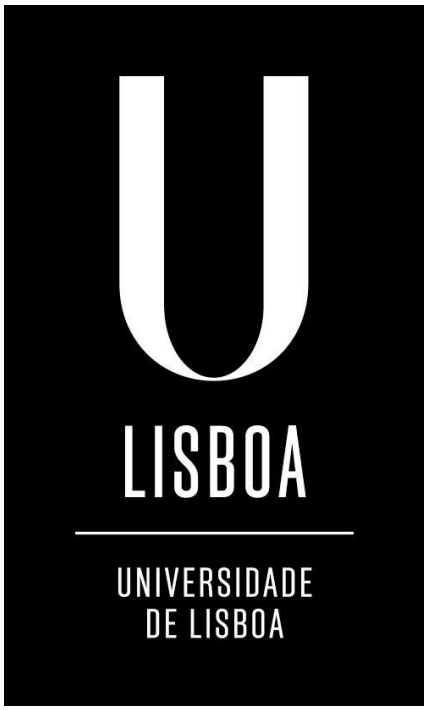


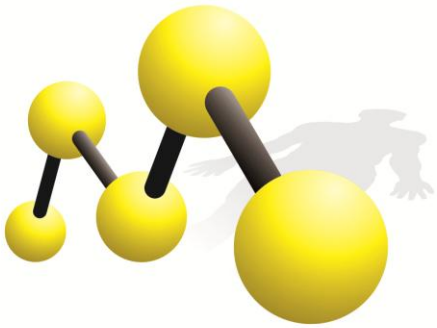

INSTITUTO DE
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# Characterization of a new malaria vaccine candidate against *Plasmodium vivax* using genetically modified rodent *Plasmodium* parasites

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Dissertação para a obtenção do Grau de Mestre orientado por: Doutor António Mendes (Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa) e Professora Doutora Deodália Dias (Faculdade de Ciências da Universidade de Lisboa)

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# Abstract

Malaria is an infectious disease transmitted by *Anopheles* mosquitoes and caused by protozoan parasites of the genus *Plasmodium*. Despite countless efforts, there is still no effective vaccine against any of the human infective *Plasmodium* parasites of which *P. falciparum* and *P. vivax* are the most clinically significant.

A new whole organism-vaccine has been proposed which is based on the use of genetically modified rodent parasites (*P. berghei*) as platforms for the delivery of immunogenic antigens of human infective *Plasmodium* species. The efficacy and safety of *P. berghei* parasites as a platform to deliver immunogenic antigens is warranted by their ability to infect human hepatocytes without being able to develop inside human erythrocytes. A genetically modified *P. berghei* expressing *P. falciparum* circumsporozoite (CS) protein, under the control of the *P. berghei* UIS4 promoter, (*Pb*(*Pf*CS@UIS4)), was already developed and characterized as a potential vaccine candidate. Immunization of mice with *Pb*(*Pf*CS@UIS4) parasites has successfully elicited an immune response capable to recognize and bind to *P. falciparum* sporozoites, and inhibit infection by this parasite.

*P. vivax* malaria is the most widespread of the human-infective *Plasmodium* species and leads to a great socioeconomic burden worldwide. In light of this fact, and given the promising results obtained for the vaccine candidate *Pb*(*Pf*CS@UIS4), a new genetically modified *P. berghei* expressing the *P. vivax* CS protein, *Pb*(*Pv*CS@UIS4) was created, as a vaccine candidate against *P.vivax* malaria. This parasite was generated using the GIMO (Gene Insertion Marker Out) transfection method.

The work presented in this thesis aims to characterize *Pb*(*Pv*CS@UIS4) parasites in terms of infectivity and development across the sporogonic and pre-erythrocytic stages of the parasite life cycle, both *in vitro* and *in vivo*. Sporogonic parasite development and infectivity in the mosquito was assessed using *Anopheles stephensi* mosquitoes by counting oocysts in the midgut and sporozoites in the salivary glands at 10 and 21 days post infectious blood meal, respectively. No significant differences were observed between the development of *Pb*(*Pv*CS@UIS4) and *Pb*GIMO (wild-type *P. berghei* - transfection motherline) parasites. Pre-erythrocytic development was evaluated *in vitro* on Huh7 and HepG2 human hepatoma cell lines (48h p.i) and *in vivo* experiments on C57BL/6J mice (44 hpi), revealing that *Pb*(*Pv*CS@UIS4) sporozoites can infect and develop within hepatocytes to a similar extent to *Pb*GIMO sporozoites. Blood stage development experiments also revealed that *Pb*(*Pv*CS@UIS4) parasites infectivity and development during this stage is comparable to *Pb*GIMO. These results indicate that the insertion of the *Pv*CS gene in *P. berghei* 230p neutral locus does not appear to have an impact on the parasite's ability to infect and develop throughout its life cycle. Additionally, CS expression was also assessed on *Pb*(*Pv*CS@UIS4) sporozoites and exoerythrocytic forms (intrahepatic forms of the parasite), showing that both the endogenous *Pb*CS and the exogenous *Pv*CS are indeed being co-expressed on both stages.

This characterization represents one of the first steps on the development of this new vaccine candidate against *P.vivax* malaria and the results here presented

provide valuable insight in order to proceed to future studies regarding the immunogenicity and efficacy of *Pb*(*Pv*CS@UIS4) as a vaccine candidate.

**Keywords**: Malaria, *Plasmodium vivax*, *Plasmodium berhgei*, whole-organism vaccine, transgenic, circumsporozoite protein.

# Resumo

A malária é uma doença infecciosa causada por um parasita protozoário do género *Plasmodium* que causa a morte entre 650.000 a 1.200.000 pessoas todos os anos, das quais aproximadamente 85% são crianças com menos de 5 anos de idade.[1] Existem 5 espécies de *Plasmodium* capazes de causar malária em humanos, sendo *P. falciparum* e *P. vivax* as espécies responsáveis pela grande maioria dos casos.[1] Estes parasitas são transmitidos sob a forma de esporozoíto através da picada de mosquitos fêmea do género *Anopheles*.[1] Após a picada, os esporozoítos invadem a corrente sanguínea do hospedeiro e migram até ao fígado onde, após atravessarem vários hepatócitos, acabam por invadir e se desenvolver dentro de um.[10,11] No interior do hepatócito, o parasita replica-se, dando a origem a milhares de merozoítos.[12] Estes são libertados para a corrente sanguínea no interior de uma estrutura denominada merosoma, terminando assim a fase pré-eritrocitária da infeção.[10] Uma vez na corrente sanguínea, o merosoma rompe-se e liberta os merozoítos que por sua vez vão infetar os eritrócitos, dando origem à fase sanguínea e sintomática da doença.[10] Dentro dos eritrócitos os parasitas vão novamente replicar-se, dando origem a novos parasitas capazes de perpetuar o ciclo de infeção na corrente sanguínea mas dando igualmente origem a formas sexuais (gametócitos) aptas para serem ingeridas aquando da picada por novos mosquitos e de se desenvolverem dentro dos mesmos, dando origem a um novo ciclo de infeção.[13] Apesar da malária causada por *P. falciparum* ter uma sintomatologia mais severa, a malária causada por *P. vivax* tem uma maior distribuição geográfica e está geralmente associada a longos períodos de morbilidade devido à capacidade que o parasita possui de gerar formas adormecidas (hipnozoítos), que podem levar a uma reincidência dos sintomas da doença.[5,6]

Atualmente, as medidas de combate à malária passam pelo uso de inseticidas para diminuir as populações de mosquitos transmissores, uso de redes mosquiteiras e a prescrição de fármacos profiláticos contra a malária. No entanto, a eficácia tanto dos inseticidas como dos fármacos tem vindo a diminuir ao longo do tempo devido ao aparecimento de "perfis" de resistência quer nas populações de mosquitos contra os inseticidas, quer nas populações de *Plasmodium* contra os fármacos existentes.[1,15,17]

Dadas as limitações das medidas existentes, tornou-se consensual entre a comunidade científica de que a criação de uma vacina é uma componente essencial no combate à malária, uma vez que esta permitiria não apenas prevenir a sintomatologia da doença mas também a sua transmissão. Contudo, até ao momento não existe uma vacina licenciada contra a malária.[17]

A RTS,S, a vacina que se encontra actualmente no estadio mais avançado de desenvolvimento, é uma vacina de subunidade, que consiste na administração de um fragmento da proteína CS do parasita *Plasmodium falciparum* fundido com uma matriz transportadora proveniente da superfície do vírus da hepatite B, mostrou oferecer um nível de proteção bastante modesto em humanos.[27] Em particular, as respostas imunitárias observadas após vacinação com RTS,S foram maioritariamente mediadas por células T CD4+ e anticorpos contra a proteína CS, sem que os níveis de células T

CD8+ detectáveis fossem muito significativos, as quais estão demonstradas como sendo as principais células efectoras na proteção imunitária contra a malária.[22,30,31]

Como alternativa, outros tipos de vacina contra a malária estão a ser desenvolvidos, como por exemplo, as vacinas de organismo inteiro. As vacinas de organismo inteiro consistem na administração de parasitas inteiros, previamente atenuados, com o objetivo de espoletar uma resposta imunitária capaz de inibir a infeção causada por organismos não atenuados. Vários estudos realizados com esporozoítos atenuados (*P. falciparum*), têm demonstrado que estas estratégias de vacinação podem garantir um alto nível de proteção em humanos.[33-35,45] Além disso, as respostas espoletadas com esta estratégia de vacinação são maioritariamente mediadas por células T CD8+.[32] Foi também observado que o nível de proteção é tanto maior quanto maior for a progressão do desenvolvimento do parasita no fígado.[32]

No entanto, estas vacinas baseiam-se na assunção que todos os parasitas administrados estão totalmente atenuados, e como tal, não progridem para a fase sanguínea da infeção. Dito isto, é fácil perceber que este princípio representa um risco para os indivíduos vacinados, uma vez que basta que um parasita escape ao processo de atenuação, para que haja progressão da infeção para a fase sintomática da doença.

Como resposta a esta limitação inerente às vacinas que se baseiam no uso de parasitas atenuados, a equipa do Prudêncio Lab no IMM está a desenvolver uma nova estratégia de vacinação que se baseia no uso de parasitas de roedores (*P. berghei*) geneticamente modificados, como plataforma de apresentação de antigénios pertencentes às espécies de *Plasmodium* que causam malária em humanos, de forma a promover imunidade contra os mesmos quer por mecanismos de proteção cruzada entre espécies quer por mecanismos de proteção especifica contra os antigénios dos parasitas causadores de malária em humanos.

Com o intuito validar esta nova estratégia, foram realizadas experiências no sentido de verificar que *P. berghei* consegue invadir e desenvolver-se em hepatócitos humanos sem progredir para a fase sanguínea, e que parasitas *P. berghei* geneticamente modificados para expressar a proteína CS de *P. falciparum*, são capazes de espoletar respostas imunológicas capazes de reconhecer e inibir *P. falciparum*. Foi então desenvolvido e caracterizado um novo candidato a vacina, que consiste em usar *P. berghei* geneticamente modificado de forma a que este expresse a proteína CS de *P. falciparum* sob o controlo do promotor UIS4 de *P. berghei*, o parasita *Pb*(*Pf*CS@UIS4).

Os resultados deste estudo demonstraram que *P. berghei* consegue de facto infetar hepatócitos humanos sem causar doença, o que faz deste parasita uma potencial plataforma de administração de antigénios imunogénicos para humanos. Por outro lado, a imunização de ratinhos com *Pb*(*Pf*CS@UIS4) mostrou conduzir a respostas imunitárias capazes de reconhecer e ligar-se a esporozoítos de *P. falciparum*, inibindo a infeção por este parasita.

Dados os resultados satisfatórios obtidos com a caracterização do parasita *Pb*(*Pf*CS@UIS4), foi produzido um novo candidato a vacina contra *P. vivax*, o parasita *Pb*(*Pv*CS@UIS4), que de um modo semelhante ao candidato anterior, consiste em

usar *P. berghei* geneticamente modificado de forma a expressar a proteína CS de *P.vivax* sob o controle do promotor UIS4 de *P. berghei*.

Deste modo, esta tese de mestrado teve como objetivo principal caracterizar o desenvolvimento deste novo parasita durante o seu ciclo de vida dentro do mosquito assim como caracterizar a sua infetividade e desenvolvimento em culturas celulares *in vitro*, e *in vivo* com ratinhos C57BL/6J. Adicionalmente, a sequência do gene *Pv*CS usada na criação deste parasita foi analisada em detalhe e comparada com sequências referencia para o mesmo gene disponíveis na base de dados PlasmoDB de modo a identificar diferenças significativas entre a sequência encontrada no isolado de *P. vivax* utilizado e as diferentes populações existentes mundialmente. Ao compararmos a sequencia da *Pv*CS usada na criação do *Pb*(*Pv*CS@UIS4) com as sequencias existentes na base de dados, concluímos que as regiões N-terminal e C-terminal da proteína se mantêm conservadas, enquanto que na zona "central de repetição", existe alguma variabilidade. De acordo com estudos anteriores, concluímos que a variabilidade encontrada no nosso isolado não é superior à que normalmente se verifica para este gene e que as alterações especificas encontradas são muito provavelmente características comuns das populações de *P. vivax* existentes na região geográfica de onde foi obtido o isolado utilizado neste estudo (Tailândia).[47,51]

De forma a caracterizar o desenvolvimento dos parasitas *Pb*(*Pv*CS@UIS4) dentro do mosquito, foram contados quer os oocistos presentes no estômago do mosquito ao dia 10 após infeção quer esporozoítos presentes nas glândulas salivares entre o dia 20 e 22 após infeção. Estes números foram então comparados com os números obtidos com parasitas não-transfetados, pertencentes à linha mãe (*Pb*GIMO) usada na transfeção dos parasitas *Pb*(*Pv*CS@UIS4). Os resultados mostraram que não existem diferenças significativas entre os parasitas *Pb*(*Pv*CS@UIS4) e *Pb*GIMO, o que nos leva a concluir que a inserção da *Pv*CS não aparenta ter qualquer impacto nesta fase de desenvolvimento do parasita.

A fim de caracterizar a capacidade deste parasita se desenvolver e invadir células *in vitro*, foram usadas 2 linhas celulares de hepatomas humanos; Huh7 e HepG2. Estas linhas celulares foram infetadas com esporozoítos recolhidos das glândulas salivares de mosquitos infetados a partir do dia 20 após infeção. Passadas 48h da infeção, as células foram fixadas e marcadas com anticorpos de marcação nuclear e anticorpos específicos contra o parasita (anti-HSP70). Desta forma, através de microscopia de fluorescência, foi quantificado o nível de infeção e comparado entre parasitas *Pb*(*Pv*CS@UIS4) e *Pb*GIMO. Não foram observadas diferenças significativas entre os níveis de infeção observados com ambos os parasitas, o que demonstra que o desenvolvimento do parasita na fase hepática não é afetado pela presença da proteína CS exógena. Resultados idênticos foram também obtidos *in vivo*, em experiencias em que ratinhos C57BL/6J foram infetados por injeção i.v. de esporozoítos. Nestas experiencias, decorridas 44h após a infeção, os ratinhos foram sacrificados e os seus fígados foram recolhidos de forma a ser possível analisar os níveis de infeção por qRT-PCR e microscopia de imunofluorescência.

Concomitantemente com as experiencias anteriores, a expressão da *Pb*CS e da *Pv*CS foi também observada com recurso a técnicas de microscopia de imunofluorescência. Como era esperado, nos parasitas *Pb*(*Pv*CS@UIS4), a *Pb*CS e a

*Pv*CS estão a ser co-expressas em esporozoítos e nas formas exoeritrocitárias presentes em hepatócitos infetados. Esta observação é extremamente relevante para a estratégia de vacinação, uma vez que é esperado que parte da imunidade gerada seja especificamente contra a *Pv*CS.

Para terminar a caracterização dos parasitas *Pb*(*Pv*CS@UIS4), foi também realizada uma experiencia no sentido de caracterizar a capacidade destes parasitas infetarem e se desenvolverem durante a fase eritrocitária da doença ao longo de 9 dias após infeção. Os resultados demonstraram que não existem diferenças significativas entre a capacidade de infectar e se replicar no interior dos eritrócitos por parte dos parasitas *Pb*(*Pv*CS@UIS4) quando comparados com parasitas *Pb*GIMO.

Concluindo, os parasitas *Pb*(*Pv*CS@UIS4) demonstraram não apresentar nenhuma diferença significativa em termos de capacidade infetiva e de desenvolvimento ao longo do seu ciclo de vida quando comparados com a linha materna usada para transgénese. No contexto de desenvolvimento de uma vacina é importante que o parasita mantenha a sua funcionalidade de forma a conseguir apresentar o maior número possível de antigénios e desta forma, tornar a vacina mais eficaz. Adicionalmente, a *Pv*CS está a ser expressa tanto em esporozoítos como durante a fase hepática, o que mais uma vez, no contexto desta estratégia de vacinação, é extremamente importante dado que a maior parte da imunidade contra *Plasmodium* é gerada durante a fase hepática. Os resultados aqui obtidos estão em concordância com os resultados previamente obtidos com o candidato a vacina contra *P. falciparum*, o *Pb*(*Pf*CS@UIS4). Os dados aqui apresentados representam um primeiro passo na caracterização deste candidato a vacina e, como tal, serão necessárias mais experiencias, nomeadamente do ponto de vista imunológico, para averiguar a sua eficácia.

**Palavras-chave**: Malaria, *Plasmodium vivax*, *Plasmodium berhgei*, vacinas de organismo inteiro, transgenico, Proteina circumsporozoite.

# Abbreviations

| | | | |
|---|---|---|---|
| **18S** | *Plasmodium* 18S ribosomal RNA | **NK** | Natural Killer cells |
| **5-FC** | *5*-fluorocytosine | **PABA** | p-aminobenzoic acid |
| **BSA** | Bovine serum albumin | ***Pb*(*Pf*CS@UIS4)** | *Plasmodium berghei* expressing *Plasmodium falciparum* circumsporozoite protein under the control of the *Pb*UIS4promoter |
| **cDNA** | Complementary Deoxyribonucleic acid | ***Pb*(*Pv*CS@UIS4)** | *Plasmodium berghei* expressing *Plasmodium vivax* circumsporozoite protein under the control of the *Pb*UIS4promoter |
| **CS** | Circumsporozoite protein | ***Pb*CS** | *Plasmodium berghei* circumsporozoite protein |
| **DMEM** | Dulbecco Modified Eagle's Medium – cell culture medium | ***Pb*GIMO** | *Plasmodium berghei* (Gene Insertion Marker Out) motherline |
| **DNA** | Deoxyribonucleic acid | **PBS** | Phosphate buffered saline solution |
| **EEF** | Exoerythrocytic forms | **PCR/qRT-PCR** | Polymerase chain reaction/quantitative real time polymerase chain reaction |
| **ER** | Endoplasmatic reticulum | ***Pf*CS** | *Plasmodium falciparum* circumsporozoite protein |
| **GAP** | Genetically Attenuated Parasites | **p.i** | Post-infection |
| **GMEP** | Global Malaria Eradication Program | ***Pv*CS** | *Plasmodium vivax* circumsporozoite protein |
| **HC-04** | Human hepatocyte cell line | **PVM** | Parasitophorous vacuole membrane |
| ***hdhfr*** | Selectable markar human dehydrofolate reductase | **RAS** | Radiation attenuated sporozoites |
| **HEPES** | 4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid | **RBC** | Red blood cell |
| **HepG2** | Human hepatoma cell line | **RNA** | Ribonucleic acid |
| **HPRT** | Hypoxanthine Guanine Phosphoribosyl Transferase | **RPMI** | Roswell Park Memorial Institute medium – cell culture medium |
| **HSPG** | Heparin sulphate proteoglycans | **RPM** | Rotations per minute |
| **Huh7** | Human hepatoma cell line | **SEM** | Standard error of the mean |
| **IFN-y** | Interferon gamma | **STAT** | Signal transducers associated with transcription |
| **iNOS** | Nitric oxide synthase | **TSR** | Thrombospondin repeat region |
| **i.p.** | Intraperitoneal | **WT** | Wild type |
| **i.v.** | Intravenous | ***yfcu*** | Yeast fcu selectable marker |
| **MHC** | Major Histocompatibility Complex | **HSP70** | Heat shock protein 70 |

# Índex

# 1. Introduction

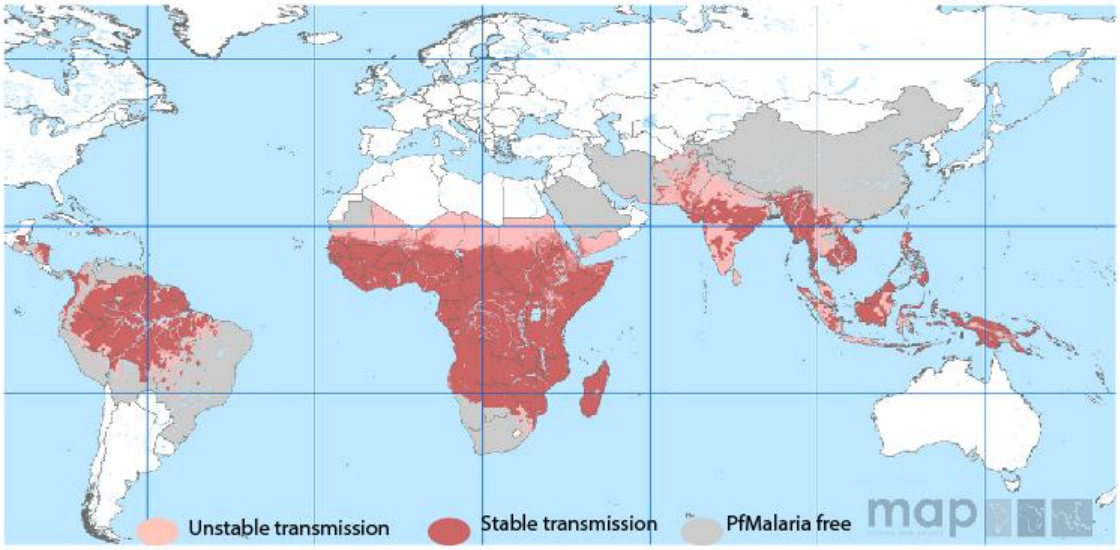
## 1.1 Socioeconomic burden

Malaria represents one of the most serious public health problems worldwide; and although malaria incidence has been decreasing over the past 14 years, every year there are more than 216 million clinical episodes resulting in 650.000 to 1.200.000 deaths, 85% of which occur in children under 5 years old. In 2012 alone, an estimated 3.4 billion people (47% of the world's total population) were at risk of malaria, of which 1.2 billion, mostly in the African and South-East Asia regions, were at high risk (>1 case per 1000 population) of contracting this disease.[1] Malaria was once present worldwide. However, between 1940 and 1969, with the establishment of the Global Malaria Eradication Programme (GMEP) by WHO, it was possible to eliminate malaria from USA and Canada, Europe, and Russia.[2] Nevertheless, the excessive use of insecticides and anti-malarial drugs, such as chloroquine, which were the core tools of the GMEP, led to a resurgence of malaria prevalence in some tropical countries on the following years.[3] Additionally, the selective pressure issued by those two factors (insecticides and anti-malarials) led to the appearance of insecticide and drug resistant mosquito and *Plasmodium* populations, respectively.[3] Nowadays, malaria is still present in 104 countries, in which it is considered endemic.[1]

## 1.2 Etiology and distribution

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium*, which are transmitted by female *Anopheles* mosquitoes. There are numerous species of *Plasmodium* parasites which can infect a plethora of vertebrate animal species, although only five *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) are known to cause disease in humans.[1] *P. falciparum* and *P. vivax* raise the greatest concerns in terms of public health as the remaining three species are reported to have much lower incidence rates.[1] *P. falciparum* malaria is indeed the most deadly form of human malaria but it predominates mostly in Africa (Fig.1.A); as for *P. vivax* malaria, although not as severe presents a wider distribution (Fig.1.B) ranging from South American to South-eastern Asian regions.[1] Despite the fact that most malaria-related deaths are due to *P. falciparum* infections, there is no denying the impact of *P. vivax* infections, mostly because *P. vivax*, unlike *P. falciparum*, is able to evolve into dormant forms (hypnozoites) that can result in disease relapses.[4] Relapses associated to *P. vivax* malaria add to the morbidity burden and make this species of *Plasmodium* even harder to eliminate.[5] The low transmission of *P. vivax* in African countries is due to the predominantly Duffy negative human populations. The Duffy receptor is a chemokine receptor present on the erythrocyte surface of Duffy positive individuals and it is known to be essential for *P. vivax* parasites to recognize and invade erythrocytes.[6] Therefore, populations with a Duffy negative trait are resistant to *P. vivax* infections.[6]

A The spatial limits of Plasmodium falciparum malaria transmission map in 2010 globally




B The spatial limits of Plasmodium vivax malaria transmission map in 2010 globally

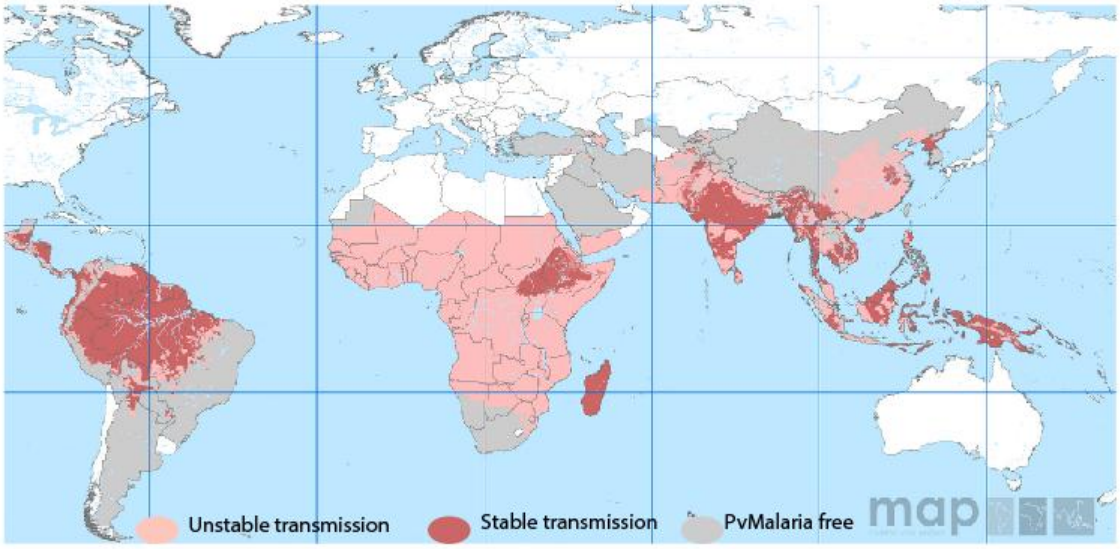



Figure 1 – **Malaria transmission map** – World map showing the areas with *P. falciparum* (top map) and *P. vivax* malaria transmission. Regions coloured in pink represent areas with unstable malaria transmission (annual case incidence reported is lower than 0,01%). Regions colored in red represent areas with stable malaria transmission (annual case incidence reported is higher than 0,01%).[68,69]

## 1.3 Symptoms and diagnosis

The first malaria symptoms usually appear about 7-9 days post infection (depending on the *Plasmodium* species). In endemic areas, malaria is the most common cause of fever, although uncomplicated malaria includes other flu-like symptoms, such as headache, fatigue, muscle ache, anemia, nausea, and after a few days, a palpable spleen.[7] Severe malaria may have other manifestations, most commonly in children or immunocompromised adult individuals, such as acute pulmonary oedema, kidney injury, jaundice, generalized seizures and ultimately coma (cerebral malaria).[8]

The most common method for malaria diagnosis is by the microscopic observation of Giemsa-stained blood films. However, depending on resource availability, there are other methods such as antigen detection kits or by polymerase chain reaction (PCR).[9]

## 1.4 *Plasmodium* life cycle

Female *Anopheles* mosquitoes regularly bite mammalian hosts to take a blood meal and gather the required proteins to generate eggs. During a bite, the mosquito injects saliva in order to generate an anesthetic, vasodilator and anti-coagulant effect.[10] When a mosquito is infected, a *Plasmodium* form called sporozoite migrates to the mosquito's salivary glands and, upon a blood meal, will be injected into the host's skin (Fig.2.1).[11] Some of the injected parasites will use gliding motility to move to the nearest blood vessel and enter the bloodstream. Upon reaching the bloodstream, sporozoites remain in circulation until they eventually reach the liver sinusoids, where they traverse endothelial, Kupffer cells and a few hepatocytes until productively infecting a final hepatocyte (Fig.2.2). When hepatocyte infection occurs, sporozoites will form a parasitophorous vacuole and, within it, transform into spherical hepatic stages called exoerythrocytic forms – EEFs.[12] *P. vivax* and *P. ovale* parasites can develop into dormant hepatic forms (hypnozoites) that can be reactivated later and proceed with the normal course of infection (Fig.2.3). Parasites multiply inside the cell, ultimately forming a schizont, which is made up of thousands of merozoites (Fig.2.4).[10] After the process of maturation, merozoites are released inside merosomes, which eventually burst and release the merozoites to the bloodstream where they will infect erythrocytes (Fig.2.5). Inside the erythrocytes, making use of the infected cell's resources, more merozoites will develop. After reaching erythrocyte capacity, the cell will burst and release all the merozoites into the bloodstream, leading to the symptoms of malaria (Fig.2.6).[10] In some cases, infected erythrocytes will lead to the formation of gametocytes, the sexual forms of the parasite (Fig.2.7).[13]The parasite's life cycle is eventually perpetuated when a new mosquito bites an infected individual and collects *Plasmodium* gametocytes in its blood meal. Upon reaching the mosquito midgut, *Plasmodium* male gametocytes are exposed to mosquito-specific factors and environmental factors forming eight motile male gametes (exflagellation) that undergo a process of fusion with female gametes leading to a new form, the zygote.[13] The zygote will mature into a motile ookinete (24h post-blood meal) that will penetrate the midgut epithelium, moving from the lumen to the basal side, beginning the transformation into

an oocyst (Fig.2.8). Oocyst development takes between 7 and 14 days (depending on mosquito-parasite species combination). About 11-18 days after mosquito's infection, thousands of sporozoites have been produced inside the oocysts (through budding process), leading to the oocyst burst and sporozoite release into mosquito's hemolymph.[13] When in the hemolymph, sporozoites will migrate and infect the salivary glands along with the formation of a parasitophorous vacuole.[10] Sporozoites will then be ready to start a new cycle of infection upon the mosquito's next blood meal (Fig.2.9).

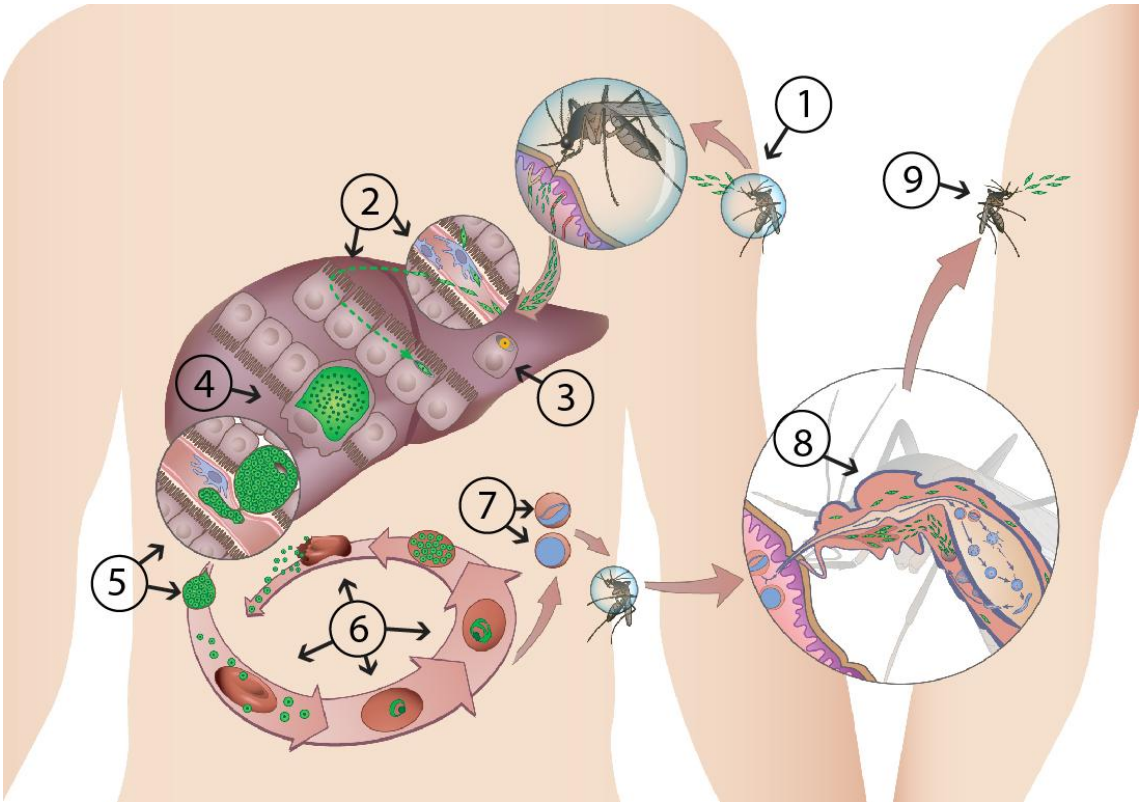



Figure 2 – ***Plasmodium*spp life cycle** – 1 – During the bite of an infected mosquito, spozoroites are injected into the host's skin. 2 – The sporozoites migrate to the liver where they traverse several hepatocytes until effectively infecting one. 3 (Only in *P. vivax* and *P. ovale* infections) – The parasites inside the hepatocyte develop into a dormant form, the hipnozoite. 4 – Merozoites replicate inside the hepatocyte, forming a schizont. 5- After finishing the development inside the hepatocyte, the merozoites grouped inside the merosome, will be released into the blood stream, where the merosome membrane will burst releasing the infectious merozoites. 6 – The merozoites will infect erythrocytes where they will replicate, leading to the formation of sexual (gametocytes) and asexual forms. Asexual forms will then perpetuate the cycle of infection inside the host by infecting more erythrocytes. 7 – Gametocytes in circulation are taken by uninfected mosquitoes that bite an infected host. 8 – Inside the mosquito, a zygote is formed and matured into motile ookinetes which will invade the midgut epithelium and develop into oocysts. As oocysts mature, sporozoites are formed within. When sporozoite's development is complete the oocyst will burst and the sporozoites will migrate to the mosquito salivary glands. 9 – The mosquito will infect a new host upon its next blood meal.

## 1.5 Control measures

Nowadays the most relevant malaria control measures are the use of indoor residual spraying to control *Anophelian* populations, the distribution of insecticide-treated bednets and the prescription of prophylactic/therapeutic drugs.[1] However, the efficacy of these measures is dramatically decreasing.[14] Specifically, the intensive process of positive selection created by the uncontrolled use of insecticides led to the appearance of *Anophelian* populations which are more likely to have multiple mechanisms of resistance against common insecticides[15], and bednets are insufficient since some mosquitoes have peak-biting hours before bedtime.[16]Likewise, many of the existing drugs are becoming ineffective as resistance is developed by *Plasmodium* populations.[17] The World Health Organization (WHO) currently recommends artemisinin-based combination therapies for uncomplicated *P. falciparum* malaria. Chloroquine, the malaria wonder drug during the 1950s and 1960s, is still a valid option for some countries (especially against *P. vivax*), although with a lower efficacy due to chloroquine-resistant *Plasmodium* profiles.[1]

## 1.6 Vaccines against Malaria

Given all the limitations associated with vector control measures and anti-malarial drugs, it became consensual within the malaria scientific community that a vaccine will always be an essential component of any malaria eradication/control strategy, as it would prevent malaria symptoms from occurring effectively, reducing morbidity and mortality on malaria endemic regions.[17] For the past 75 years, efforts have been made to create a vaccine that would grant immunity against *Plasmodium* parasites, although no licensed vaccine has been developed so far. The main reasons for such a delayed progress toward an effective malaria vaccine are the complexity of the parasite, its genetic diversity, the incomplete and temporary nature of naturally acquired immunity and also the fact that parasite material must be obtained from infected hosts or mosquitoes, as only *P. falciparum* can be grown in continuous culture.[18] To put all its complexity into context, the *P. falciparum* genome has more than 23 million bases of DNA organized into 14 chromosomes and about 5.000 genes. This is a significantly more complex genome than that of many of the pathogens against which vaccines have already been developed.[18] Despite all the hardship on malaria research, and particularly on malaria vaccine development, in the past couple of decades, with the increased funding and the development of new molecular biology techniques, there has been an increase of viable vaccine candidates. Malaria vaccines can fall into three groups based on their target in the parasite's life cycle; blood-stage vaccines, transmission-blocking vaccines and pre-erythrocytic vaccines.[19]

### 1.6.1 Historical background

The first attempts to generate a malaria vaccine took place during the early 1930s. These studies used avian animal species as a model and used inactivated, killed, whole parasites or parasite extracts as vaccines, often accompanied by immune-boosting adjuvant systems, though with no success.[18] In 1945, Jules Freund reported that he had partially protected ducks against intravenous challenge with the avian malaria *P. lophurae* by immunizing them with formalin-inactivated malaria-infected blood cells and an adjuvant system consisting of a lanolin-like substance, paraffin oil, and killed tubercle bacilli.[18] Shortly afterwards, a similar experiment was done with *Rhesus* monkeys and *P. knowlesi*, also successfully. These initial studies showed that whole-organism vaccines appeared to be a good and viable approach and that it is important to use good adjuvants to elicit an immune protection against the parasite.[18] It was only in 1967 that the first successful pre-erythrocytic targeting vaccine was reported; Ruth Nussenweig's group showed that radiation-attenuated *P. berghei* sporozoites injected intravenously in mice could indeed elicit protective immunity.[20] These findings already suggested that the capacity of the parasite to infect is important to elicit protective immunity, as past attempts showed that immunizations with dead sporozoites grant no protection against subsequent infections.[21]

### 1.6.2 Immunity in Malaria

Despite all the years of human and animal research in malaria, the basis of protective immunity against malaria is still poorly understood. However, some recent studies have been aiming to establish which specific immune responses have an essential role in protective immunity. For a long time, it was believed that the mechanism responsible for the protection induced by the radiation-attenuated sporozoites was mainly antibody-meadiated.[22] Nowadays, not excluding the importance of the humoral response, it is commonly accepted that CD8+ T cell specific for the parasite-derived peptide/class I MHC molecule complexes on the infected hepatocyte surface may be the primary immune effectors in protective immunity.[22] CD4+ T cells also seem to play a role in recognizing parasite-derived peptide/class II MHC molecule complexes.[22,23] The mechanism through which CD8+ T cells were initially believed to eliminate infected hepatocytes was by direct cytolysis, but more recent data revealed that infected hepatocyte elimination is mediated by the release of interferon-gamma(IFN-γ) by CD8+ T cells (Fig.3).[24]

Individuals living in malaria-endemic regions are constantly exposed to *Plasmodium* infections leading to some naturally acquired immunity; however this immunity is slowly developed and only seems to attenuate the severity of the disease symptoms. [25] A possible explanation for this could be that the dose of sporozoites inoculated is too low to induce and boost immune responses or that suppression of anti-sporozoite immunity may occur due to concurrent skin infecting sporozoite stages and concurrent blood stage infections.[25]

In addition to acquired immunity, there are also some genetic factors that can offer some degree of protection against malaria, although through completely different mechanisms. Sickle cell trait and other haemoglobinopathies are examples of genetic

traits that protect against malaria[26]; or in the case of *P. vivax* malaria, as previously mentioned, individuals that lack erythrocyte Duffy receptors are also protected against blood-stage infection.

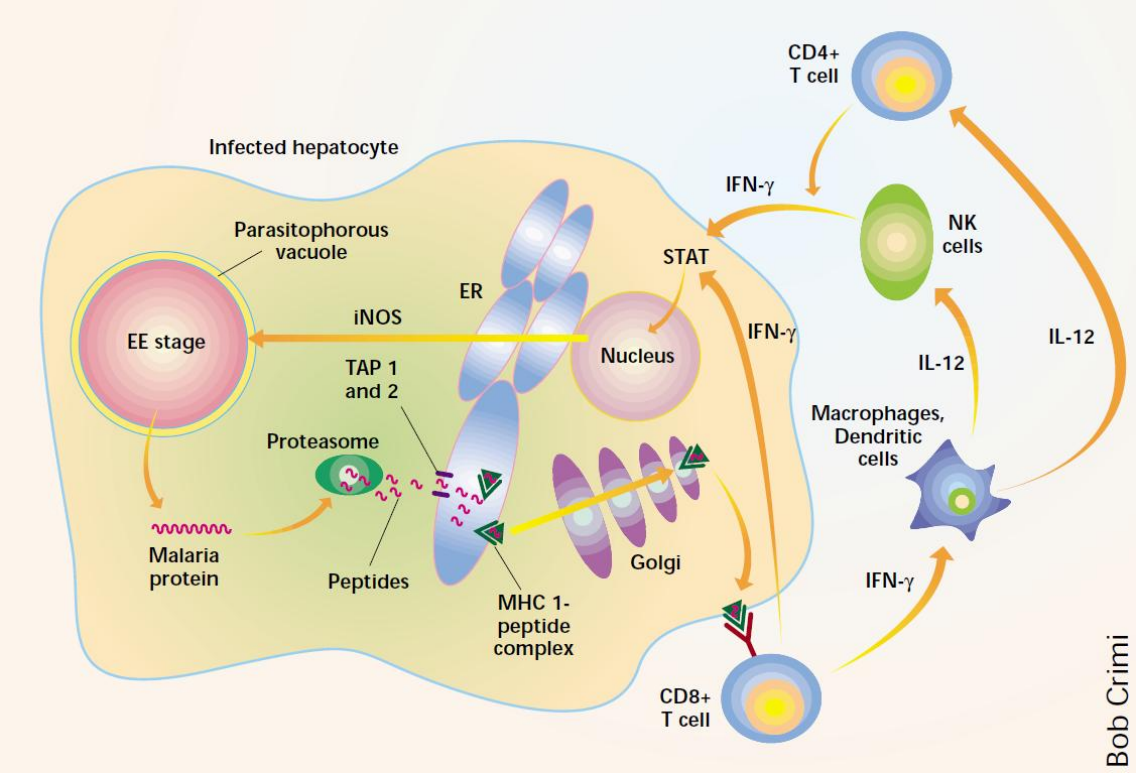



Figure 3 - **Proposed mechanism of protective immunity directed against the *Plasmodium* infected hepatocyte.[Image adapted from [22]] -** Within the infected hepatocyte, cytoplasmic malaria proteins are transformed into short peptides through a proteolytic process by proteosomes. These peptides are imported into the endoplasmatic reticulum (ER) via transporters associated with antigen processing, TAP1 and TAP2. At the ER, peptides are associated with MHC class I molecules and pass through the Golgi apparatus to the cell surface. When on the surface, the peptide/MHC complex is recognized by antigen-specific CD8+ T cells, the primary immune effectors in malaria protective immunity. Upon activation, CD8+ T cells will produce IFN- *γ* which may be upregulated by a positive feedback loop involving dendritic cells, macrophages, NK cells or CD4+ T cells. IFN- *γ* will then activate nitric oxide synthase (iNOS), via signal transducers associated with transcription (STAT), inducing the Larginine dependent nitric oxide pathway to eliminate the hepatocyte/intrahepatic schizonts.[22]

### 1.6.3 Malaria pre-erythrocytic vaccines

Pre-erythrocytic vaccines can belong to one of 2 groups: subunit vaccines and whole-organism vaccines.

*Subunit vaccines*

Subunit vaccines consist of delivering a specific parasite antigen (without introducing any organism) to the vaccinee in order to elicit immune protection against the parasite. This type of vaccine's major handicap is their lack of immunogenicity, and therefore, they often require the use of immunostimulating adjuvants or other similar strategies to elicit strong immune response against that specific antigen. RTS,S, the most well-known and advanced vaccine candidate against *P. falciparum* malaria to date is an example of this; consisting of a central repeat (NANP) of the circumsporozoite (CS) protein of the parasite (R), fused to a region know to contain T cell epitopes (T), fused in turn to the hepatitis B surface antigen as a carrier matrix (S), self-assembled with unfused S antigen ('S)(Fig.4).[27] This subunit particle is administered in combination with a potent adjuvant (AS01B) that contains the immunostimulants, toll-like receptor 4 agonist, and QS21.[28] RTS,S has advanced to Phase III testing and the results showed a 55% reduction on clinical malaria's acquisition and a 35% reduction on progression to severe malaria episodes.[29] A study performed on volunteers, immunized with RTS,S, showed that 6 months post-immunization, none of the vaccinees was protected. The vaccine induced both antibody and CD4+ T cell responses, but CD8+ T cell responses were not detectable.[30,31] Despite the modest results from RTS,S Phase III trials, there are ongoing studies to find a stronger antigen-adjuvant combination.

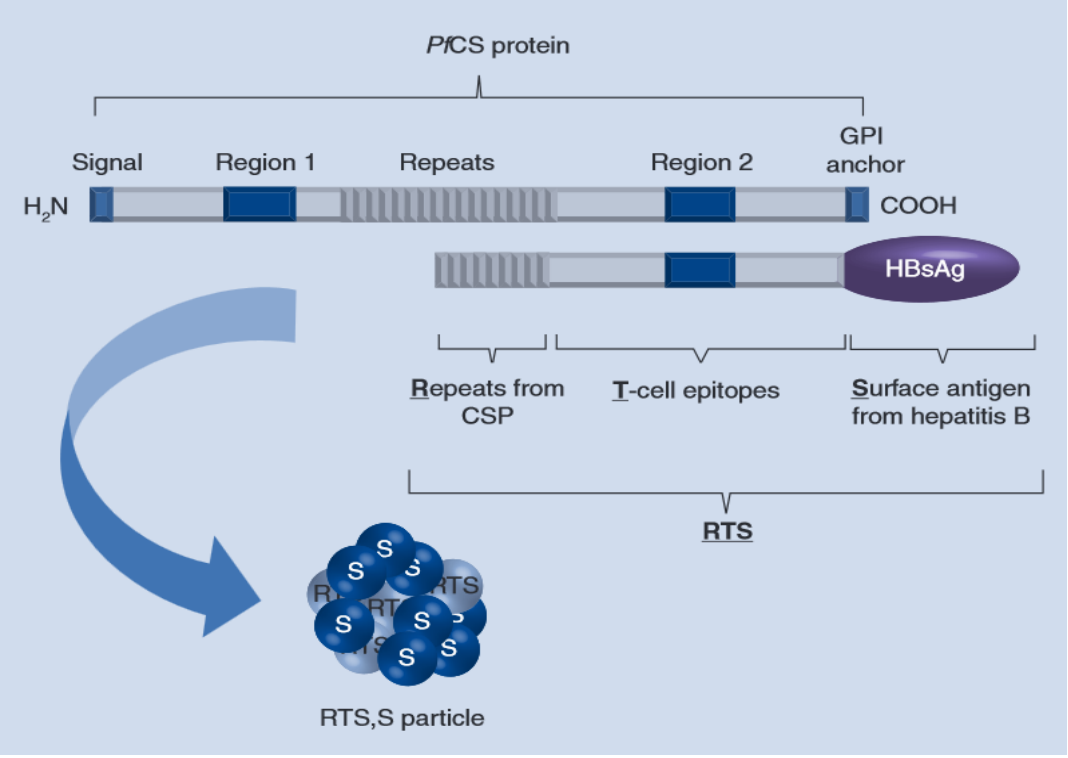



Figure 4 – **Schematic representation of the RTS,S particle**. [Image adapted from [27]]

*Whole-organism vaccines*

Whole-organism vaccines consist of administering an attenuated organism in order to elicit immune protection against the same organism or a very close related organism. In this case, the parasitic form used on whole organism vaccine strategies is the sporozoite.

However, as previously mentioned, it has been shown that dead *Plasmodium* sporozoites fail to induce protection; which can be explained mostly because the parasite needs to invade the liver cells in order to elicit differential protection and CD8+ T cell responsiveness, in addition to this, it was also shown that the later the liver-stage arrest of the parasites, the more efficacious is the protection elicited.[32]

**Radiation-attenuated sporozoites**

The whole idea of using radiation-attenuated sporozoites (RAS) as a vaccination strategy originated on the previously mentioned work of Ruth Nussensweig's group, showing that immunization of mice with X-irradiated *P. berghei* sporozoites successfully elicits protective immunity.[20] In this first approach, sporozoites were subjected to a radiation dose between 8.000 and 10.000 rads and 75.000 sporozoites were injected per mouse. Control and immunized mice were then challenged with 1.000 sporozoites per mouse and, strikingly, only 37% of the immunized mice developed patent parasitemia as compared with 90% of the controls.[20] Given these encouraging results, in the following years, immunization studies with human volunteers, showed that sterile immunity against *P. falciparum* and *P. vivax* was possible with RAS administered by infected mosquito bites, although in order to be effective it would require hundreds of mosquito bites to deliver enough sporozoites to elicit protection, therefore it was for many years considered impractical as a vaccination strategy.[33] More recent studies aimed to compare the efficacy of two administration routes for aseptic purified RAS in human volunteers; subcutaneously and intradermally.[34] The immunization protocol, varied in terms on immunization dose (7.500, 30.000 or 135.000 sporozoites per immunization) and number of immunizations (4 and 6). When challenged, 3 weeks after last immunization, not only all the immune response levels were low but merely 2 of the 80 immunized volunteers developed immune protection; 1 immunized 4 times intradermally with 30.000 sporozoites and 1 immunized 4 times subcutaneously with 30.000 sporozoites.[34] Later, another study was performed using the same formulation of aseptic purified RAS in humans injected intravenously.[35] A similar experimental setup to the previous study was performed (same immunization doses administered in 4 or 5 immunizations). The results showed that the 6/9 volunteers who received 135.000 sporozoites on the 4-dose group and all of the 6 volunteers who received 135.000 sporozoites on the 5-dose group were protected and did not develop parasitemia while groups that received lower doses were not significantly protected.[35] This led to the conclusion that not only the administration route seems to be of most importance for eliciting protection, but also the number of sporozoites used as well as the number of doses received by each volunteer.[34,35] Another concern is that this vaccine candidate uses whole *P. falciparum* sporozoites as the immunogen, leading to possible breakthrough infections to the vaccinees. Based on the human trials, it is believed that sporozoite exposure to 15.000-20.000 rads is

optimal, as no breakthrough infections were observed and still elicited immune protection, although, this approach still represents a risk, as it would require only one sporozoite to escape attenuation in order to cause disease.[33,36]

**Genetically-attenuated sporozoites (GAP)**

With the advancements in the genetic engineering field, the increased knowledge of the *Plasmodium* genome and the possibility of its manipulation, it was demonstrated that sporozoites lacking certain stage-specific genes become impaired to complete their development inside hepatocytes and confer protective immunity. [37] Studies with rodent malaria parasites, *P. berghei* and *P. yoelii*, showed that these parasites could be attenuated by deleting pre-erythrocytic stage-specific genes, such as UIS3 and UIS4, and confer sterile protection against wild type parasites.[38] These genes are involved in the formation of the parasitophorous vacuole membrane during the hepatocyte invasion; therefore, their deletion completely arrests parasite development during the pre-erythrocytic stage.[37] However, unlike in the case of uis3- parasites, the use of uis4- parasites led to occasional breakthrough infections, raising concerns about the complete attenuation of these parasites.[39,40] Other examples include the p52-/p36- *P. berghei* mutants which also showed an early arrest on liver stage development, while inducing protective immunity against wild type parasites.[41] However, further studies showed that these parasites are not effectively attenuated and that breakthrough infections may occur depending on the mice strain used.[42] The only GAPs clinical trial performed so far used p52-/p36- attenuated *P. falciparum* sporozoites as a vaccine candidate.[43] The immunization protocol consisted on the administration of sporozoites by the bite of infected mosquitoes (5 bites on the first immunization and 200 on the second). After the second immunization, 1 out 6 volunteers developed parasitemia after being immunized with 263 bites of infected mosquitoes. This event triggered a stopping rule for the clinical trial that led to its abortion.[43] A PCR was performed and confirmed that the parasites present on the volunteer's blood were indeed p52-/p35- parasites, showing that even with a successful deletion of these genes, rare events may occur whereas the attenuated parasites can still progress to blood stage and cause disease.[43] Considering all the available GAS vaccine candidates, effective attenuation is still a limitation and therefore none has successfully undergone phase I trials yet.

**Live-parasite immunization under antimalarial therapy**

This type of vaccine is based on the administration of a drug-susceptible, non-attenuated parasite along with the drug itself, to elicit immunization without development of disease. This approach has proven successful in mice with administration of azithromycin with concomitant transmission of *P. berghei*, eliciting protection against further challenges.[44] The same kind of study was performed on human volunteers with administration of *P. falciparum* (NF54 chloroquine-sensitive strain) and chloroquine, achieving similar long-term immune protection. Subjects were exposed to 12-15 infected mosquito bites once a month for 3 months while taking chloroquine.[45] Individuals were then challenged 2 months after last immunization and none of them developed parasitemia, another challenge was performed 28 months after the first challenge and only 2 out of 6 individuals developed (delayed)

parasitemia.[45] Although this vaccination strategy has shown to be efficacious, it is difficult to be implemented in the field, given the long immunization protocol and the fact that it relies on the administration of live, infectious, non-attenuated *P. falciparum* sporozoites.[45] Nonetheless, it encourages further studies regarding the development of more whole-organism malaria vaccines.

**Rodent *Plasmodium* parasites as a vaccination strategy**

Given all the limitations presented by the existing whole-organism, pre-erythrocytic vaccine candidates, either in terms of safety (use of *P. falciparum* as immunogen), or efficacy, the Prudêncio Lab of the Instituto de Medicina Molecular (IMM) has been working on the development of a new whole-organism vaccine using fully infectious, genetically modified, *P. berghei* parasites as a platform for the delivery of the immunogenic antigens of human-infective *Plasmodium* species.

This new vaccination strategy allows the administration of non-attenuated *P. berghei* sporozoites expressing a protein that is known to be immunodominant in human-infective *Plasmodium* parasites. When comparing the RAS vaccine strategy with live-parasite immunization under antimalarial therapy in terms of immunization dose required to elicit protection, we can easily understand that in some cases attenuated parasites may constitute a suboptimal immunogen as they require a much higher immunization dose in order to be effective.[43] Therefore, when using non-attenuated *P. berghei* sporozoites expressing *P. falciparum* or *P. vivax* immunodominant proteins we expect to unfold a higher antigenic potential than RAS without causing a blood stage infection.

To validate this new strategy as a potential vaccine candidate against malaria, the initial project consisted of(I) evaluating if rodent *P. berghei* can infect and develop in human hepatocytes, therefore following a natural route of infection needed for a pre-erythrocytic vaccine; (II) showing that *P. berghei* parasites are unable to cause human pathology, thereby ensuring a high safety level. (III) Determining whether a genetically modified *P. berghei* expressing a highly immunogenic human infective *Plasmodium* antigen is able to elicit an immune response able to recognize and inhibit a subsequent infection with a similar human parasites.

In order to assess if *P. berghei* could or not infect human hepatocytes, several *in vitro*, *in vivo* and *ex vivo* experiments were performed. *In vitro* infection assessment was performed using 2 hepatoma cell lines (HepG2 and Huh7) and one immortalized hepatocyte cell line (HC-04). *Ex vivo* infection was performed using primary hepatocyte/fibroblast co-cultures. Lastly, *In vivo* infection was assessed in liver-humanized mice with wild-type (WT) *P. berghei* sporozoites. Results showed that *P. berghei* is able to infect human hepatocytes and therefore is a valid option to deliver immunizing antigens to human liver cells, where the immunization process is initiated.

*P. berghei* is the most commonly used laboratory model of malaria and is not known to cause disease in humans. Nonetheless, in order to demonstrate that *P. berghei* cannot complete its development and multiply inside red blood cells, blood-humanized mice (engrafted with human erythrocytes) were infected with this parasite. Results showed that *P. berghei* has a very low ability to infect human erythrocytes and,

most relevantly, the few merozoites that are able to infect these cells, are unable to multiply and cause disease.

In order to establish the proof-of-principle of the proposed immunization strategy, a highly immunogenic antigen expressed by *Plasmodium* parasites during sporogonic and intrahepatic stages of infection was selected; the circumsporozoite (CS) protein.

The CS protein is a major surface multifunctional protein, expressed on sporozoites and early liver stage forms.[46] It is believed to be required for the hepatocyte invasion process through interaction with heparin sulphate proteoglycans (HSPGs) but also appears to be extremely important in the invasion of mosquito's salivary glands by sporozoites and their exit from mature oocysts.[46] The structure of the CS protein is highly conserved among *Plasmodium* species infecting rodents, primates, and humans.[47] It consists of a central repeat region that varies greatly in repeat sequence and repeat number throughout *Plasmodium* species, flanked by 2 highly conserved domains; Region I at the N-terminus and a thrombospondin repeat (TSR) motif at the C-terminus, region II.[48] A model has been suggested where CS protein seems to have 2 conformational states at the sporozoite's surface; an adhesive state where the TSR motif is exposed, which is displayed during development inside oocysts and hepatocyte invasion, and a non-adhesive state where the TSR motif is masked by the N-terminus, displayed by sporozoites during salivary gland invasion and migration to the liver.[46] Previous studies have shown that the CS protein is the immunodominant protein of the humoral response to *Plasmodium* infections.[49] CS protein's non-conserved repeat region varies in terms of repeat sequences and number of repeats for different *Plasmodium* species but there is also some variation within the same species.[50–52] *P. vivax* CS protein for instance, exists in 3 variations of the predominant central repeat sequences; VK210 (GDRA(A/D)GQPA), VK247 (ANGA(G/D)(N/D)QPG) and *P. vivax*-like (APGANQ(E/G)GGAA). [52]

The first approach to the proposed *P. berghei*-based vaccination strategy employed an already existing transgenic *P. berghei* line, which had its endogenous CS protein replaced by *P. falciparum*'s CS protein (*Pb*(*Pf*CS)).[48] Results revealed that the parasite had relatively low mosquito salivary gland, *in vitro*, *ex vivo* and *in vivo* infectivity. A justification for this observation is that the endogenous CS protein might be necessary for infection of both mosquito salivary glands and host hepatocytes.[48,53] In light of this, a new transgenic parasite was generated, a *P. berghei* line expressing *P. falciparum* CS protein under the control of the *P. berghei* UIS4 promoter (*Pb*(*Pf*CS@UIS4)). This latter approach differs from the previous *Pb*(*Pf*CS) as the *Pf*CS@UIS4 genomic cassette was inserted on an inert genomic locus (230p) rather than replacing *Pb*CS with *Pf*CS, allowing both CS proteins (*Pb* and *Pf*) to be expressed. UIS4 is known to be expressed by sporozoite and liver stages; therefore it was expected to lead to the expression of *Pf*CS during those same stages. Also, the results showed that salivary gland and hepatocyte infectivity are similar for wild-type (WT) and *Pb*(*Pf*CS@UIS4) parasites, overcoming the initial limitations of the *Pb*(*Pf*CS) parasite. Post-immunization results revealed that sera from *Pb*(*Pf*CS) and *Pb*(*Pf*@UIS4) immunized mice; contains high titers of *Pf*CS specific antibodies, are able to elicit a cellular immune response; and those antibodies are also able to

recognize and bind with high avidity to *P. falciparum* sporozoites. Additionally there is some evidence that there is some cross-species protection between *P. berghei* and *P. falciparum* conserved epitopes, at the cellular immunity level.[54]

The results available so far indicate that this approach can potentially be more versatile, efficient and safer than other malaria vaccination strategies.

Recently, Inês Albuquerque from IMM's Prudêncio Lab, using a blood sample obtained from a *P. vivax*-infected patient in the field (Thailand), was able to isolate the gene encoding the *P. vivax* CS protein and insert it on a plasmid containing the 230p targeting region and the promoting regions of the UIS4. In order to generate a new *P. berghei* line expressing the *P. vivax* CS protein, *Pb*(*Pv*CS@UIS4), a transfection was performed according to the GIMO (Gene Insertion Marker Out) method [55], similarly to what was done in order to generate the *Pb*(*Pf*CS@UIS4) transgenic parasite (Fig.5).

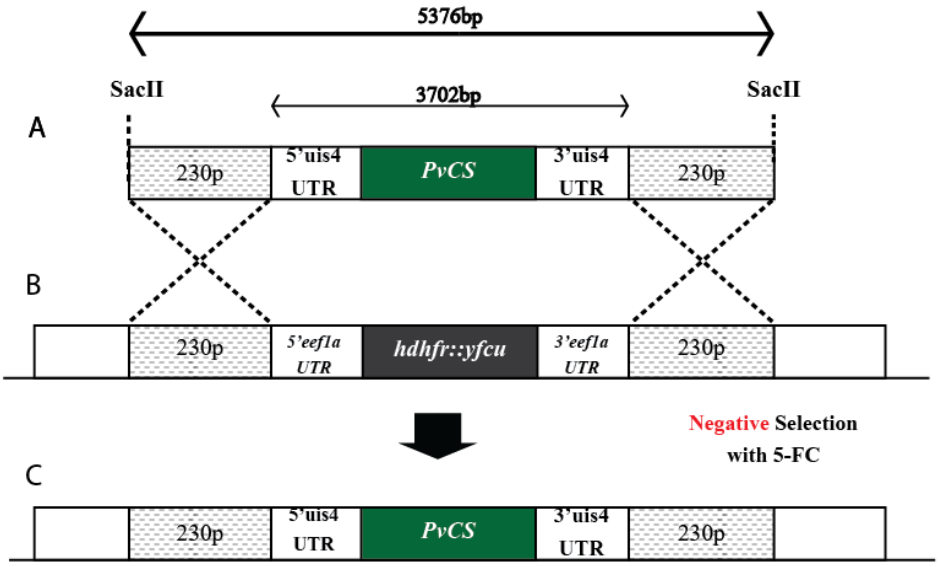



Figure 5 – **Schematic representation of *Pb*(*Pv*CS@UIS4) transfection through GIMO's transfection method** – A – Linearized plasmid containing the *Pv*CS@UIS4 gene cassette inside the 230p targeting regions. B – By double cross-over homologous recombination, *Pb*GIMO parasites' positive/negative selectable marker (*hdhfr::yfcu*) is replaced by the *Pv*CS@UIS4 cassette. C – Transfected parasites are negatively selected using the 5-FC drug, which will eliminate all parasites that still have the selectable marker on their genome, leading to populations where all parasites are *Pb*(*Pv*CS@UIS4).

The GIMO transfection method consists of using a genetically modified *P. berghei* or *P. yoelii* as recipient motherline for transfection. These transgenic parasites have a positive/negative selection marker (*hdhfr::yfcu*) stably integrated into the silent 230p locus. Using a linearized plasmid with a gene of interest fused to the 230p targeting region (Fig.5.A), is possible to transfect the recipient motherline by double cross-over homologous recombination leading to simultaneous replacement of the

selection marker with the gene of interest (Fig.5.B). After this, the parasites are negatively selected with 5-fluorocytosine (5-FC). The 5-FC drug will eliminate any parasite that still has the *yfcu* selectable marker, resulting on the generation of parasites with the integrated gene of interest and no drug resistance gene (Fig.5.C).[55]

Based on the results obtained with *Pb*(*Pf*CS@UIS4) parasites, this new vaccination strategy represents a good alternative to the ones that have and are being developed so far. However the newly generated transgenic parasite, *P. berghei* expressing *P. vivax* CS under the control of the *Pb*UIS4 promoter (*Pb*(*Pv*CS@UIS4)), requires developmental characterization in order to proceed to more advanced immunological studies. After the parasite's transfection, 2 clones of *Pb*(*Pv*CS@UIS4) were selected for characterization; *Pb*(*Pv*CS@UIS4) clone 2 and *Pb*(*Pv*CS@UIS4) clone 4.

# 2. Aims

As mentioned before, in order to proceed with further, immunological studies, the vaccine candidate *Pb*(*Pv*CS@UIS4), must be fully characterized in terms of infectivity and development. . In the specific context of this vaccine candidate, it is also important to characterize the expression of the inserted CS gene, to ensure that it is being expressed correctly throughout various development stages. Therefore, in order to characterize the new vaccine candidate *Pb*(*Pv*CS@UIS4), the following aims were established for this thesis:

- To compare the *Pv*CS gene sequence from the *P. vivax* isolate used in this study with sequences of the same gene found in isolates from different geographic regions across the world. This will ensure that the CS protein being inserted into our vaccine candidate is not significantly different from the CS protein found in most *P. vivax* populations worldwide. To do this we shall perform a sequence alignment between our *Pv*CS sequence against reference sequences found on a *Plasmodium* genome database (PlasmoDB).
- To characterize *Pb*(*Pv*CS@UIS4) parasite development and infectivity during sporogonic development. This task is to be performed by registering oocyst numbers in the mosquito midgut at 10 days post infectious blood meal and sporozoites in the mosquito salivary glands at 21 days post infectious blood meal.
- To assess *Pb*(*Pv*CS@UIS4) parasite's ability to infect and develop hepatocytes *in vitro*. In order to do this, two different hepatoma cell lines, Huh7 and HepG2, will be infected with freshly isolated sporozoites. Infection and development will be assessed by immunofluorescence microscopy at 48h post infection.
- To assess the ability of *Pb*(*Pv*CS@UIS4) parasites to infect and develop *in vivo*. C57BL/6J mice will be infected through i.v. injection of sporozoites. Infected livers will be analyzed by qRT-PCR and immunofluorescence microscopy.
- To observe CS protein expression on sporozoites and exo-erythrocytic parasite forms, *in vitro* and *in vivo*. This will be done by immunofluorescence microscopy, using specific antibodies against *Pb*CS and *Pv*CS.
- Blood-stage development of *Pb*(*Pv*CS@UIS4) will be analyzed throughout time to assess the parasite's ability to exit the liver, infect and replicate inside erythrocytes.

Throughout the whole study, *Pb*(*Pv*CS@UIS4) results will be compared to *Pb*GIMO parasites as the latter represent the non-transfected, wild-type parasites.

# 3. Materials and Methods

## 3.1 Mice (C56BL/6J AND BALB/C)

*In vivo* experiments were performed on C56BL/6J and BALB/C mice, ordered from Charles River Laboratories international. Ages ranged between 4-6 weeks and were housed, upon arrival, in specific pathogen-free conditions, at the animal house facility of Instituto de Medicina Molecular (IMM). All the experimental work involving animals was performed according to the EU regulations and was approved by the Animal Care and Ethical Committees of IMM.

## 3.2 Parasite lines

-*Plasmodium berghei* GIMO (Gene Insetion Marker Out)

In order to produce our transgenic parasites, *Pb*GIMO was used as transfection motherline, and therefore used as control on characterization experiments. *Pb*GIMO has a positive/negative selection marker cassette (*hdhfr::yfcu*) on the 230p locus.

-*Plasmodium berghei* expressing the *Plasmodium vivax* CS protein under the control of the *Pb*UIS4 promoter (*Pb*(*Pv*CS@UIS4))

This transgenic parasite was produced through the GIMO transfection method (by Inês Albuquerque). It expresses *P. vivax*'s CS protein under the control of the *P. berghei* UIS4 promoter. Confirmation of integration will be shown in the results section of this thesis. After confirmation, 2 clones were selected to proceed with characterization; *Pb*(*Pv*CS@UIS4) Clone 2 and *Pb*(*Pv*CS@UIS4) Clone 4.

## 3.3 Rearing of *Anopheles stephensi* mosquitoes

*Anopheles stephensi* mosquitoes were reared in IMM's insectary at 27ºC and 80% humidity. Larvae developed in trays with water and standard fish food while adult mosquitoes were maintained on a 10% sucrose solution with 0,05% of p-aminobenzoic acid (PABA), on a 12h light/dark cycle, according to standard rearing conditions.[56] The rearing schedule is described below (Figure 6);

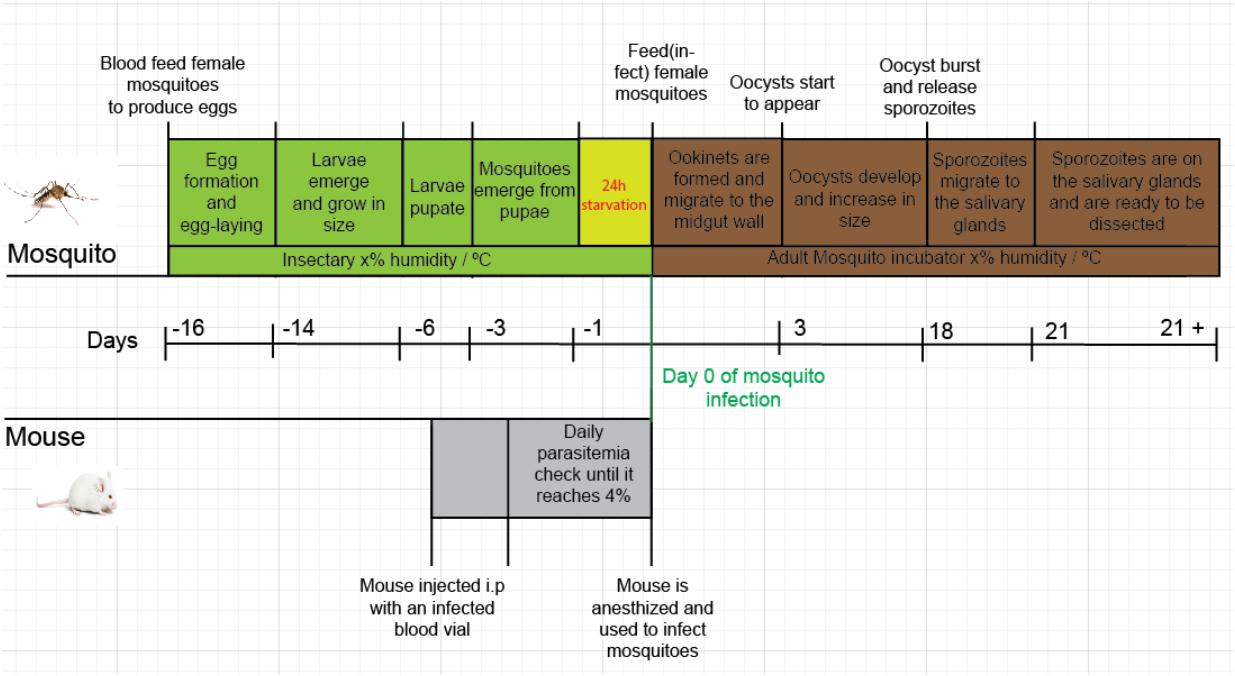



Figure 6 – **Mosquito rearing and infection Schedule** – Day 0 corresponds to the day that mosquitoes were fed with an infectious blood meal. (Schedule was repeated on a weekly basis) Adult female mosquitoes were fed with rodent blood in order to produce eggs (16 days before the infectious blood meal). Through the 2 days after feeding, female mosquitoes laid their eggs. Eggs hatched and larvae emerged (-16 to -14). Larvae grew in size through the following 7-8 days (-14 to -6). At day -6, larvae began to pupate. Also, on the same day, mice were injected i.p. with an vial of frozen parasite stabilates. At day -3, mosquitoes started to emerge from pupae. Mouse parasitemias were checked daily until 4% parasitemia. Male gametocyte exflagellation was accessed as it is a strong indicator of transmission capacity of infected mice. At the day previous to blood infection, adult mosquitoes were moved from the insectary to a mosquito incubator and left on starvation for 24h. The following day (day 0), infected mice were anesthetized and used to feed starved mosquitoes. Within the mosquito, ookinets are formed and develop into oocysts on the first 3 days post infectious blood meal. From day 3 to 18, oocysts grow in size. At day 18, oocysts start to burst and release sporozoites, which migrate to the salivary glands through the following 3 days. At day 21, sporozoites are already on the salivary glands and ready for dissection.

## 3.4 Maintenance of *Plasmodium berghei* infections

*Sporozoite collection*

Sporozoites were freshly extracted from the salivary glands of female *Anopheles stephensi* mosquitoes by hand-dissection and collected in RPMI medium. To obtain free sporozoites the salivary gland suspension was mechanically homogenized and filtered through a 70 µm strainer. Sporozoites were counted in a Neubauer-chamber.

*Monitoring of parasitemias*

A small incision was made on the tip of the mouse tail and a small drop of blood was placed on a glass slide. The blood was smeared by using a second glass slide, dried in air, fixed in methanol for 30 seconds and stained in Giemsa solution for 10 min. The smear was dried in air and observed with a x100 oil immersion objective.

*Exflagellation test*

Similarly to what is done for monitoring parasitemias, a drop of blood is collected to a microscope slide and a coverslip is placed on top of it and left at room temperature for 7 min. The slide was observed on an optical microscope with phase or interference contrast with a ×40 objective. Exflagellation is noted by the violent lashing movements of the male gamete flagella.

*Oocyst counts*

For oocyst counts, mosquito midguts were hand-dissected 10 days after the infectious blood meal. To enhance contrast, midguts were mounted on a glass slide with a drop of mercurochrome. Oocysts were then counted using an Olympus CX41 optical microscope with a 20x objective.

*Mouse infection*

BALB/C mice were infected through intraperitoneal injection with $10^6$ parasited red blood cells, from either *Pb*GIMO or *Pb*(*Pv*CS@UIS4) clone 2/4, collected from a previously infected mouse (direct blood transfer) or from a stored frozen parasite stabilate. Normally, “mouse-to-mouse” blood transfers never exceed 5 passages (due to telomeric erosion of the parasite), after which a blood vial is injected instead.

*Mosquito infection*

Infected mouse parasitemias were counted 3-4 days after passage. When parasitemias range between 4%-20% and the exflagellation test shows more than 2 exflagellation events per field it means that the gametocytes present on the blood are potentially infectious to the mosquitoes. Before the blood meal, the mouse was anesthetized (ketamine-xylazine) and placed on the top of a cage with previously starved female mosquitoes. The mouse remained on top of the cage (belly down) for at least 30min, so most of the mosquitoes present on the cages had enough time to bite. During the blood meal, the cage was maintained at 19ºC-21ºC on a darkened environment. After the feeding, mosquitoes were left in incubators at 21ºC and 80% humidity and 12h light/dark cycle until sporozoite collection.

## 3.5 *In vitro* culture of hepato-cellular carcinoma celllines

For *in vitro* characterization of *Pb*(*Pv*CS@UIS4) parasites, cultures of 2 human adherent hepatoma cell lines, Huh7 and HepG2, were employed.

Huh7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS),1% non-essential amino acids, 1% penicillin/streptomycin,1%

glutamine and 10mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), pH 7, and maintained at 37ºC with 5% $CO_2$.

HepG2 were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 1% penicillin/streptomycin, 1% glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES), pH 7, and maintained at 37 ºC with 5%$CO_2$.

## 3.6 *In vitro* infection

In order to analyze infection, cells were seeded 1 day before infection, on 24-well plates (with coverslips), with a confluence of 50.000 cells/well for Huh7 and 70.000 cells/well for HepG2. On the day of infection, each well was infected with 30.000 sporozoites obtained from infected mosquito's salivary glands (21-29 days after infectious blood meal), plates were then centrifuged (3000 RPMs for 5 min) and incubated at 37ºC for 48h. At 48h post-infection, cells were fixed with paraformaldehyde (4%) for 10 min, washed with 1x PBS and stored at 4ºC.

## 3.7 *In vivo* experiments

C56BL/6J mice were anesthetized with isofluorane and then inoculated by retro orbital injection with sporozoites obtained from infected mosquitoes (21 days after the infectious blood meal). Each mouse was injected with 30.000 sporozoites. At 44h post inoculation, all mice were sacrificed and their livers collected. The left liver lobes were snap-frozen in liquid nitrogen and stored at -80ºC (to be used for qRT-PCR analysis); the remaining was fixed on 4% paraformaldehyde and stored at 4ºc (for microscopy).

## 3.8 RNA extraction and cDNA synthesis

With a blender rod, mouse livers were homogenized in denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% n-lauryl sacosomine) supplemented with 0.1M β-mercaptoethanol. After homogenization, RNA was extracted using a Quiagen RNA extraction kit (volume of elution, 35µl).

RNA was quantified on a NanoDrop 1000 Spectrophotometer and dilutions were made so that all samples were at final RNA concentration 200 µg/µl. One µg (5µl) of each sample's RNA was used to synthesize cDNA according to the following reaction:

| cDNA reaction mixture | Volume µL/reaction |
|---|---|
| 10x Buffer | 2 |
| 50µg/mL Random Hexamer | 1 |
| 10mM dNTP mix | 1 |
| 40 U/µL RNAse inhibitor | 0,5 |
| 200 U/µL Reverse transcriptase | 1 |
| $H_2O$ | 9,5 |
| Template RNA (1µg) | 5 |
| Final Volume | 20 |

**Table 1 – cDNA reaction mixture**

cDNA synthesis program was as follows: 25ºC – 10 min; 55ºC – 30 min; 85ºC – 5 min. Reaction performed on a Biometra Personal Thermo cycler.

## 3.9 Real Time quantitave PCR

Malaria infection was quantified using quantitative real-time PCR with primers specific for *P. berghei* 18S and normalized to an endogenous mouse housekeeping gene, HPRT (Hypoxanthine Guanine Phosphoribosyl Transferase). The primers were designed to span exon-exon junctions, to avoid genomic DNA amplification, and so, RNA-DNAse treatment was not required. Two µl of cDNA from each sample were added per well on a 96-well PCR-plate (2 wells per sample) along with the qRT-PCR Mixture (see table below).

| 18S RT-PCR Mixture | Volume µL/reaction | HPRT RT-PCR Mixture | Volume µL/reaction |
|---|---|---|---|
| SYBR® Green Real-Time PCR Master Mix | 10 | SYBR® Green Real-Time PCR Master Mix | 10 |
| 18S Forward primer | 0,4 | HPRT Forward primer | 0,4 |
| 18S Reverse primer | 0,4 | HPRT Reverse primer | 0,4 |
| $H_2O$ | 7,2 | $H_2O$ | 7,2 |
| cDNA Template | 2 | cDNA Template | 2 |

**Table 2 – qRT-PCR mixture components**

| qRT-PCR Reaction Program | |
|---|---|
| 50ºC | 2 min |
| 95ºC | 10 min |
| 95ºC | 15 s |
| 60ºC | 1 min |
| 95ºC | 15 s |
| 60ºC | 1 min |
| 95ºC | 30 s |
| 60ºC | 15 s |

40 Cycles

**Table 3 – qRT-PCR reaction program**

To analyze infection, gene expression was analyzed by the comparative $C_T$ (cycle threshold) method and a ratio between 18S and HPRT was calculated for each sample; $2^{HPRT}/2^{18S}$ and compared between samples.

## 3.10 Genomic DNA extraction from blood stage parasites

Parasite DNA used in the PCR for confirmation of transgenic integration was extracted from infected whole-blood vials by the phenol-chloroform method. The blood sample was centrifuged at 13.000 RPM for 1 min in order to create a cell pellet. Supernatant was discarded and added 350µl of RBC lysis buffer to the cell pellet. The pellets were resuspended and vortexed in order to lyse the cells efficaciously. The sample was transferred to a new tube and added 10µL of RNaseA (10mg/mL) and incubated for 10min at 37ºC. After incubation, 10µL of proteinase K (20mg/mL) were added to the tube and incubated for 1h at 37ºC. Phenol was added to a final volume of 750 µl. Tube was inverted several times and centrifuged at maximum speed for 5 min. The supernatant was transferred to a clean tube. Phenol/chloroform/isoamylalcohol (25:24:1) was added up to 1,5 ml. The tube was inverted several times and centrifuged at 13.000 for 5 min. The supernatant was again transferred into a clean tube and chloroform/isoamylalcohol (24:1) was added up to 1,5 ml. Tube was inverted several times and centrifuged at 13.000 for 5 min. The supernatant was transferred into a new tube and added 1/10 volume of 3M Sodium Acetate and 96% ethanol for precipitation. The tube was then centrifuged 13.000 speed for 10min. Supernatant was discarded and the pellet was washed with 70% ethanol, air-dried and dissolved in MiliQ water. DNA was then quantified on a Nanodrop 2000.

## 3.11 Confirmation PCR for genomic integration

In order to confirm that the transfection occurred as expected and that the *Pv*CS@UIS4 cassette was successfully inserted in the 230p genomic locus of the

parasite, 3 different PCR reactions were performed; 5' integration, 3' integration and selectable marker (see results for details).

| PCR Reaction Mixture | Volume µL/reaction |
|---|---|
| 10x PCR buffer | 5 |
| 50mM MgCl2 | 3 |
| NZYTaq DNA polimerase | 0,5 |
| 0.1M dNTP | 1,25 |
| DNA template | 50ng |
| Forward primer | 2,5 |
| Reverse primer | 2,5 |
| $H_2O$ | until 50µL of total volume |

**Table 4 – PCR reaction mixture components**

| 5' and 3' Integration PCR Program | |
|---|---|
| 95ºC | 2 min |
| 95ºC | 45 s |
| 48ºC | 10 s |
| 50ºC | 10 s |
| 52ºC | 10 s |
| 72ºC | 2 min |
| 72ºC | 2 min |

35 Cycles

**Table 5 – 5' and 3' confirmation of integration PCR reaction program**

| Selectable marker PCR Program | |
|---|---|
| 95ºC | 2 min |
| 95ºC | 45 s |
| 50ºC | 10 s |
| 54ºC | 15 s |
| 56ºC | 10 s |
| 72ºC | 45 s |
| 72ºC | 2 min |

35 Cycles

**Table 6 – Selectable marker confirmation of integration PCR reaction program**

After the reaction, PCR products were run on a 1% agarose gel and revealed on a Chemidoc XRS+.

## 3.12 Antibody production and acquisition

In order to produce antibodies against *Pv*CS and *Pb*CS, 2 hydridoma cell lines, 2F2 and 3D11 respectively, were ordered from MR4 (Malaria Research and Reference Reagent Resource Center) and cultured in the lab:

Cells were thawed at 37ºC and ressuspended in 5 mL of DMEM medium. After cell resuspension, these were transfered on to a culture flask with 15 mL of fresh DMEM medium supplemented with 10-15% FBS and left in the incubator at 37ºC.

Every two days, the cells were aspirated from the culture flask and centrifuged at 1800 RPM for 5 min. After centrifugation, the supernatant containing the antibodies were collected and stored at 4ºC. Cells were then resuspended and based on the confluence were split into 2 or 3 culture flasks with fresh DMEM medium. This process was repeated until obtaining 2L of antibody-containing supernatant. Cells in the supernatant were removed by centrifugation at 10000 RPMs for 20 min. In order to precipitate the antibodies present in the supernatant, a 2L saturated solution of ammonium sulfate were previously prepared (900g per 1L of $H_2O$). Ammonium sulfate was slowly added to the supernatant and left overnight with constant stirring. On the following day, the solution was transferred to 250mL flasks and centrifuged at 10.000 RPM for 20min (4ºC). The supernatant was discarded and the pellet was resuspended in PBS. This solution was then transferred to a dialysis membrane and dialyzed against PBS. The solution was finally stored at -80ºC and sent to Dr. Ricardo Franco's laboratory (FCT-UNL) where the final steps of the purification were performed.

## 3.13 Imunofluorescence microscopy

### Sporozoites

Following sporozoite collection, 30.000 sporozoites /well were placed on a "10-well slide" (ThermoScientific Diagnosis Microscope slides) and left at room temperature until the wells were completely dry. After drying, sporozoites were fixed with 4% paraformaldehyde for 10min and then washed with PBS. The CS protein is found at the parasite's membrane, so no permeabilization was required. Sporozoites were then incubated with 3D11 (against *P. berghei* CS) and 2F2 (against *P. vivax* CS). Both antibodies were obtained from mouse hybridomas, therefore the staining had to be done in 2 different wells for each parasite. Both antibodies were diluted on PBS with 0,25% gelatin, 1:200 3D11 and 1:500 2F2. Twenty-five µl of each antibody solution were used per well. The slides were left inside a wet chamber at 37ºC for 30min. Slides were washed with PBS and 25 µl of a secondary antibody solution containing Alexa-Fluor 488 anti-mouse, Donkey IgG (1:500) and Hoechst(1:150) were placed on each well. Slides were left on a wet chamber for 30min at 37ºC. After that, each slide was mounted with Fluoromount G (MARCA) and a cover slide. Immunofluorescent CS protein localization and expression images were acquired on a Zeiss LSM 710 confocal point-scanning microscope with a 63x objective, 1660x1660 resolution, 4 scans, scanspeed 6.

### Coverslips

Coverslips obtained from *in vitro* culture and infection of hepatoma cell lines were fixed with 4% paraformaldehyde for 10 min (as stated above). After that, cells were permeabilized with PBS, 0.25% Triton X-100 and 1% Bovine Serum Albumin (BSA) for 1h. Primary antibodies used were either 3D11 and a-UIS4 or 2F2 and a-UIS4. These were diluted on the solution used for permeabilization; 3D11 (1:200), 2F2 (1:500), UIS4 (1:450). Each coverslip was incubated with a single drop of antibody solution in a wet chamber for 1,5h at room temperature. Coverslips were then washed and incubated with secondary antibodies; Alexa-Fluor 488 (anti-mouse, Donkey IgG), Alexa-Fluor 555 (anti-goat, Donkey IgG) and Hoechst (for nuclei labeling). Coverslips

were left in a wet chamber for another 1,5h. Coverslips were then washed thrice and mounted with Fluoromount G (Southern Biotech). EEFs were counted on a Zeiss Axiovert200MWidefield fluorescence microscope, with a 20x objective, and normalized to the total number of nuclei. EEF size was also measured on the same microscope with a 40x objective. Immunofluorescent CS protein localization and expression images were acquired on a Zeiss LSM 710 confocal point-scanning microscope with a 63x ojective. *Images acquired* with 1750x1750 resolution, 4 scans*,* scanspeed 6*.* Images of immunofluorescence-stained sections were analyzed with ImageJ 1.49b software.

**Liver slices**

Livers previously collected and fixed in 4% paraformaldehyde for 10 min, were washed and then sliced into 50-µm sections using a vibratome (VT1000S, Leica). Sections were permeabilized and blocked overnight in 0,5% Triton X-100, 1% Bovine Serum Albumin (BSA) and IgG anti-mouse (1:150), washed thrice with PBS, and incubated with primary antibodies for 2h; Primary antibodies employed were 3D11(1:200) and anti-UIS4(1:450) or 2F2(1:500) and anti-UIS4 (liver slices were divided into 2 groups for each parasite, in order to perform both stainings). After primary antibody incubation, sections were washed thrice with PBS and incubated with the following secondary antibodies; Alexa-Fluor 488 1:500(anti-mouse, Donkey IgG), Alexa-Fluor 555 1:500(anti-goat, Donkey IgG), Alexa-Fluor 660–phaloidin 1:50 and Hoechst 1:150. Sections were mounted on slides with Fluoromount G (Southern Biotech). EEFs were counted on a Zeiss Axiovert200M Widefield fluorescence microscope, with a 10x objective, and normalized to the total area observed of the slices. EEF size was also measured on the same microscope with a 40x objective. Immunofluorescent CS protein localization and expression images were acquired on a Zeiss LSM 710 confocal point-scanning microscope using a 63x objective*.* Images were acquired with 1024x1024 resolution, 4 scans, scanspeed 6. Images of immunofluorescence-stained sections were analyzed with ImageJ 1.49b software.

## 3.14 Statistical analyses

Results were expressed in terms of mean ± standard error of the mean (SEM). Statistical comparisons were made using the Mann-Whitney non-parametric test.

# 4. Results

## 4.1 Characterization of the *Pv*CS sequence

Before the transfection of *Pb*GIMO parasites in order to originate *Pb*(*Pv*CS@UIS4) parasites, vector plasmids were generated, containing the *Pv*CS gene sequence flanked by the *Pb*UIS4 promoter sequences forming a cassette fused to the 230p targeting region. These vector plasmids were amplified by transforming competent bacteria and subsequently sequenced (Stabvida sequencing services). Consensus of the different sequenced bacterial colonies can be viewed on Appendix 2. Using these sequencing results, the *Pv*CS gene selected for parasite transfection was aligned against reference *Pv*CS gene sequences present on PlasmoDB (Sal-1, Brazil I and India VII), in order to evaluate the divergence of the *Pv*CS gene amplified from our *P. vivax* isolate from Thailand in terms of mutations/polymorphism and central region repeat sequence number and diversity. Reference sequences used for alignment belong to isolates from Salvador, Brazil and India, respectively (Fig.7).

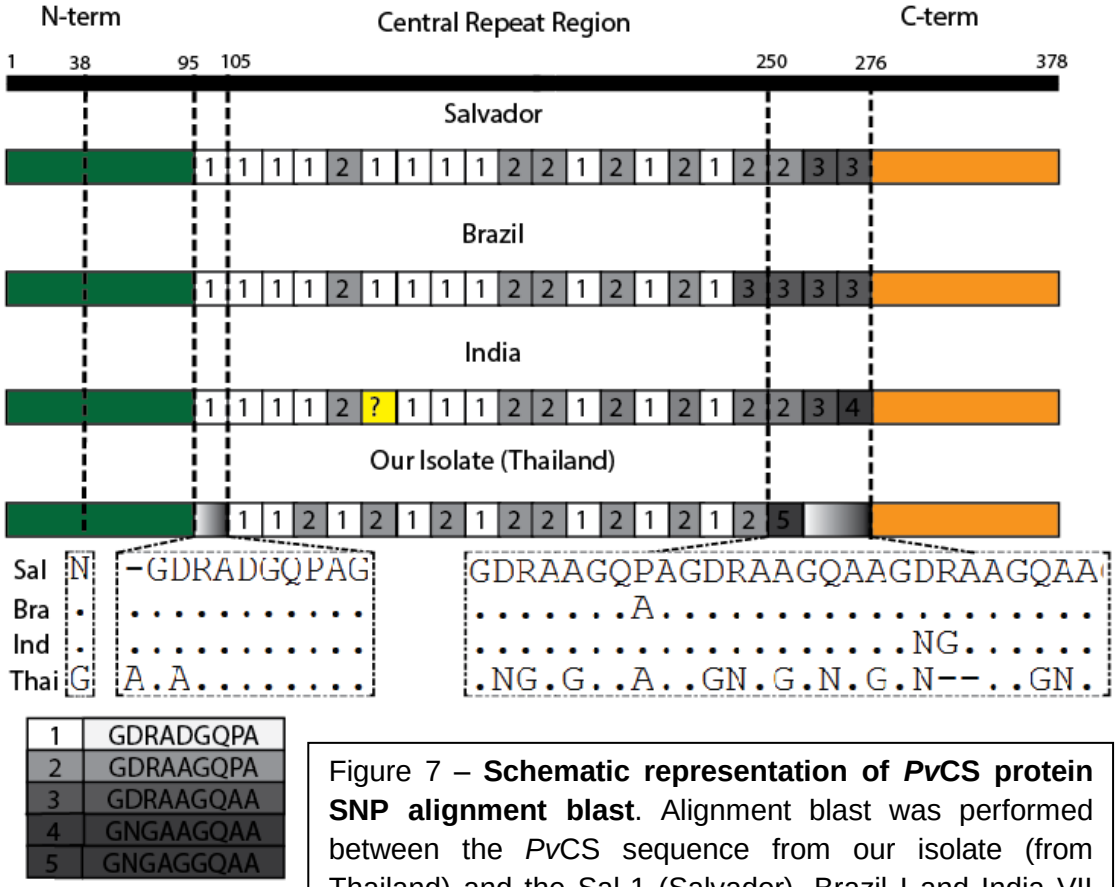



Figure 7 – **Schematic representation of *Pv*CS protein SNP alignment blast**. Alignment blast was performed between the *Pv*CS sequence from our isolate (from Thailand) and the Sal-1 (Salvador), Brazil I and India VII isolates. N-terminus and C-terminus regions are represented in green and orange, respectively. Each numeric square represents a central region repeat. Non-synonymous SNPs are highlighted below.

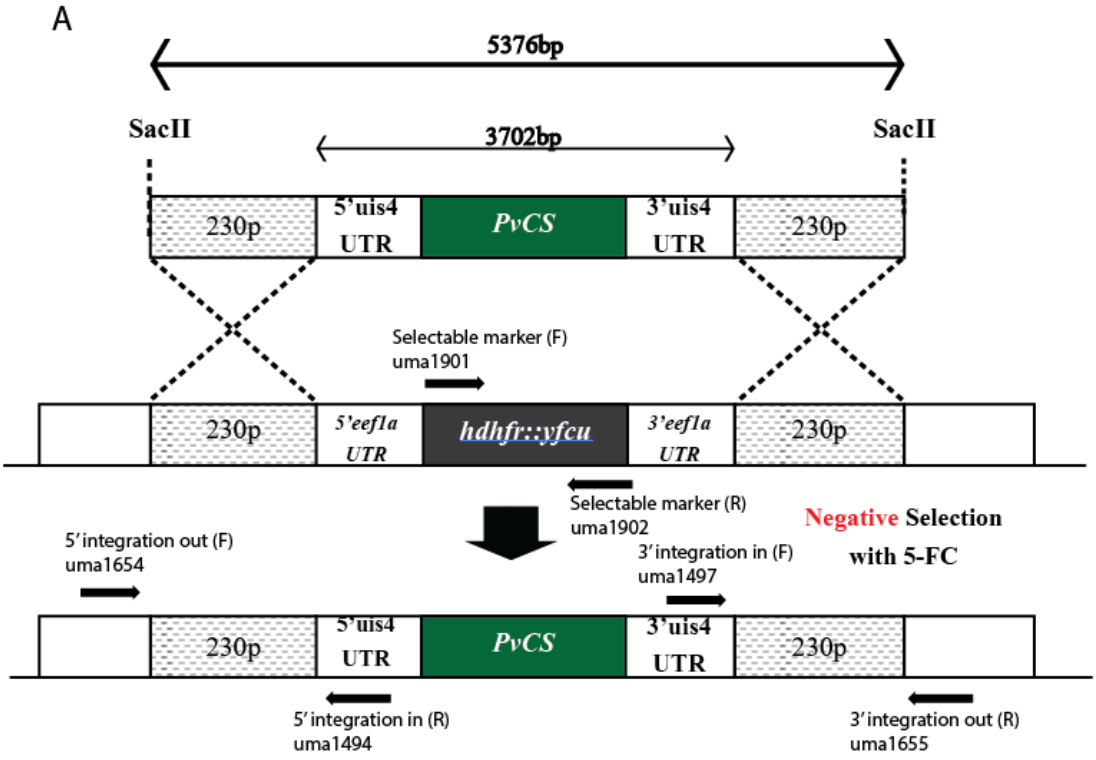
As expected, both the N-terminus and C-terminus regions were extremely conserved in relation to the reference *Pv*CS sequences present on PlasmoDB, with a single non-synonymous polymorphic event at position 38, originating a transition from an asparagine to a glycine. However, a great variability on the central repeat region can be observed.

As previously mentioned, three major variants of the central repeat regions of the *P. vivax* CS have been described; VK210 (GDRA(A/D)GQPA), VK247 (ANGA(G/D)(N/D)QPG) and a much less common variant named *P. vivax*-like (APGANQ(E/G)GGAA).[52] Both VK210 and VK247 variants are globally distributed, but some geographic biases have been described on several studies.[47,51,52,57] Our results indicate that the *Pv*CS sequence used in our study is similar to the most common and well adapted variant of the *P. vivax* CS protein, the VK210. Besides being the most widely spread, previous studies revealed that *Anopheles* mosquitoes appear to be more easily infected by the VK210 variant of *P. vivax*.[58] Despite VK210 variant's most common peptide repeat motifs being GDRADGQPA and GDRAAGQPA, our isolate has one other peptide repeat motif; GNGAGGQAA. This central repeat has also been found on two other studies performed on Sri Lanka's and Brazil's *P. vivax* populations.[47,51] Insertions and deletions in the central repeat domain generate different CS protein variants that may be positively selected if the mutant parasites evade host immunity.[51] In theory, these variations can affect the structure of the CS protein and may be responsible for parasite's evasion of natural variant-specific immunity. However, there is no proof of whether or not that is the case.

## 4.2 PCR confirmation of the genomic integration for the *Pv*CS@UIS4 gene cassette

After GIMO-transfection, parasite populations consist in a mix between transfected and non-transfected parasites despite the negative selection process performed with 5-FC; therefore, parasite cloning after negative selection is an essential step in GIMO-transfection in order to obtain a clonal population originated from a single transfected parasite that express the transgene (with no selectable marker). Parasite cloning consists in injecting a dilution of mouse blood infected with a mixed population of transfected and non-transfected parasites that is equivalent to approximately a single parasite being injected per mouse so that only a clonal population develops from a single parasite. In order to ensure that our clonal populations of *Pb*(*Pv*CS@UIS4) (clone 2 and clone 4) parasites consist only of successfully transfected parasites a series of PCR reactions were performed (Fig. 8.A/B). First, PCR reaction primers, were designed to amplify only in case of proper 5' integration; the uma1654 forward primer binds outside the 230p targeting region and the uma1494 reverse primer binds inside the integrated region at 5'UIS4 UTR (expected amplicon size – 1240bp). Similarly to the first reaction, the second PCR reaction would only amplify if proper 3' integration occurred; the uma1497 forward primer binds inside the integrated region at 3'UIS4 UTR and the uma1655 reverse primer binds outside the 230p locus targeting region (expected amplicon size – 1989bp). As mentioned before, upon transfection, the selectable marker present in the 230p locus of *Pb*GIMO parasites is replaced by the *Pv*CS@UIS4 cassette. In order to ensure that there are no mixed populations, the

reaction performed with uma1901 (*hdhfr* forward primer) and uma1902 (*yfcu* reverse primer), only amplifies if the parasite contains the selectable marker cassette, thereby demonstrating whether or not the *Pb*(*Pv*CS@UIS4) clones consist in mixed populations of transfected and non-transfected (*Pb*GIMO) parasites (no amplification expected for *Pb*(*Pv*CS@UIS4) clones). Our results show that both clones have correct 5' and 3' integration and none of them showed amplification of the selectable marker cassette; therefore no untransfected parasites are present on *Pb*(*Pv*CS@UIS4) clone 2 and 4 populations (Fig 8.C). See appendix 1 for primer sequences.




B

| b/ground | region | primers | | amplicon |
|---|---|---|---|---|
| *Pb*GIMO (hdhfr::yfcu/230p) | 5'int | uma1654 | F outside 5' TR | No Amp. |
| | | uma1494 | R inside *Pb*UIS4 5' | |
| | 3' int | uma1497 | F inside *Pb*UIS4 3' | No Amp. |
| | | uma1655 | R outside 3' TR | |
| | SM (hdhfr) | uma1901 | F inside hdhfr | 1108 |
| | | uma1902 | R inside hdhfr | |
| *Pb*(*Pv*CS@UIS4) (PvCS@UIS4/230p) | 5'int | uma1654 | F outside 5' TR | 1240 bb |
| | | uma1494 | R inside *Pb*UIS4 5' | |
| | 3' int | uma1497 | F inside *Pb*UIS4 3' | 1989 bp |
| | | uma1655 | R outside 3' TR | |
| | SM (hdhfr) | uma1901 | F inside hdhfr | No Amp. |
| | | uma1902 | R inside hdhfr | |

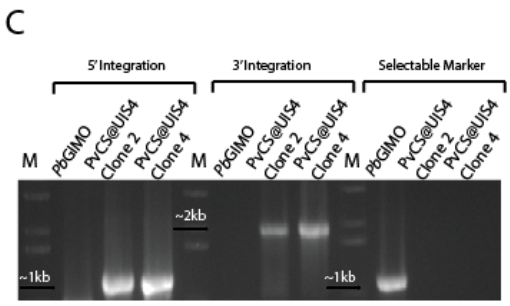



Figure 8 – A – Schematic representation of the GIMO transfection method used to generate *Pb*(*Pv*CS@UIS4) parasites with integration PCR primer binding sites. B – Table with primers and respective reaction amplicons. C- Agarose gel showing PCR results that confirmed correct integration and absence of selectable markers for both *Pb*(*Pv*CS@UIS4) clones.

## 4.3 Mosquito stage development of *Pb*(*Pv*CS@UIS4) parasites

After demonstrating correct integration of the *Pv*CS@UIS4 cassette into the *Pb*(*Pv*CS@UIS4) parasites genome, mosquito stage characterization experiments were performed, to compare the growth kinetics of these parasites with those of the *Pb*GIMO (WT) parasite .

### 4.3.1 Oocyst Development of *Pb*(*Pv*CS@UIS4) parasites

In order to evaluate the ability of *Pb*(*Pv*CS@UIS4) parasites to infect and develop inside the mosquito, oocysts were counted at day 10 post-infected blood meal. Oocyst numbers were compared between *Pb*(*Pv*CS@UIS4) clones 2 & 4 and *Pb*GIMO parasites. Results presented on Fig.9.A indicate that *Pb*(*Pv*CS@UIS4)clones and *Pb*GIMO parasites present a similar capacity to invade, adhere and develop inside *A. stephensi* midguts. For each group, 30 midguts were analyzed in total (3 experiments); [*Pb*GIMO] 83,3% midguts infected, 172,7 ± 26,19 oocysts; [*Pb*(*Pv*CS@UIS4) Clone2] 90% midguts infected, 171,2 ± 22 oocysts; [*Pb*(*Pv*CS@UIS4) Clone 4] 80% midguts infected, 195,8 ± 24,71 oocysts (Fig.9.A). Results are expressed in mean number of oocysts per midgut, accompanied by the standard error of the mean.

These findings suggest that the presence of the *Pv*CS gene on the *Pb*(*Pv*CS@UIS4) transgenic parasites has no effect on the initial development stages of the parasite inside the mosquito.

### 4.3.2 Sporozoite development and salivary gland infectivity of *Pb*(*Pv*CS@UIS4) parasites

Mosquitoes used for sporozoite collection were selected 24h after the infected blood meal, to ensure all mosquitoes used for this experiment were fed. Twenty to 22 days after the blood meal, mosquitoes were dissected and sporozoites present on the salivary glands were collected. The number of sporozoites inside the mosquito salivary glands was recorded for 5 different experimental infections, with 25-35 mosquitoes dissected for each group of infected mosquitoes. Results show that both *Pb*(*Pv*CS@UIS4) clones 2/4 and *Pb*GIMO present similar numbers of sporozoites in mosquito salivary glands 20-22 days post mosquito infection (Fig 9.B); [*Pb*GIMO] 48231 ± 5017; [*Pb*(*Pv*CS@UIS4) Clone 2] 54753 ± 9948; [*Pb*(*Pv*CS@UIS4) Clone 4] 55801 ± 6558 (Fig.9.B). Results are expressed as mean number of sporozoites per mosquito ± standard error of the mean. These results suggest that the presence of the *Pv*CS protein on the transgenic *Pb*(*Pv*CS@UIS4) parasite, has no effect on its capacity to complete oocyst development, form and release sporozoites, and on the ability of sporozoites to migrate to and invade salivary glands.

As previously stated, salivary gland infection was greatly reduced on a previous transgenic parasite in which the endogenous *Pb*CS gene was replaced by the CS gene of the *P. falciparum* parasite. An possible explanation for this is that *Pf*CS has reduced function within *P. berghei* background, probably because region II of the CS protein (which has been shown to be essential for salivary gland invasion) is slightly different between *P. berghei* and *P. falciparum* or simply because the replacement of *Pb*CS by

*Pf*CS could have altered the sequences around the conserved regions and somehow impaired sporozoite motility. Either way, by inserting *Pv*CS in the 230p silent locus, that limitation has been successfully overcome.

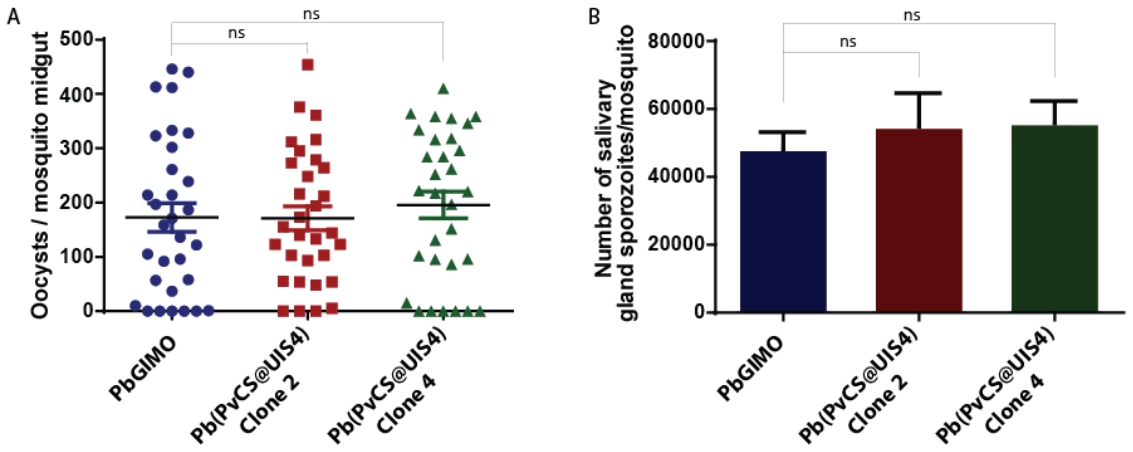



Figure 9 - ***Pb*(*Pv*CS@UIS4) Development inside the mosquito compared to *Pb*GIMO motherline**– A – Number of oocyst per mosquito midgut at day 10 post infectious blood meal. B – Number of sporozoites inside mosquito salivary glands 20-22 days after infectious blood meal. Bars indicate the mean of all observations and error bars represent standard error of the mean. Mann-Whitney test was applied between *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO indicating no significant differences between the parasite lines (p>0,05).

### 4.3.3 *PbCS and PvCS* expression in *Pb*(*Pv*CS@UIS4) parasites in mosquito stages

To determine the expression of the *Pb*CS and *Pv*CS proteins on *Pb*(*Pv*CS@UIS4) parasites, immunofluorescence assays were performed on sporozoites collected from infected mosquito salivary glands. In order to confirm expression and rule out possible cross reactivity from the antibodies, for each group (*Pb*GIMO and *Pb*(*Pv*CS@UIS4) clones 2&4), sporozoites were labeled with either 3D11 (anti-*Pb*CS) or 2F2 (anti-*Pv*CS) mouse monoclonal primary antibodies and a secondary anti-mouse antibody conjugated with a 488 nm fluorochrome. These parasites were imaged on a confocal point-scanning microscope using an excitation laser (488 nm) as well as the differential interference contrast (DIC) optical microscopy illumination technique, to enable visualization of sporozoites regardless of labeling (Fig.10). The results show that both *Pb*(*Pv*CS@UIS4) clones 2 & 4 express both *Pb*CS and *Pv*CS (Fig.10.B/C), while *Pb*GIMO sporozoites only exhibit a fluorescent signal for *Pb*CS (Fig 10.A), showing that 2F2 antibodies are specific for *Pv*CS and have no cross reactivity with *Pb*CS. Expression of *Pv*CS at this stage was expected because in *Pb*(*Pv*CS@UIS4), the *Pv*CS gene is being expressed under the control of the promoter regions for the*Pb*UIS4 (a protein expressed from sporozoite stage until late liver stage EEFs).[39] Also, *Pb*CS expression on both *Pb*(*Pv*CS@UIS4) clones is similar to the expression of *Pb*CS in *Pb*GIMO, which was also expected, as it is essential for the parasite to conclude its cycle inside the mosquito efficaciously.[48]

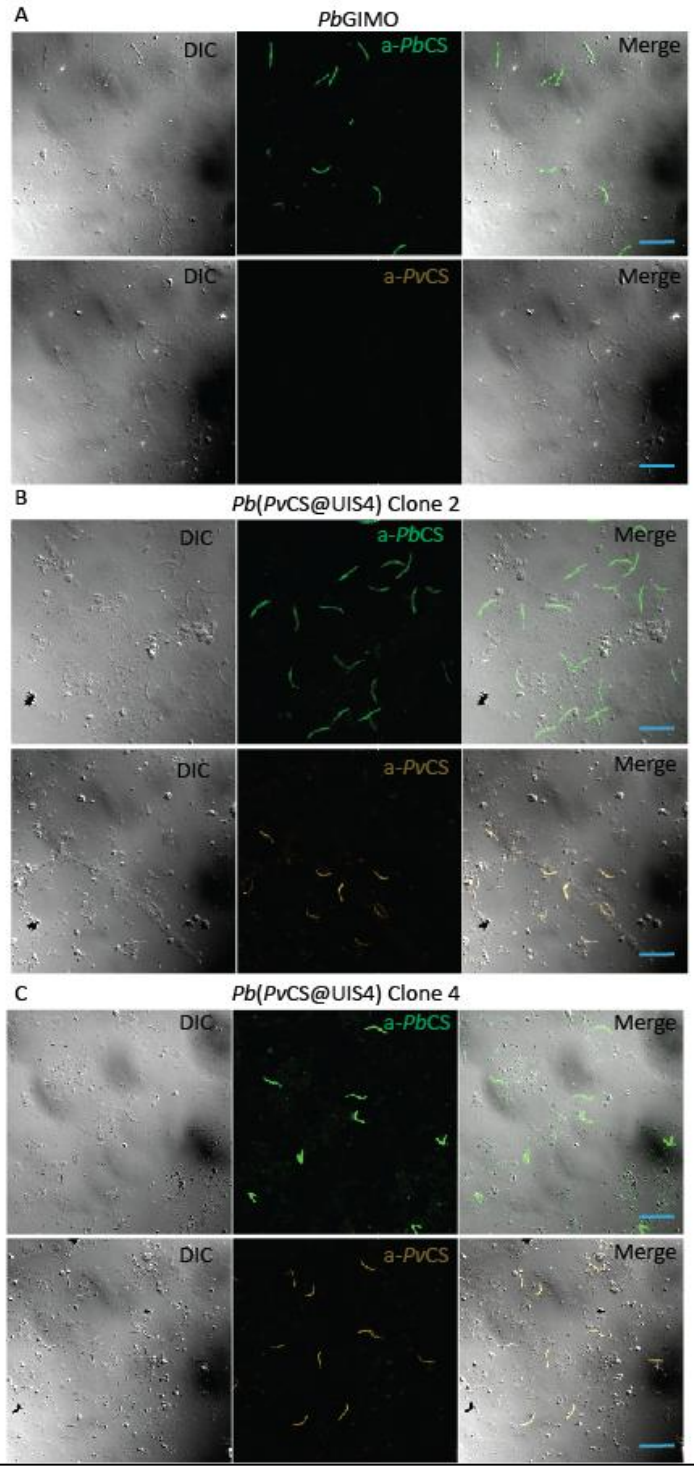



Figure 10 – **Differential interference contrast (DIC) and immunofluorescence microscopy imaging of sporozoites** – A – *Pb*GIMO sporozoites labeled with 3D11 (green) for *Pb*CS expression (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). B - *Pb*(*Pv*CS@UIS4) clone 2 sporozoites labeled with 3D11(green) for *Pb*CS expression (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). C- *Pb*(*Pv*CS@UIS4) clone 4 sporozoites labeled with 3D11(green) for *Pb*CS expression (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). Microsopy settings remained unchanged for all images (laser intensity, scan speed, resolution). Scale bar (Cyan blue) = 21 µm

## 4.4 *In vitro* characterization of *Pb*(*Pv*CS@UIS4) parasites

Having characterized the capacity of *Pb*(*Pv*CS@UIS4) to infect and develop within the *Anopheles* mosquitoes, it was important to characterize the parasite's capacity to infect and develop inside hepatic cells. To that end, two human hepatoma cell lines, Huh7 and HepG2 cells, were used to perform *in vitro* infectivity assays.

### 4.4.1 *In vitro* infection assays in Huh7 hepatoma cells

To assess the infectivity and development of *Pb*(*Pv*CS@UIS4) parasites, three independent infections were performed. In each experiment two microscopy coverslips were seeded with 50.000 cells and infected with 30.000 sporozoites for each of the three parasite groups (*Pb*GIMO, *Pb*(*Pv*CS@UIS4) clones 2&4). Cells were fixed and labeled 48h post infection. Labeling was performed with 2E6 antibodies for *Pb*HSP70 and Hoechst for nuclei (Fig.11).

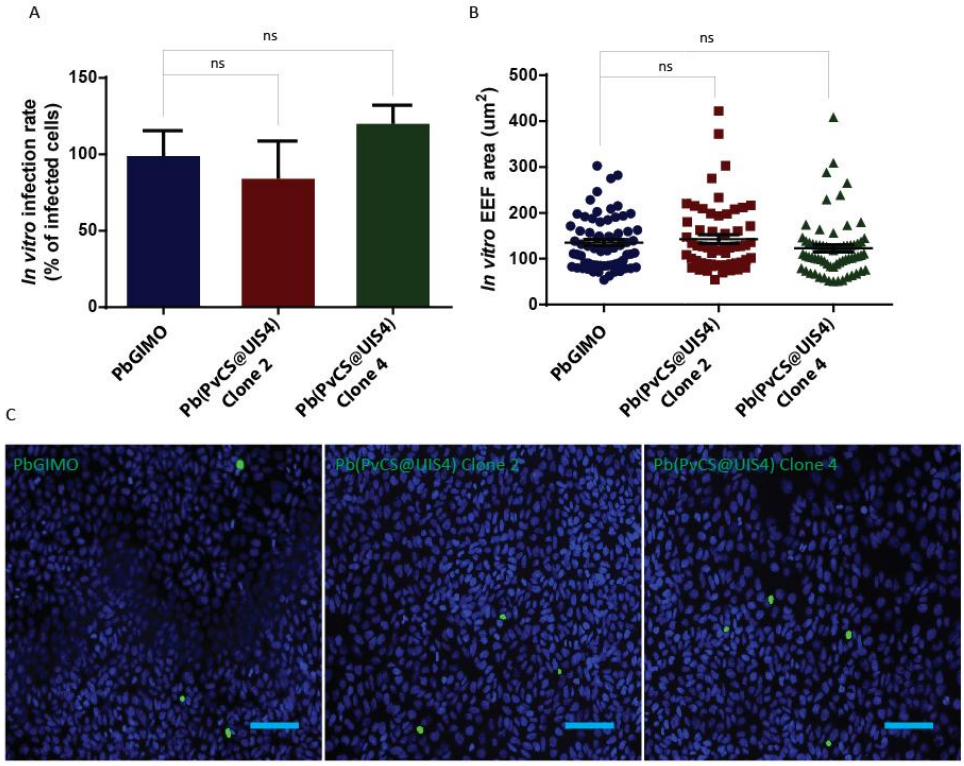



Figure 11 – **Infectivity and development of *Pb*(*Pv*CS@UIS4) in Huh7 cells** – A – *Pb*(*Pv*CS@UIS4) clones infectivity in Huh7 cells, 48h post infection, relative to control (*Pb*GIMO). B – Parasite development inside Huh7 cells measured as EEF area 48h post infection. Results are expressed as means and error bars represent standard error of the mean. Mann-Whitney test was applied between *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO showed no significant difference (p>0,05). C – Representative immunofluorescence microscopy pictures of Huh7 cells infected with the different parasite lines 48h p.i. EEFs labeled with 2E6 primary antibodies (green) and cell nuclei labeled with Hoechst (blue). Scale bar (Cyan blue) = 100 µm

In order to determine infectivity, coverslips were analyzed on an Axiovert 200M wide field microscope. Total infectivity was calculated as the ratio between infected and non-infected hepatocytes. Infectivity was then normalized to 100% of *Pb*GIMO infection, showing no significant statistical difference between *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO (p>0,05); [*Pb*GIMO] 0,1038% ± 0,01616% ;[*Pb*(*Pv*CS@UIS4)Clone 2] 0,8828% ± 0,02447% (85% of control) ;[*Pb*(*Pv*CS@UIS4) Clone 4] 0,1256% ± 0,01164% (121% of control) (Fig.11.A).

Development was assessed through microscopy by measuring the total area of approximately 60 EEFs of each parasite; [*Pb*GIMO] 135,1 ± 7,078 $\mu m^2$; [*Pb*(*Pv*CS@UIS4)Clone 2] 142,6 ± 9,535 $\mu m^2$; [*Pb*(*Pv*CS@UIS4) Clone 4] 122,9 ± 7,921 $\mu m^2$ (Fig.11.B). These results are expressed as mean area of EEFs ± standard error of the mean.

### 4.4.2 Expression assays in Huh7 hepatoma cells

Expression assays for *Pb*CS and *Pv*CS have been performed on Huh7 cells, 48h post infection with *Pb*GIMO or *Pb*(*Pv*CS@UIS4) clones 2&4 parasites, respectively. Coverslips containing infected cells were incubated with either 3D11(anti-*Pb*CS) + anti-*Pb*UIS4 or 2F2(anti-*Pv*CS) + anti-UIS4 primary antibodies, and corresponding secondary antibodies; Alexa-Fluor 488 anti-mouse IgG (for 3D11 and 2F2), Alexa-Fluor 568 anti-goat IgG (for anti-UIS4) and Hoechst for nuclei labeling. *Pb*UIS4 works as a control labeling, as it enables visualizing EEFs regardless of CS expression. Images were acquired on a Zeiss LSM710 confocal microscope and all settings remained unchanged for each channel. As expected, the results indicate that both *Pb*(*Pv*CS@UIS4) clones express both *Pv*CS and *Pb*CS, while *Pb*GIMO expresses only *Pb*CS (Fig.12).

### 4.4.3 *In vitro* infection assays in HepG2 hepatoma cells

Similarly to Huh7 assays, three independent infections were performed with HepG2 cells. Each experiment employed two microscopy coverslips seeded with 70.000 cells and infected with 30.000 sporozoites for each of the three parasite groups (*Pb*GIMO, *Pb*(*Pv*CS@UIS4) clones 2&4). HepG2 cells grow slower than Huh7 cells, hence the higher initial number (70000 cells instead of 50000 cells). Cells were fixed and labeled 48h post infection. Labeling was performed with 2E6 antibodies (anti-HSP70) and Hoescht (Fig.13.C). To determine infectivity, coverslips were analyzed on an Axiovert200M widefield microscope. Total infectivity was calculated as the ratio between EEFs and non-infected hepatocytes, infectivity was then normalized to 100% of *Pb*GIMO infection, with no significant differences found between them (p>0,05); [*Pb*GIMO] 0,8% ± 0,09416%; [*Pb*(*Pv*CS@UIS4) clone 2] 0,7691% ± 0,1153 (92,366% of control);[*Pb*(*Pv*CS@UIS4) clone 4] 0,8188% ± 0,1799% (98,337% of control) (Fig.13.A).

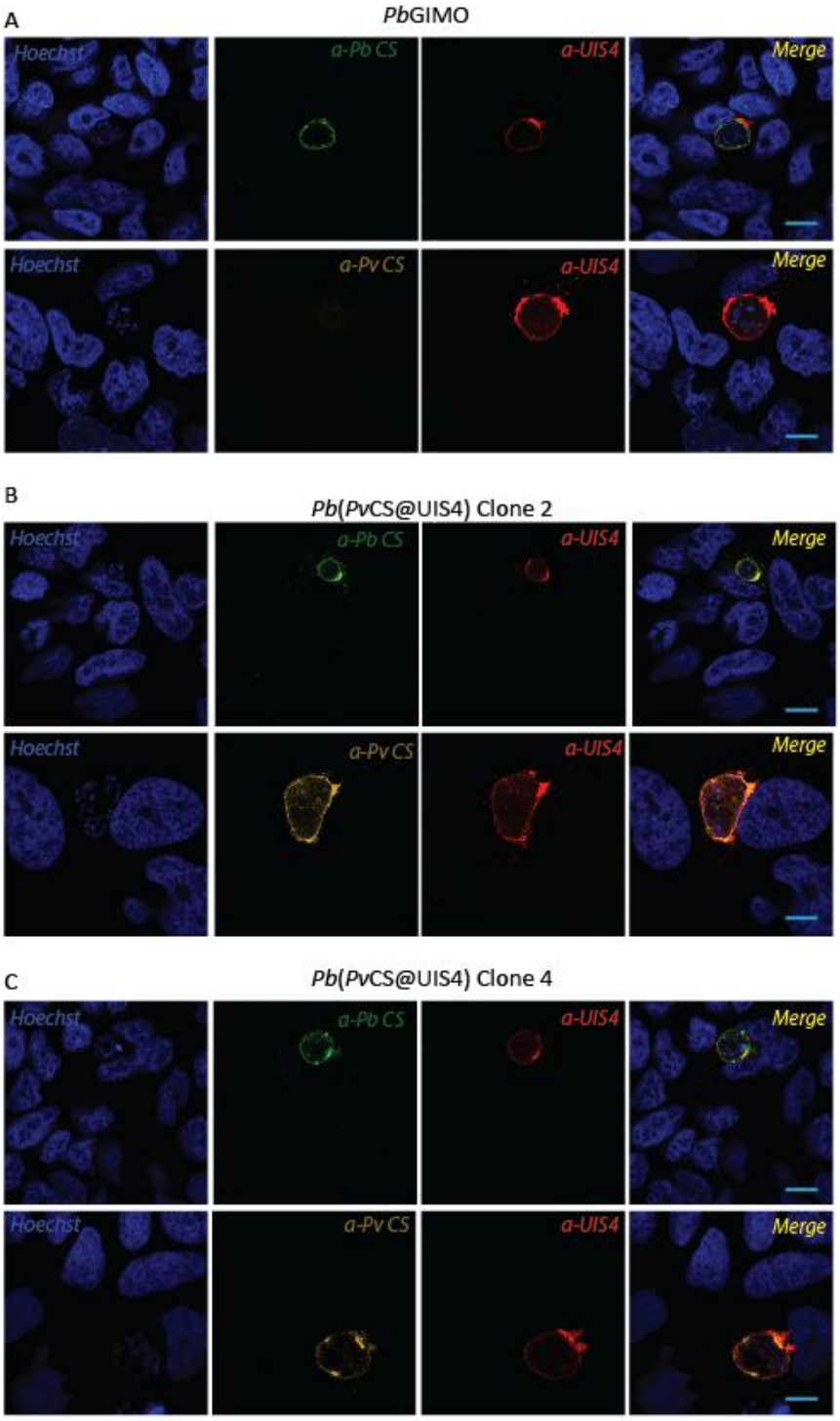



Figure 12 - **Immunofluorescence microscopy of exoerythrocytic forms in Huh7 cell culture** – A- *Pb*GIMO EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red) (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). B -*Pb*(*Pv*CS@UIS4) clone 2 EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red) (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). C - *Pb*(*Pv*CS@UIS4) clone 4 EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red) (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). Cell nuclei labeled with Hoechst (blue).All cultures were fixed at 48 hpi. Scale bar (Cyan blue) = 21 µm

Parasite development was assessed through microscopy by measuring the total area of about 120 EEFs of each parasite group; [*Pb*GIMO] 269,7 ± 15,27 $\mu m^2$;[*Pb*(*Pv*CS@UIS4) clone 2] 290,8 ± 15,85 $\mu m^2$;[*Pb*(*Pv*CS@UIS4) clone 4] 222,8 ± 12,48 $\mu m^2$ (Fig.13.B). These results are expressed as mean area of EEFs ± standard error of the mean.

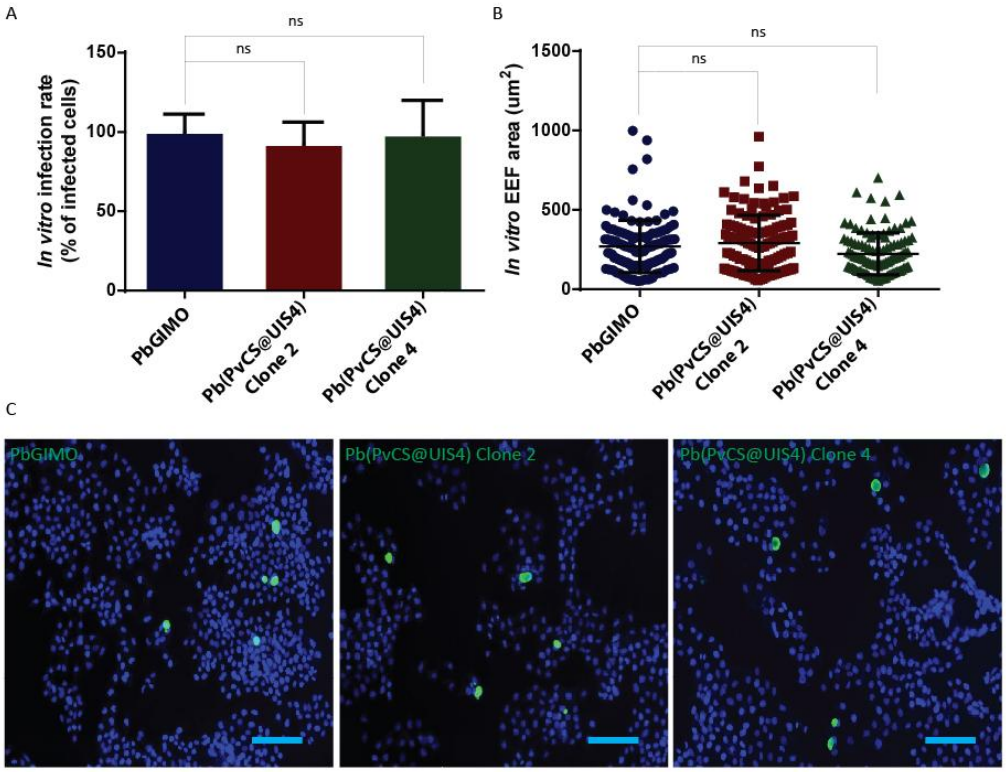



Figure 13 – **Infectivity and development of *Pb*(*Pv*CS@UIS4) in HepG2 cells** – A – *Pb*(*Pv*CS@UIS4) clones infectivity in HepG2 cells, 48h post infection, relative to control (*Pb*GIMO). B – Parasite development inside HepG2 cells measured as EEF size 48h post infection. Results are expressed as means and error bars represent standard error of the mean. Mann-Whitney test was applied between *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO showed no significant difference (p>0,05). C – Representative immunofluorescence microscopy images of HepG2 cells infected with the different parasite lines 48h p.i. EEFs labeled with 2E6 primary antibodies (green) and cell nuclei labeled with Hoechst (blue). Scale bar (Cyan blue) = 100 µm

### 4.4.4 Expression assays in HepG2 hepatoma cells

*Pb*CS and *Pv*CS expression on HepG2 cells was determined 48h post infection with *Pb*GIMO or *Pb*(*Pv*CS@UIS4) clones 2&4 parasites, respectively (Fig.14). Coverslips containing infected cells were incubated with either 3D11(*Pb*CS) + anti-*Pb*UIS4 or with 2F2(*Pv*CS) + anti-*Pb*UIS4 primary antibodies, and corresponding secondary antibodies; Alexa-Fluor 488 anti-mouse IgG (for 3D11 and 2F2), Alexa-Fluor 568 anti-goat IgG (for anti-UIS4) and Hoechst for nuclei labeling. Anti-*Pb*UIS4 was used as a control labeling. Images were acquired on a Zeiss LSM710 confocal

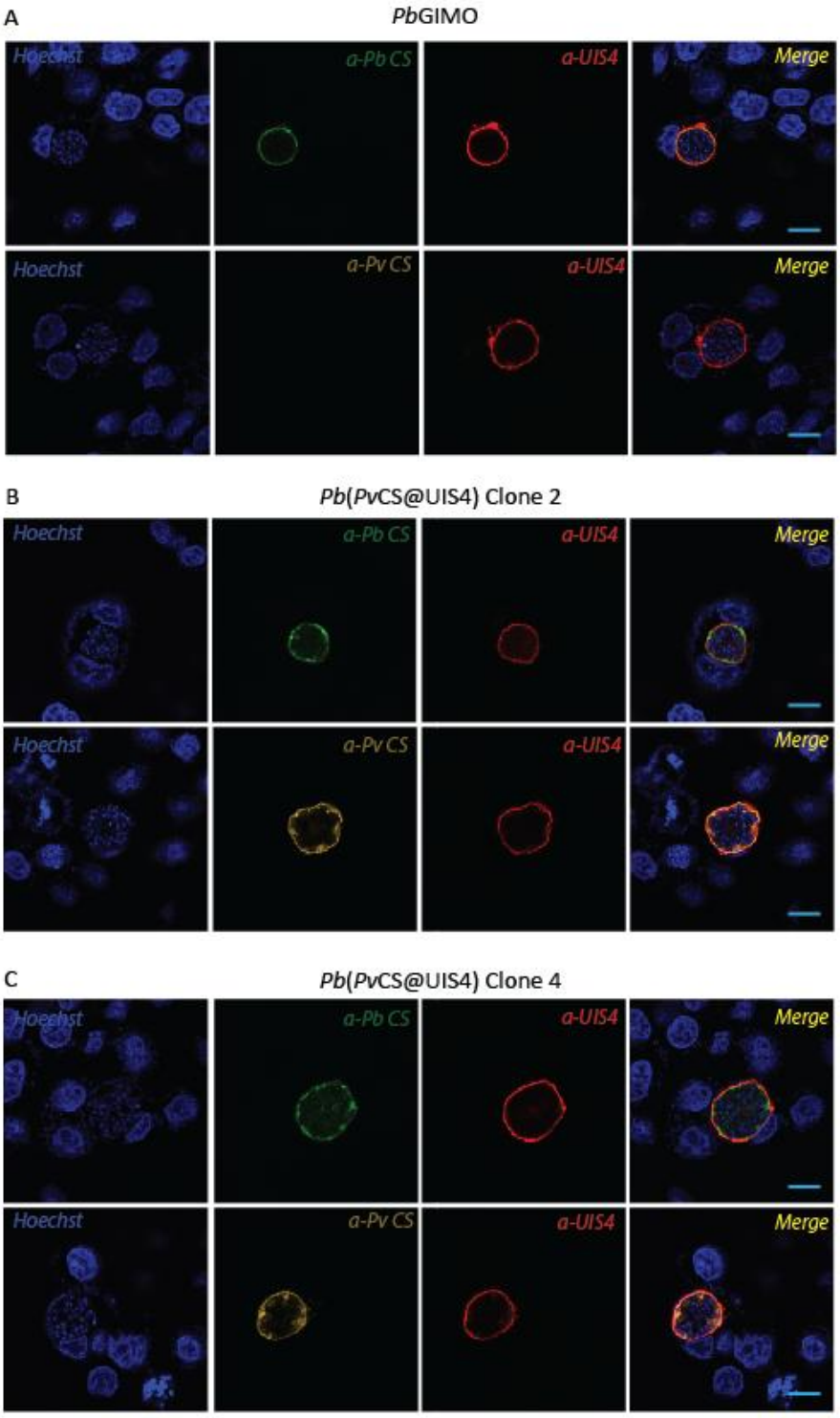



Figure 14 - **Immunofluorescence microscopy of exoerythrocytic forms in HepG2 cell culture 48h pi**– A- *Pb*GIMO EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red)(top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). B -*Pb*(*Pv*CS@UIS4) clone 2 EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red)(top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). C - *Pb*(*Pv*CS@UIS4) clone 4 EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red)(top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). Cell nuclei labeled with Hoechst (blue). Scale bar (Cyan blue) = 21 μm

microscope and all settings remained unchanged for each channel. As expected, and similarly to what was observed in the assays employing Huh7 cells, our results show that both *Pb*(*Pv*CS@UIS4) clones express both *Pv*CS and *Pb*CS while *Pb*GIMO expresses only *Pb*CS (Fig.14).

*In vitro* experiments with either Huh7 or HepG2 cells clearly indicated that liver-stage development of *Pb*(*Pv*CS@UIS4) parasites is similar to that of *Pb*GIMO parasites. *Pb*(*Pv*CS@UIS4) liver-stage parasite forms also seem to express both *Pb*CS and *Pv*CS to similar extents. This was expected due to the abundant expression of *Pb*UIS4 during *Plasmodium* liver stage development (Fig.14, in red), therefore having *Pv*CS being expressed under that promoter would lead to its expression during this stage.

## 4.5 *In vivo* characterization of *Pb*(*Pv*CS@UIS4) parasites

Upon completion of the *in vitro* characterization of *Pb*(*Pv*CS@UIS4) infectivity, we proceeded with the characterization of its infectivity and development *in vivo*.

To evaluate *in vivo* infectivity and development of *Pb*(*Pv*CS@UIS4), 9 C57BL/6 mice (3 for each group) were infected i.v. with 30.000 sporozoites (3 experiments). At 44h post infection, mice were sacrificed and their livers collected. A section of each liver was collected for microscopy analysis and the remaining was used for qRT-PCR quantification of *Pb* infection.

### 4.5.1 Infection load quantified by qRT-PCR

The first *in vivo* assay consisted in evaluating *Pb*(*Pv*CS@UIS4) parasites ability to infect hepatocytes using an *in vivo* model. Using the RNA extracted from infected livers, the infection load was quantified by qRT-PCR. Quantification was carried out using the comparative threshold (cT) method, based on the ratio between the mouse HPRT as a housekeeping gene and the *P. berghei* 18S mRNA levels ($2^{HPRT}/2^{18S}$) (Fig.15). The following results were obtained from 2 independent experiments.

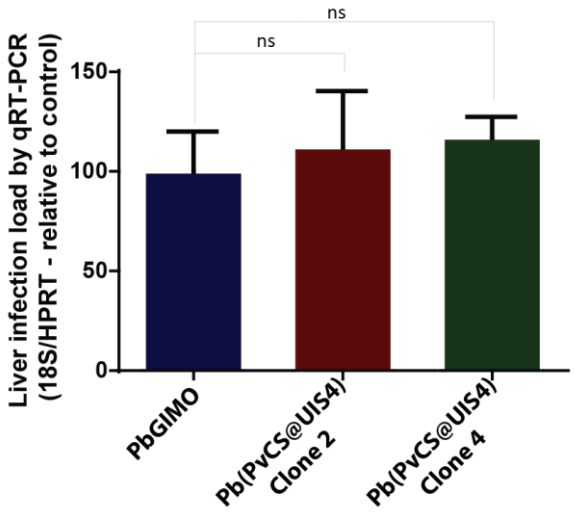



Figure 15- **Determination of liver infection load by qRT-PCR**– A total of 3 mice were infected with each parasite (*Pb*GIMO, *Pb*(*Pv*CS@UIS4) clone 2&4).Quantification of parasite liver load at 44h post infection with 30.000 sporozoites. Results are expressed as means of the infection load and error bars represent standard error of the mean. Mann-Whitney test showed no significant differences between *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO. (p>0,05).

### 4.5.2 Infectivity and development analysis by immunofluorescence microscopy

*Pb*(*Pv*CS@UIS4) infectivity was also assessed by immunofluorescence microscopy. To this end, 50-µm sections of the livers collected from all 3 groups of mice were stained and analyzed by immunofluorescence microscopy. Liver slices were stained with anti-*Pb*UIS4 antibodies (and the corresponding secondary antibody, anti-goat Alexa Fluor 568). Liver slices were then observed on an Axiovert200M widefield microscope and the total number of EEFs was counted. Infectivity was calculated as the number of EEFs by total area of the liver slice (EEF/mm$^2$); infectivity was then normalized to 100% of *Pb*GIMO infection, with no significant differences found between them (p>0,05); [*Pb*GIMO] 0,074 ± 0,0145 EEF/mm$^2$; [*Pb*(*Pv*CS@UIS4) clone 2] 0,0674 ± 0,0112 EEF/mm$^2$ (91,44% of control);[*Pb*(*Pv*CS@UIS4) clone 4] 0,0711 ± 0,0097 EEF/mm$^2$ (96,44 % of control) (Fig.16.A).

Likewise, development was assessed as a function of EEF size. The following results were obtained from 3 independent experiments; [*Pb*GIMO] 1082,4 ± 82,8 µm$^2$; [*Pb*(*Pv*CS@UIS4) clone 2] 917,7 ± 47,6 µm$^2$; [*Pb*(*Pv*CS@UIS4) clone 4] 1063,6 ± 48,4 µm$^2$ (Fig.16.B). These results are expressed as mean area of EEFs ± standard error of the mean.

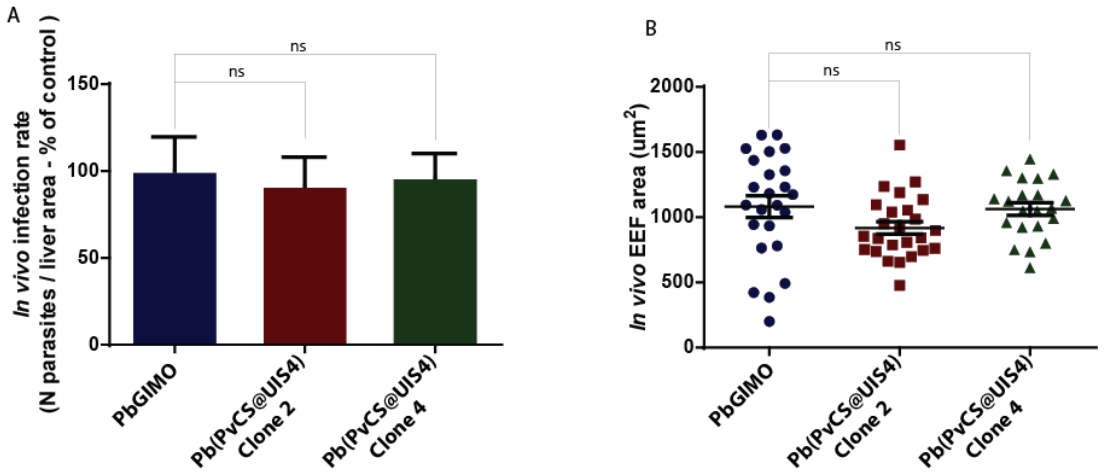



Figure 16 –***In vivo*** **liver-stage infectivity and development of *Pb*(*Pv*CS@UIS4) assessed by immunofluorescence microscopy**– A – *Pb*(*Pv*CS@UIS4) clones infectivity 44h post infection, with 30.000 sporozoites, relative to control (*Pb*GIMO). B – Parasite development inside mouse hepatocytes measured as EEF size 44h post infection. Results are expressed as mean of EEF area and error bars represent standard error of the mean. Mann-Whitney test showed no significant difference between *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO (p>0,05).

### 4.5.3 *In vivo* expression assays

To evaluate *Pb*CS and *Pv*CS expression by *Pb*(*Pv*CS@UIS4) parasites in an *in vivo* model, 3 C57BL/6J mice (1 for each *Pb*(*Pv*CS@UIS4) clone and *Pb*GIMO) were infected with 100.000 sporozoites injected intravenously. At 44h post infection, livers were collected and 50 µm thick liver sections were prepared for immunofluorescence analysis. For each mouse, liver slices were incubated with either 3D11(anti-*Pb*CS) + anti-UIS4 or 2F2 (anti-*Pv*CS) + anti-UIS4 primary antibodies. In addition to secondary antibodies (Alexa-Fluor 488 anti-mouse IgG (for 3D11 and 2F2), Alexa-Fluor 568 anti-goat IgG (for anti-UIS4)), liver slices were also incubated with Phaloidin Alexa-Fluor 660 for cellular F-actin labeling and Hoechst for nuclei. Imaging was performed on a Zeiss LSM710 microscope and channel settings were kept unchanged for all images. As was shown *in vitro*, our results show that *Pb*(*Pv*CS@UIS4) parasites also express both *Pb*CS and *Pv*CS during liver-stage (EEFs) in this *in vivo* model (Fig.17).

The *in vivo* experiments performed in C57BL/6J mice showed that *Pb*(*Pv*CS@UIS4) parasites can infect and develop inside mouse hepatocytes to the same extent as the control parasite (*Pb*GIMO). We could also observe that *Pb*(*Pv*CS@UIS4) expresses both *Pb*CS and *Pv*CS as expected from this parasite. These last experiments conclude the pre-erythrocytic stage characterization of *Pb*(*Pv*CS@UIS4) parasites. However, none of the experiments above have addressed *Pb*(*Pv*CS**@**UIS4)'s ability to cause blood-stage infection in mice. Thus, in order to address this, we decided to conduct a time course experiment of blood-stage development of the parasites.

## 4.6 Characterization of blood-stage development of *Pb*(*Pv*CS@UIS4) parasites

To characterize the ability of *Pb*(*Pv*CS@UIS4) parasites to progress to blood-stage forms and develop parasitemia in rodents, a total of nine mice (three for each parasite) were infected by i.v. injection of 30.000 sporozoites. This experiment is intended to determine whether *Pb*(*Pv*CS@UIS4) merozoites can reach the bloodstream, infect red-blood cells and replicate to the same extent as wild type parasites. Blood smears were checked for parasitemias every day starting two days post-infection. All mice were kept in the same environment to avoid possible bias. As shown on Fig.18, the observed blood-stage development of all three groups was almost identical. These results show us that the presence of *Pv*CS in *Pb*(*Pv*CS@UIS4) parasites, does not seem to influence the parasite's capacity to exit hepatocytes and develop inside red blood cells.

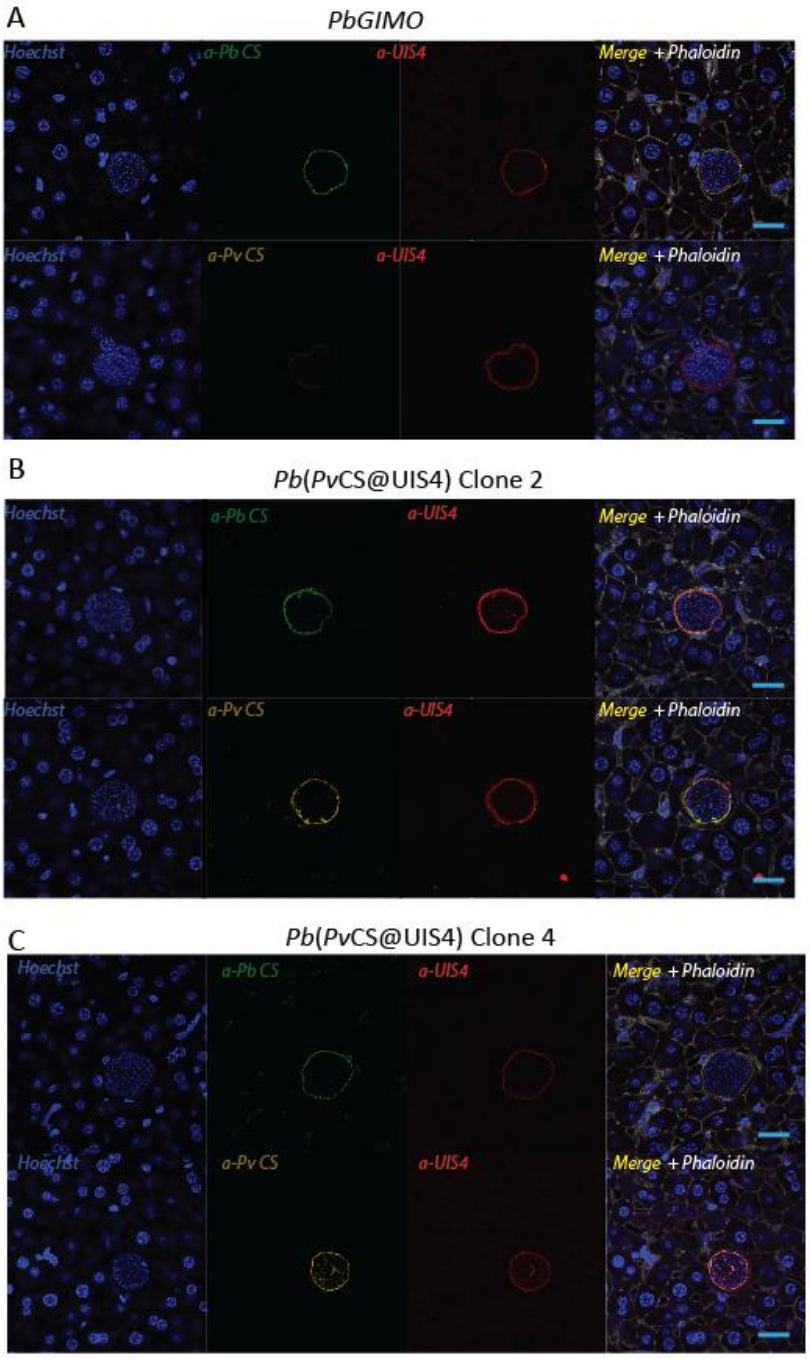



Figure 17 - **Immunofluorescence microscopy analysis of EEF forms on mouse livers, 44h post sporozoite infection**– A- *Pb*GIMO EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red) (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). B -*Pb*(*Pv*CS@UIS4) clone 2 EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red) (top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). C - *Pb*(*Pv*CS@UIS4) clone 4 EEFs labeled with 3D11 (green) for *Pb*CS expression and anti-UIS4 (red)(top row). Labeling with 2F2 (gold) for *Pv*CS expression (bottom row). Cell nuclei were labeled with Hoechst (blue) and cellular F-actin was labeled with phalloidin (white). Scale bar (Cyan blue) = 21 µm.

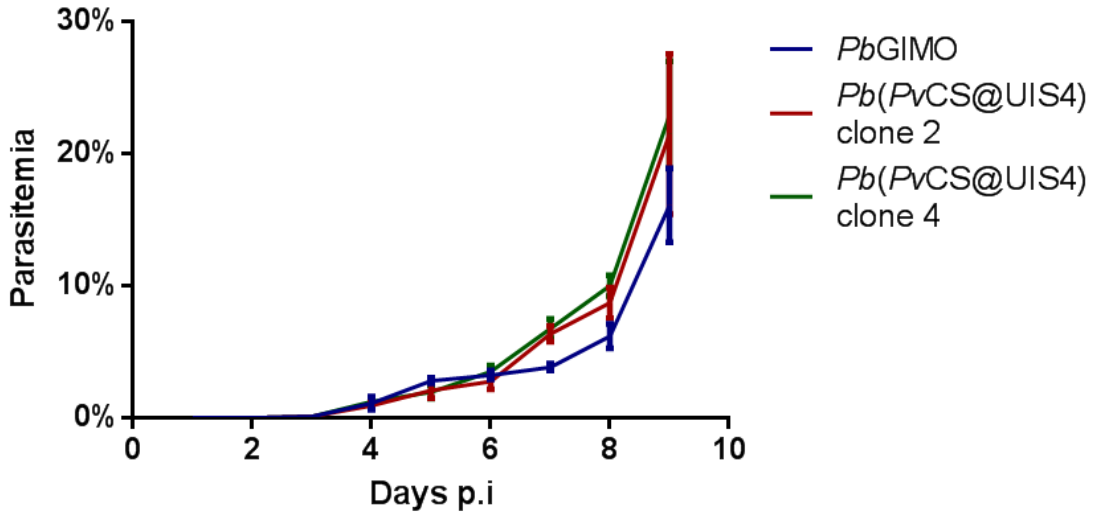



Figure 18–**Time course of parasitemia in C57BL/6J mice infected by i.v. injection of 30.000 sporozoites–** Three mice were infected with each parasite; *Pb*GIMO, *Pb*(*Pv*CS@UIS4) clone 2 and *Pb*(*Pv*CS@UIS4) clone 4. Parasitemias were checked daily. The results are expressed as means of the parasitemias for each infection group at each day. Vertical bars represent the standard error of the mean for each daily parasitemia check.

With this last experiment, we concluded the characterization of *Pb*(*Pv*CS@UIS4) parasites during all of its developmental stages. No significant differences were observed between the fitness of both *Pb*(*Pv*CS@UIS4) clones and *Pb*GIMO parasites during sporogonic, pre-erythrocytic and blood-stage development. Additionally, CS expression was also observed, showing that *Pb*CS expression was identical between *Pb*GIMO and *Pb*(*Pv*CS@UIS4) parasites during sporogonic and pre-erythrocytic stages while only *Pb*(*Pv*CS@UIS4) parasites expressed *Pv*CS.

# 5. Discussion

Malaria remains one of the most deadly infectious diseases worldwide, with no vaccine licensed so far. Despite many years of intense research, the most advanced vaccine candidate against *P. falciparum* malaria, RTS,S, has shown to confer only modest and short lasting protection.[29] RTS,S is a subunit vaccine and consists on the administration of part of the *P. falciparum* CS protein fused to a carrier matrix and immunostimulants.[27] As an alternative, other vaccination strategies are also in development, such as whole-organism vaccines. Studies using radiation-attenuated *P. falciparum* sporozoites have already proven that whole-organism vaccines can elicit effective and long-lasting protection against non-attenuated parasites in humans. However, whole-organism vaccination strategies that have been under development thus far rely on the use of human-infective *Plasmodium* as a vaccination agent and therefore present the risk of breakthrough infections, leading to pathology upon administration of the vaccine.[33,36,43] The host lab proposed an alternative method to circumvent some of difficulties associated to previously proposed whole vaccination strategies against malaria:

We proposed the use of *P. berghei* as a vaccination agent against human-infective *Plasmodium* species. We hypothesize that this strategy can confer protection against infection with human malaria parasites through the induction of an heterologous immune response against conserved antigens between rodent and human malaria parasites, as well as serve as delivery platform of specific antigens from human infecting *Plasmodium*, namely the immunodominant CS protein.

The phenomenon of cross-protection between different *Plasmodium* species has been previously reported in mice.[54,59,60] Mice immunized with radiation-attenuated *P. berghei* sporozoites have shown to have a high level of protection against *P. yoelii* infections (79%) and *vice-versa* (63%).[59] A different study showed that mice immunized with *P. falciparum* were protected against *P. berghei* infections.[54] The immune responses responsible for heterologous protection in these cases were mainly CD8+ and CD4+ T cell-mediated.[59–61]

In order to prove the validity of the proposed strategy, the host lab has demonstrated that *P. berghei* can infect and develop in human hepatocytes without manifestations of blood stage infection and that a *P. berghei* parasite expressing *P. falciparum* CS can elicit an immune response capable of recognizing and inhibiting *P. falciparum* parasites.

Given the fact that *P. vivax* is the most widespread human-infective *Plasmodium* species, and that research on a vaccine against this parasite species is hampered by technical limitations, such as the inability to produce *in vitro* cultures and the unavailability of animal models[62], we decided to use the *P. berghei* platform to express *P. vivax* CS in order to create *P. berghei* parasites that express *P. vivax* CS in addition to their endogenous *Pb*CS protein, *Pb*(*Pv*CS@UIS4).

A previous study showed that chimeric *P. berghei* parasites which had *Pb*CS central repeats replaced by *Pv*CS central repeat region (*Pb-Pv*), could infect and

develop inside hepatocytes to the same extent as wild type *P. berghei*.[63] Moreover, these parasites were shown to successfully present the introduced *Pv*Cs repeats on the sporozoite surface and during their hepatic development, in such a way that rendered them susceptible to neutralization by anti-*Pv*Cs sera.[63] On the other hand, *Pv*CS repeats in these parasites are flanked by *Pb*CS termini regions and therefore lack the *Pv*CS C-terminal and N-terminal regions, which have been shown to be responsible for both CS-specific and non-specific T cell responses.[64–66] In light of this, we aimed to create a parasite that expresses the full *Pv*CS in a way that is non-disruptive to the parasite's development, in order to elicit both humoral and cellular responses against *Pv*CS as well as heterologous protection against other *P. vivax* antigens.

All the results presented in this thesis aimed to characterize and clarify any relevant features regarding the new vaccine candidate, *Pb*(*Pv*CS@UIS4), throughout its life cycle. Firstly, after generating this new transgenic parasite, we were concerned about how different our *Pv*CS sequence would be from the *Pv*CS proteins found worldwide. Our analysis showed that the N-terminus and C-terminus regions of our *Pv*CS sequence are almost totally conserved when compared to the reference *Pv*CS sequences found in PlasmoDB. If the protein sequence was changed in any way, it could acquire a different conformation, preventing *Pv*CS antigens from being properly recognized. As previously mentioned, the N-terminus and C-terminus regions of our *Pv*CS sequence contain smaller regions that have been shown to be critical for the parasite invasion and protein conformational changes throughout the parasite's development, namely the TSR motif and region I. [46] Therefore, the integrity of these regions is very important in the context of the proposed vaccine candidate. On the other hand, and most importantly in the context of a vaccine candidate, our *Pv*CS protein's central repeat region has the GDRA(A/D)GQPA repeat as its most prevalent repeat, identically to the most common type worldwide, the VK210 type; however, within the VK210 type of *Pv*CS there is some variability in terms of repeat numbers (normally range between 9 and 18 repeats)[47,51,52,57] and repeat sequences[58]. The last repeat sequence in the transgenic parasite used in this work appears to differ from the ones present on the database (GNGAGGQAA). However, the same repeat has previously been found in parasite population studies from Sri Lanka, in the same location as in the *Pv*CS gene sequence amplified here.[47] Despite the biological significance (if any) of this specific repeat remaining unknown, within the context of a vaccine candidate, repeat variability may represent an advantage as it contributes with one more *P. vivax* specific antigen for the immune system to recognize.

During the mosquito stage characterization of *Pb*(*Pv*CS@UIS4) we have observed that the development of these parasites inside the mosquito was similar to that of *Pb*GIMO parasites, the "transfection motherline". Like *Pb*CS, the inserted CS protein, *Pv*CS, is also expressed on sporozoites. Despite the fact that recent studies indicate that most CD8+ T cell immune responses are elicited during liver-stage infection and are essential for a long-lasting protection against infection[32], the possible contribution given by pre-hepatic stages should not be overlooked. Our *in vitro* experiments, consisting of infecting Huh7 and HepG2 cells with live, freshly collected, sporozoites, allowed us to demonstrate that *Pb*(*Pv*CS@UIS4) parasites have a capacity to invade and develop inside hepatocytes similar to that of *Pb*GIMO parasites.

The same results were observed *in vivo* by injecting 30.000 sporozoites i.v. in C57BL/6J mice. In the context of vaccination it is important that the *Pb*(*Pv*CS@UIS4) parasites develop as much and as long as possible inside the hepatocytes, as it has been shown that the further the parasites develop in the liver, the higher the overall CD8+ T cell response, and the more likely it is for a vaccine to elicit effective protection.[32] A possible explanation for this may be due to the full antigenic unfold on non-attenuated parasites during liver-stage, which may not happen if the parasites are attenuated by radiation.[43] In light of this, our vaccination approach may prove to be a better alternative to vaccine candidates with an early parasite arrest during liver-stage development, as *Pb*(*Pv*CS@UIS4) parasites are not attenuated in any way and seem to have no impairment in liver-stage infection and development without leading to blood-stage infection in humans. Our *in vitro* and *in vivo* findings also show that *Pb*(*Pv*CS@UIS4) parasites express *Pb*CS and *Pv*CS during liver stage infection (observed at 46h post infection). Both CS proteins seem to be expressed on the parasitophorous vacuole membrane (PVM). This shows that *Pv*CS is being correctly translocated to the membrane, despite being expressed on a *P. berghei* background. When evaluating blood-stage development of the transgenic parasites, parasitemia developed similarly in mice infected with each of the three parasites (*Pb*GIMO, *Pb*(*Pv*CS@UIS4) clone 2 and 4), indicating that the insertion of *Pv*CS has no impact on blood stage development for these parasites. No CS (or UIS4) expression is expected during blood-stage, therefore no expression assays were performed for this developmental stage.

From various immunization studies with the RTS,S vaccine, it became evident that, the CS protein can elicit a significant degree of protection against *Plasmodium* infection. The protection elicited is mainly antibody and CD4+ T cell mediated and none of the individuals vaccinated were protected six months after immunization.[30,31] Despite CD4+ T cell and antibody responses being able protect against malaria, these have shown to not be sufficient to achieve long term protection against a new infection.[31] On the other hand, human trials with *P. falciparum* irradiated sporozoites, showed some degree of CD8+ T cell mediated response against CS.[35] These two studies together suggest that whether or not the CS is presented within the context of a whole-organism influences the type of cellular response elicited against it.

All the efforts in creating vaccination strategies against malaria provided valuable insights on what type of responses seem to be essential to successfully elicit immune protection against *Plasmodium* infections. By employing *Pb*(*Pv*CS@UIS4) sporozoites we believe to have overcome safety limitations of using human-infective *Plasmodium* species as a vaccination agent. Moreover, protection against malaria has shown to be possible through various cellular and humoral responses. With *Pb*(*Pv*CS@UIS4) sporozoites, we believe that it is possible to elicit a strong and long-lasting immunity against *P. vivax* infections as a result of the summation of *P. vivax*-specific and non-specific cellular and humoral responses against *Pv*CS and heterologous antigens present on *P. berghei*.

A number of questions remain open at the end of this study:

- Are *Pb*(*Pv*CS@UIS4) parasites able to trigger an immune response against *P*. vivax?

- What kinds of response are elicited (cellular/humoral)?

- Are these responses able to recognize *P. vivax* parasites and inhibit infection?

- Are the responses elicited by *Pb*(*Pv*CS@UIS4), able to protect against *P. vivax* infections in humans?

- Which responses are the main effectors of that protection? (The ones against *Pv*CS or against *P. berghei* heterologous epitopes)?

One possible way to tackle these questions would be through the use of an immunization protocol in mice similar to the ones performed on the "live-parasite immunization under antimalarial therapy studies" in mice[67], with *Pb*(*Pv*CS@UIS4) sporozoites and concomitant administration of chloroquine. After the immunization protocol is concluded, mice would be sacrificed and their blood and spleen collected. In order to determine if there is a humoral response capable of recognizing *Pv*CS, ELISA analyses of the collected blood sera using *Pv*CS central repeats as "bait" could be performed. Additionally, using the collected spleens, ELISpot analyses could be performed in order to detect specific cellular responses against *P. vivax* parasites and *P. berghei* parasites. This would provide an indication of what type of responses may be triggered in a real immunization scenario.

In order to know whether or not the immune responses elicited by *Pb*(*Pv*CS@UIS4) parasites are able to recognize *P. vivax*, an immunofluorescence assay could be performed using sera from *Pb*(*Pv*CS@UIS4)-immunized mice on immobilized *P. vivax* sporozoites. Additionally, to determine if these responses are able to inhibit infection, serum from *Pb*(*Pv*CS@UIS4) immunized mice could be used to incubate with *P. vivax* sporozoites; these sporozoites would then be used to infect liver-humanized mice and livers analyzed 48h post infection in order to determine whether or not the serum was able to inhibit sporozoite invasion.

The last two questions, however, would require this vaccine candidate to reach human trials. In order to tackle those, an identical immunization protocol as previously established for clinical trials performed with a vaccine candidate based on the administration radiation-attenuated *P. falciparum* sporozoites[36] could be applied. Performing a challenge with *P. vivax* in humans can be difficult, as *P. vivax* can develop into hypnozoites, which could lead to relapses later on. This way, challenge with *P. vivax* should be performed with a primaquine-sensitive strain, so that hypnozoites can be effectively eliminated, without leading to further complications. After challenge with *P. vivax*, human volunteers should be monitored for parasitemia to assess whether or not they were protected against *P. vivax* malaria. We could also perform an ELISA test to assess if the same type of humoral recognition occurs in humans. Moreover, *P. vivax* cellular immune responses should be assessed before and after immunization in both protected and non-protected individuals to assess the kind of responses that are responsible for protection against *P. vivax* with our vaccine candidate. These are only a few examples of experiments that would answer some of the open questions left after this characterization.

In conclusion, the results presented here demonstrate that *Pb*(*Pv*CS@UIS4) parasites can develop normally throughout its life cycle ,expressing an exogenous *Pv*CS as well as their endogenous *Pb*CS, without altering the parasite's fitness. When taken together, the results obtained with *Pb*(*Pv*CS@UIS4) and the former *Pb*(*Pf*CS@UIS4) parasites strongly suggest that this vaccination strategy represents a viable and safe alternative to the already existing vaccine candidates against human-infective *Plasmodium* species. Based on results obtained from several studies, we believe that delivering a CS protein from a human malaria parasite on the *P. berghei* platform may confer a great advantage in the context of a vaccine as it is the immunodominant protein on the sporozoite's surface. However, *P. berghei* can be used as a platform to express other antigens found relevant for vaccination, such as antigens with relevant functions in the blood-stage development or transmission of *Plasmodium* parasites. On the other hand, as previously mentioned, several additional experiments are still required in order to understand the nature of the immune responses induced by these vaccine candidates, before being able to proceed to human trials.

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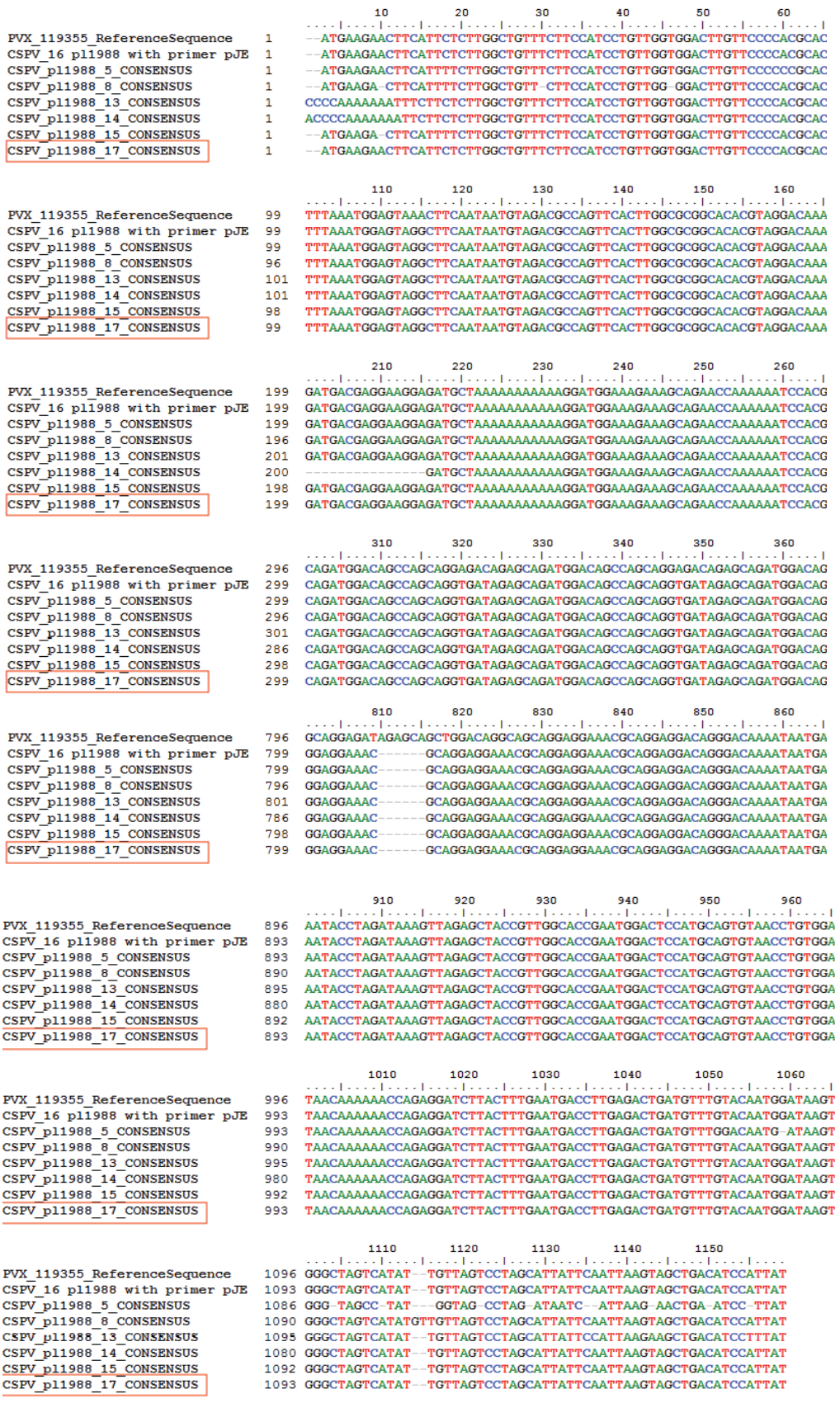
# 7. Appendix section

## Appendix-1. Sequences of primers used.

| Primer name/nº | Primer sequence |
|---|---|
| **uma1494** | AATTTAGTGGGATCCATATGC |
| **uma1497** | TATAATTCATTATGAGTAGTGTAATTCAG |
| **uma1654** | GCAAAGTGAAGTTCAAATATG |
| **uma1655** | GAAATCGCAAACATAAGTATC |
| **uma1901** | GTTCGCTAAACTGCATCGTC |
| **uma1902** | GTTTGAGGTAGCAAGTAGACG |
| ***P. berghei* 18S rRNA Forward** | AAGCATTAAATAAAGCGAATACATCCTTAC |
| ***P. berghei* 18S rRNA Reverse** | GGAGATTGGTTTTGACGTTTATGTG |
| **HPRT Forward** | TTTGCTGACCTGCTGGATTAC |
| **HPRT Reverse** | CAAGACATTCTTTCCAGTTAAAGTTG |

## Appendix -2. Consensus sequences from plasmids (With *Pv*CS@UIS4 cassette) obtained from transformed bacteria before transfection.



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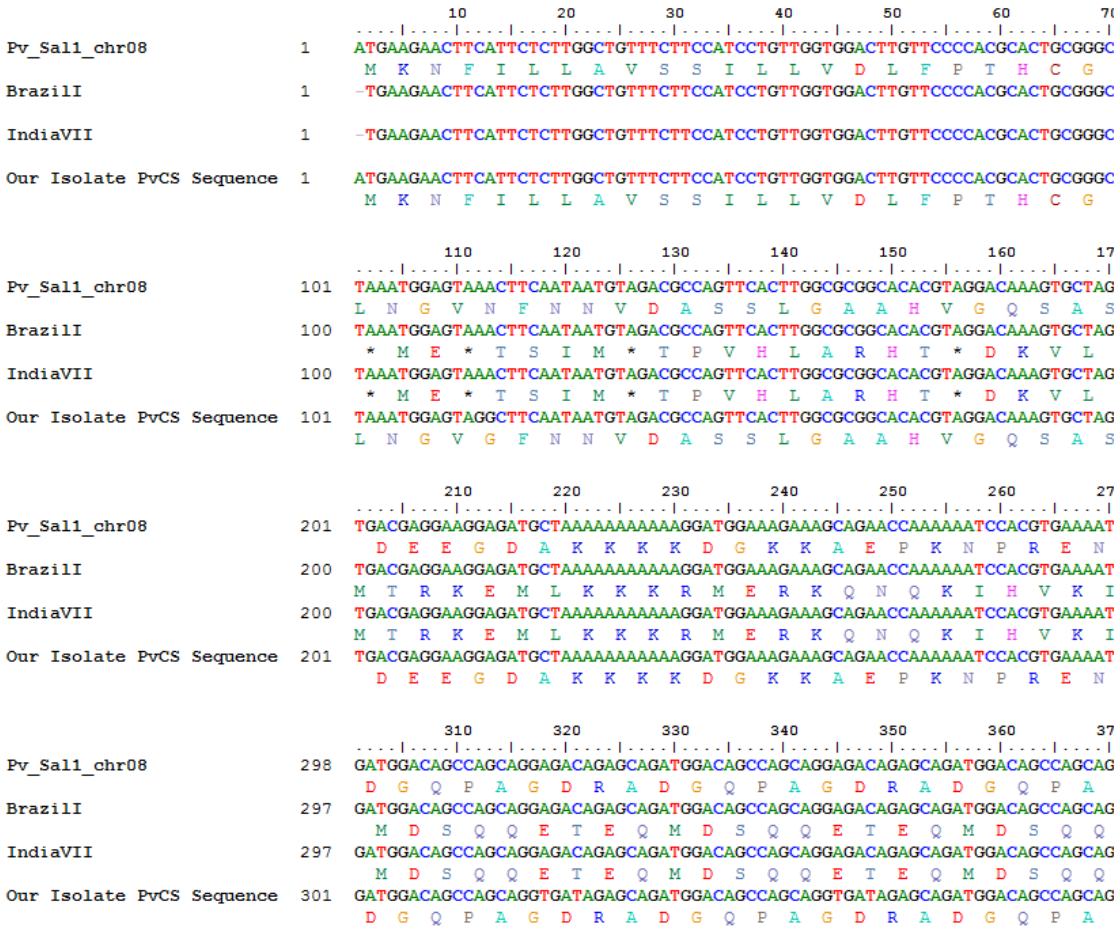
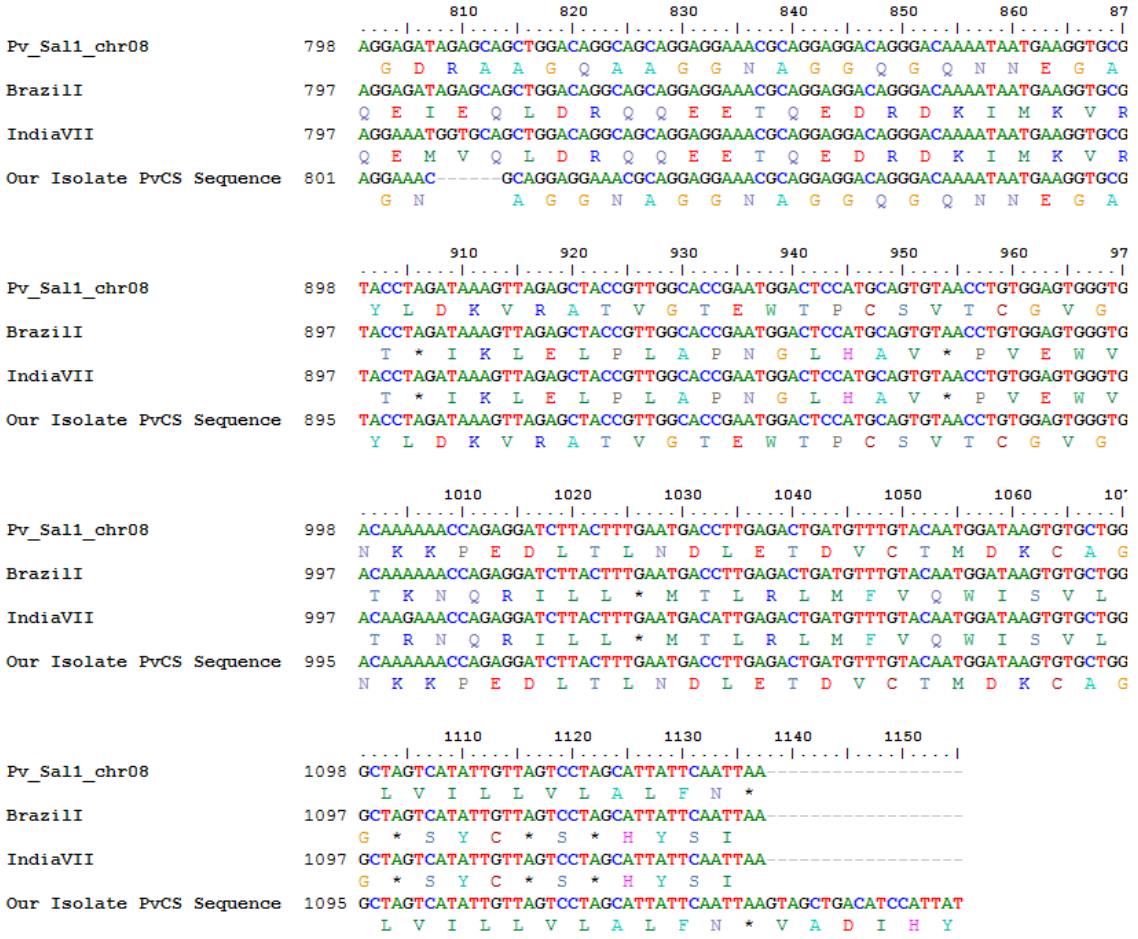
**Supplementary figure 1:** Consensus alignment between the *Pv*CS sequences present on plasmids obtained from different transformed bacteria (Colony 5, 6, 13, 14, 15 and 17). *Pv*CS sequence from colony 17 (red rectangle) was the one used for transfection as it is the one with highest homology to the reference sequence (PlasmoDB) PVX_119355.

## Appendix 3. Alignment Blast between our *Pv*CS sequence and *Pv*CS reference sequences






**Supplementary figure 2**: Alignment Blast between our *Pv*CS sequence and *Pv*CS reference sequences present on PlasmoDB; Pv_Sal1_chr08 (Salvador), BrazilI (Brazil) and IndiaVII (India).