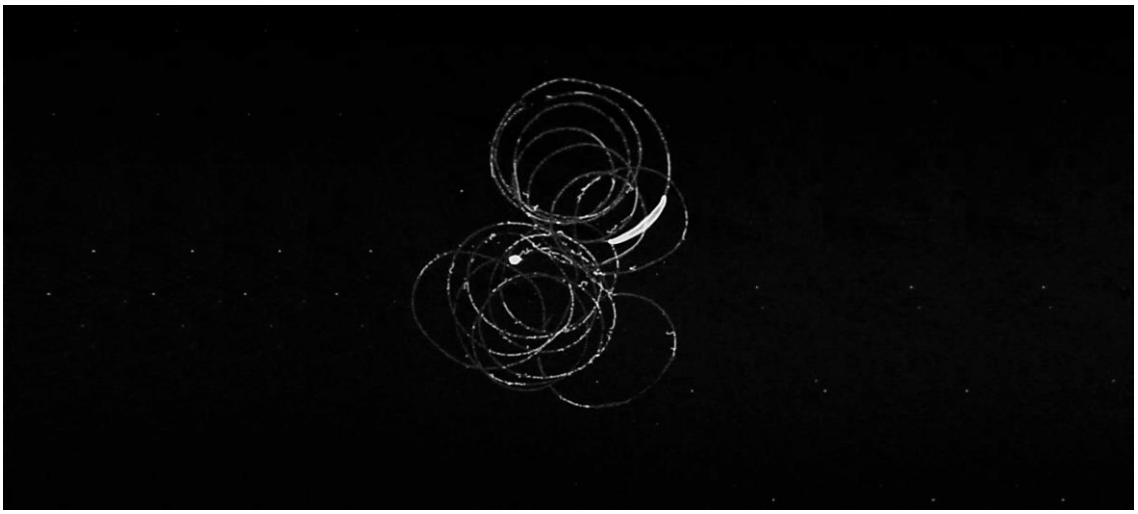
TÉCNICO LISBOA



Cryopreservation of *Plasmodium* Sporozoites for Whole-Organism Vaccination Against Malaria

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Biotechnology

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II. Resumo

As estratégias de vacinação contra a malária baseadas no uso de esporozoítos de *Plasmodium* surgem como uma abordagem promissora para desenvolver uma vacina eficaz contra esta doença. Uma das principais limitações no desenvolvimento destas vacinas é a necessidade de existir um sistema eficaz de criopreservação de esporozoítos. Este sistema iria também facilitar a investigação da infeção do parasita no fígado em laboratórios onde a criação de mosquitos e instalações de infeção não estão disponíveis. Todavia, um método de criopreservação de esporozoítos de *Plasmodium* é um processo difícil de desenvolver, sendo que o seu sucesso está dependente de vários factores, tais como a geometria do tubo de criopreservação e a composição da solução de criopreservação, que inclui crioprotectores (CPA). Neste projecto, propusemos optimizar uma metodologia de criopreservação de esporozoítos de *Plasmodium* testando uma vasta combinação de CPAs e outras condições associadas ao congelamento destes parasitas. Os nossos resultados estabeleceram VG2, como tendo a geometria apropriada para a criopreservação de esporozoítos e mostraram também que uma uma mistura racionalmente selecionada de CPAs leva a uma retenção significativa de ~50% da viabilidade de esporozoítos de parasita de roedores *P. berghei*, após criopreservação. Em paralelo, também mostramos que as nossas soluções de criopreservação são igualmente capazes de criopreservar esporozoítos do parasita humano, *P. falciparum*. Testes adicionais, *in vivo,* têm visado entender o impacto de injectar várias soluções de criopreservação *in vivo.* Estes resultados têm capacidade para revolucionar a capacidade de criopreservar esporozoítos de *Plasmodium,* estabelecendo uma ferramenta essencial para o desenvolvimento de estratégias anti-malária.

Palavras-chave: Vacina, Esporozoítos de *Plasmodium,* Criopreservação, Mistura de criopreservação

III. Abstract

Whole sporozoite-based malaria vaccination strategies appear as one of the most promising approaches to the development of an effective vaccine against this disease. One of the main bottlenecks in the deployment of such vaccines is the need for a system that enables effective cryopreservation of sporozoites, retaining their fitness and, therefore, preserving vaccine potency. Such a system would also facilitate the investigation of the liver stage of malaria infection in laboratories where insect rearing and infection facilities are not available. However, effective cryopreservation methods for *Plasmodium* sporozoites are extremely difficult to develop, and their success is highly dependent on various factors, such as vial geometry and the composition of cryopreserving solutions, including the cryoprotectants (CPAs) employed. We proposed to optimize this methodology by testing a wide range of CPAs and sporozoite freezing conditions in order to identify the optimal freezing formulation for cryopreservation of *Plasmodium* sporozoites. Our results established VG2 as presenting the most appropriate geometry for sporozoite cryopreservation and showed that a rationally selected blend of CPAs leads to a significant retention of 50% of P. berghei viability after cryopreservation and thawing. In parallel, we also showed that our freezing mixtures are also very effective to cryopreserve sporozoites of human-infective P. falciparum. Further in vivo tests have aimed at understanding the impact of injecting several freezing compositions *in vivo*, employing mouse models. These results have the potential to revolutionize our capacity to cryopreserve *Plasmodium* sporozoites, establishing an essential tool for the development of new anti-malarial interventions.

Keywords: Malaria Vaccine, *Plasmodium* Sporozoites, Cryopreservation, Freezing Mixture

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VII. Abbreviations

A. stephensis – *Anopheles stephensis*

ACT - Artemisinin-based Combination Therapy

AQ - Amodiaquine

AS - Artesunate

ATF – Anti-Freezing Proteins

BSA – Bovine Serum Slbumin

CHMI - controlled human malaria infection

CPA - Cryoprotectant

CPS - Chloroquine Chemoprophylaxis with sporozoites

CQ - Chloroquine

CS - Circumsporozoite

DHA - Dihydroartemisinin

GAP – Genetically Attenuated Parasute

HES - Hydroxyethyl Starch

HPRT – Hypoxanthine Phosphoribosyltransferase

HsHSPG - sulfated-heparan proteoglycans

I.V. - Intravenous

iMM – Instituto de Medicina Molecular

IRS - Indoor Residual Spraying programs

ITNs - insecticide-treated mosquito nets

LDL – Low Density Lipoprotein

LLNs - long-lasting insecticidal nets

MFQ - Mefloquine

npCPA – non-penetrating Cryoprotectant

P. cathemerium - *Plasmodium cathemerium*

P. falciparum – *Plasmodium falciparum*

P. gallinaceum - *Plasmodium gallinaceum*

P. kowlesi – *Plasmodium kowlesi*

P. lophurage - *Plasmodium lophurage*

P. malariae – *Plasmoium malariae*

P. ovale - *Plasmodium ovale*

P. vivax – *Plasmodium vivax*

pCPA – penetrating Cryoprotectant

PPQ – Piperaquine

PV - Parasitophorous Vacuole

qRT-PCR – Reverse transcription polymerase chain reaction quantitative real time

RAS – Radiation Attenuated Sporozoites

RDT - Rapid Diagnosis Tests

ROI – Region of Interest

RT – Room Temperature

SP - Sulfadoxine-Pyrimethamine

UIS – Up-regulated in Infective Sporozoites

WHO – World Health Organization

1. Chapter – Introduction

1.1 Malaria

1.1.1 Malaria: A World Threat

Malaria is one of the oldest documented diseases which remains a significant public health threat, responsible for almost half a million deaths worldwide and placing at risk approximately half of the world's population, accordingly to the World Health Organization (WHO)[1,2]. In 2016, throughout the world, 216 million of malaria new cases were estimated mostly in the WHO African Region (90%), followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%)[1]. Within these regions, people who reside in the poorest countries are more susceptible to malaria, especially children under 5 years old in sub-Saharan Africa (**Figure 1**)[1].

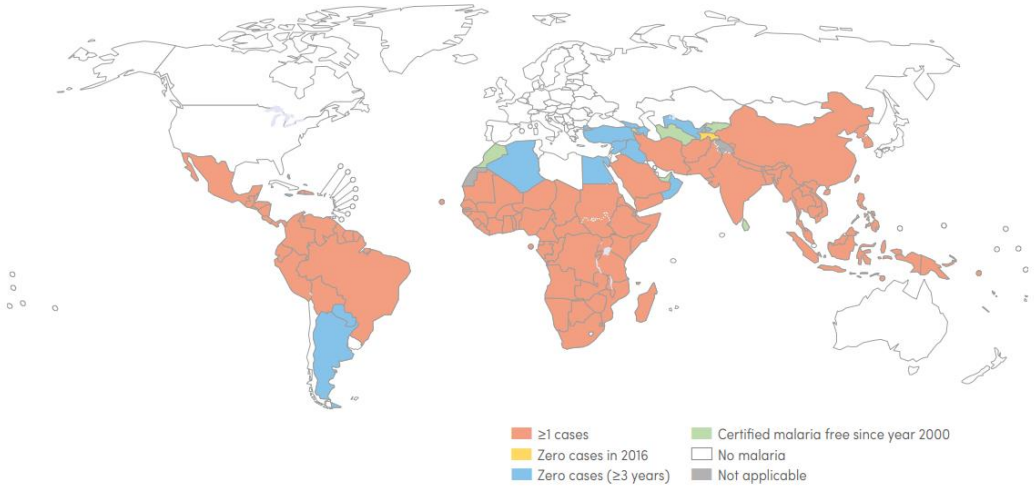


Figure 1 - Countries and territories with indigenous cases in 2000 and their status by 2016. Countries with zero indigenous cases over at least the past 3 consecutive years are eligible to request certification of malaria free status from WHO[1].

Malaria is an apicomplexan parasitic disease caused by a protozoan that infect mammalian hosts through the bite of an infected female mosquito *Anopheles* vector[3]. Five different species of *Plasmodium* genus are responsible for malaria in humans: *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*), *Plasmodium malariae* (*P. malariae*) *and Plasmodium knowlesi* (*P. knowlesi*). Among those human parasite species, almost all malaria deaths are associated to *P. falciparum,* the species responsible for the highest malaria-related mortality and morbidity worldwide[4]. Nonetheless, *P. vivax* also presents a significant risk to human health which should not be under appreciated, since it accounts for half of all malaria cases outside Africa[4]. Among the remaining human malaria parasites, *P. ovale* and *P. malariae,* are less common and cause a milder form of this disease, while *P. knowlesi,* is spread through southeast Asia, where it commonly infects macaques and may be lethal to humans in some occasions[5]. An additional problem mostly caused by *P. falciparum* and *P. vivax* malaria, is their impact on pregnant women, which leads to indirect mortality from abortion or intrauterine growth retardation[1].

1.1.2 Malaria Intervention Strategies

During the last years, malaria mortality rates have decreased, partially due to the growing funding for malaria control interventions including insecticide-treated mosquito nets (ITNs), long-lasting insecticidal nets (LLNs) and indoor residual spraying programs (IRS), which are particularly useful for pregnant women and young children[1]. Another aspect contributing to this reduction in malaria mortality regards increasing accessibility to diagnosis and treatment tools[1,6,7]. Microscopy and rapid diagnosis tests (RDT) are now commonly employed to malaria diagnosis[1,8]. A rapid and accurate malaria diagnosis is a crucial tool to define the treatment to be adopted and to ensure that a mild case does not develop into more severe disease and probable death[8].

Effective treatments are extremely important to ensure complete elimination of the *Plasmodium* from the patient's blood, reducing further complications (as chronic infection that leads to anemia)[1]. Chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) are less-effective drugs frequently used as first line or second line to fight mild malaria caused by *P. falciparum*, particularly in sub-Saharan Africa, since they are cheaper than the more effective drugs currently recommended by the WHO[9]. Between 1997 and 2004, studies showed that these two drugs had no longer efficacy to treat malaria throughout most endemic regions[10]. Amodiaquine (AQ), an antimalarial drug similar in structure and activity to CQ, remained effective although being associated to possible severe side adverse reactions[11]. A few years ago, WHO proposed some modifications on malaria treatments guidelines, changing from monotherapies to artemisinin-based combination therapy (ACT) in countries where *P. falciparum* malaria is resistant to CQ, SP and AQ[1,8]. The choice of the ACT is usually based on the therapeutic efficacy of the combination in the country or area of intended use. The five ACTs currently in use are artemether plus lumefantrine, artesunate plus AQ, artesunate (AS) plus mefloquine (MFQ), AS plus SP, and dihydroartemisinin (DHA) plus piperaquine (PPQ). Although more expensive than previous generation of effective drugs, ACTs ensure the highest cure rate and reduce the diffusion of malaria drug resistance[12].

Nonetheless, despite the availability of tools for diagnosis, prevention, control, and treatment, *Plasmodium* parasites persist.

1.1.3 Malaria Parasite Life-Cycle

Plasmodium species, that infect mammalians all have similar life-cycles successively infecting two types of host: a mammalian host and female *Anopheles* mosquitoes[13]. In mammalians, *Plasmodium* development is divided in two stages: the pre-erythrocytic stage and the blood stage (**Figure 2 (A)** and **(B)**). The pre-erythrocytic stage comprises all the steps since the mosquito bites until the release of the first generation merozoites into the bloodstream, whereas blood stage (also known as the symptomatic erythrocytic stage of infection) is characterized by the invasion of erythrocytes by the merozoites and the development of *Plasmodium* sexual forms[14,15].

Upon an infected female mosquito *Anopheles* bite, the mosquito salivary gland-resident liver-infective forms of *Plasmodium* parasites, termed sporozoites at this stage of their life-cycle, are injected in the dermis of mammalian host (**Figure 2 (A)**)[16,17]. At this stage, hundreds of injected sporozoites (170 on average) may have several fates in the mammalian host[17,18]. A small percentage of sporozoites remains

in the dermis - due to the exhaustion of their motility - while a bigger percentage leaves the site of the mosquito bite passing through the dermis cells, using random movements (termed gliding motility). These sporozoites continue with this gliding movements until they reach the host´s vasculature where they enter into the blood stream. Besides the blood vessel invasion, 15-20% of sporozoites may also migrate to lymphatic circulation, reaching the draining lymph node, where most are degraded by immune system while the remaining partially develop and eventually trigger an immune-malaria response[17]. The sporozoites that remain in the dermis (0,5-5% of the inoculated sporozoites) were shown as capable of developing into exoerythrocytic stages at the inoculation site but not able to trigger the blood stage infection, being degraded by innate immune cells and leading to the initiation of an immune response[19,20,14,18]. Thereby, only 10-25% of the inoculated sporozoites, once in the blood circulation, are able to reach the liver[21].

Once in the liver, sporozoites leave the capillaries crossing the sinusoidal cell layer composed of specialized highly fenestrated endothelial and Kupffer cells (the resident macrophages of the liver), in order to gain access to hepatocytes[16,15]. The selectivity of this process is suggested to occur as result of the interaction between the circumsporozoites (CS) protein located on the surface of sporozoites and sulfated-heparan proteoglycans (hsHSPGs) present on hepatocytes[22]. It was described that hsHSPGs on the liver cells are able to penetrate fenestrated endothelium cells leading to sporozoite sequestration from the sinusoidal lumen (**Figure 3**)[23]. After traversing the sinusoidal barrier, sporozoites traverse several hepatocytes by using gliding motility until they finally find the hepatocyte that will be invaded and will serve as host cell[24]. Although this migration remains not fully understood, it is known that

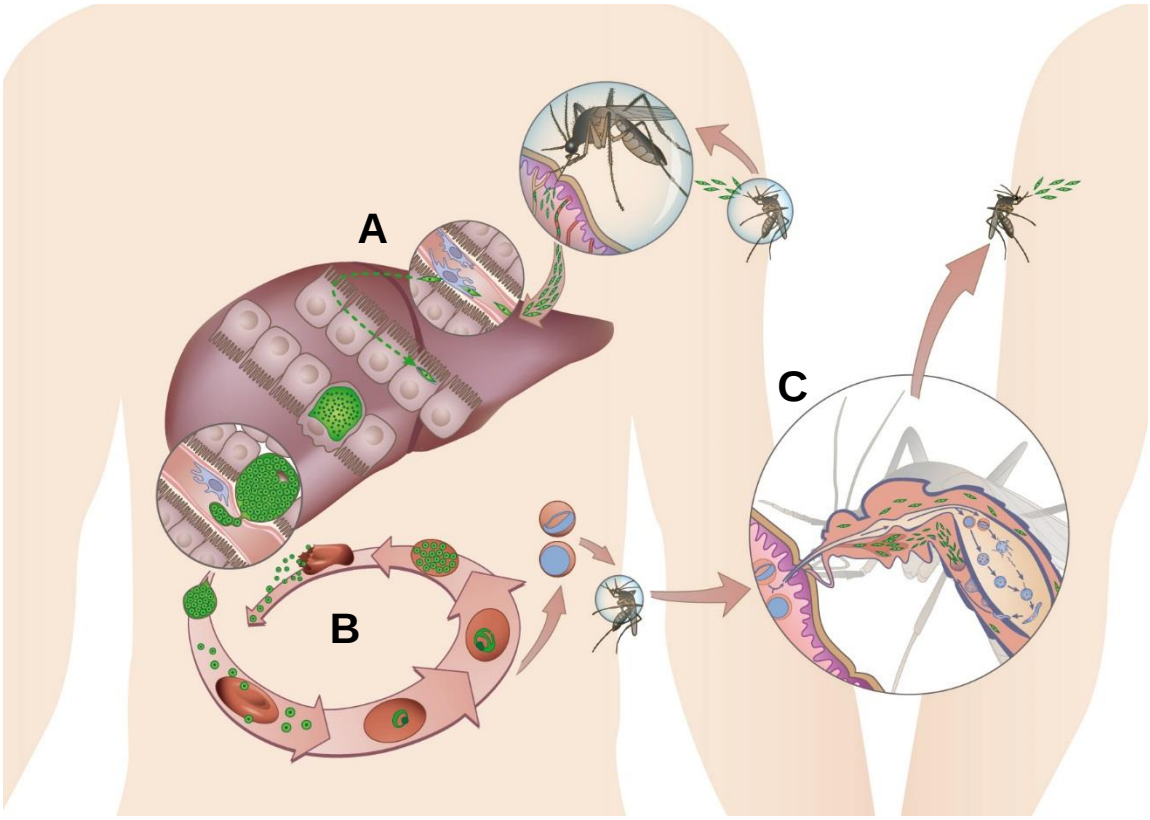


Figure 2 – Life-cycle of *Plasmodium* parasite. The life-cycle comprises 3 main stages: **(A)** Pre-erythrocyte stage; **(B)** erythrocytic stage and **(C)** transmission stage in the mosquito.

sporozoites with the ability to transverse hepatocytes are more infectious than the non-traversing ones mostly due to some molecules (potassium and hepatocyte growing factor) released during the traversing that activate sporozoites or leave the hepatocytes more susceptible to be infected[25,26,27]. The invasion process proceeds with the formation of a parasitophorous vacuole (PV) where the parasite remains during all liver stage development (**Figure 3**)[5,15]. Once the invasion is totally completed, parasites change its invasion form to a replicative one, the liver stage trophozoite[23]. To support their rapid multiplication, parasites derive nutrients from host hepatocyte both by passive diffusion through pores in the PV membrane and by active processes such as those that take up glucose , fatty acids and cholesterol[28,29]. After parasite replication and cellularization are completed, the newly formed haploid parasites (termed merozoites) are package into vesicles called merosomes which are then released into the blood stream and eventually burst in the vasculature of the lung, releasing merozoites that will initiate the erythrocytic stage of infection. Interestingly, merosomes have the ability to evade the detection by the host immune system since their membranes comprise components of the host hepatocyte plasma membrane[30].

Once the merosomes are in the blood stream, the erythrocytic stage of infection starts and each merozoite infects an erythrocyte, which is used as a site of asexual replication[14,31]. Inside the red blood cells, the parasite progresses from a ring-stage, which is the earliest form of the parasite that is established following invasion of the red blood cell by a merozoite, to a more metabolically active trophozoite and, finally, to a schizont, characterized by asexual replication and cell division[32]. At the end of each cycle, the parasites multiplied inside the red blood cells generate newly formed merozoites that rupture the cell and will infect other erythrocytes, destroying a significant amount of erythrocytes and

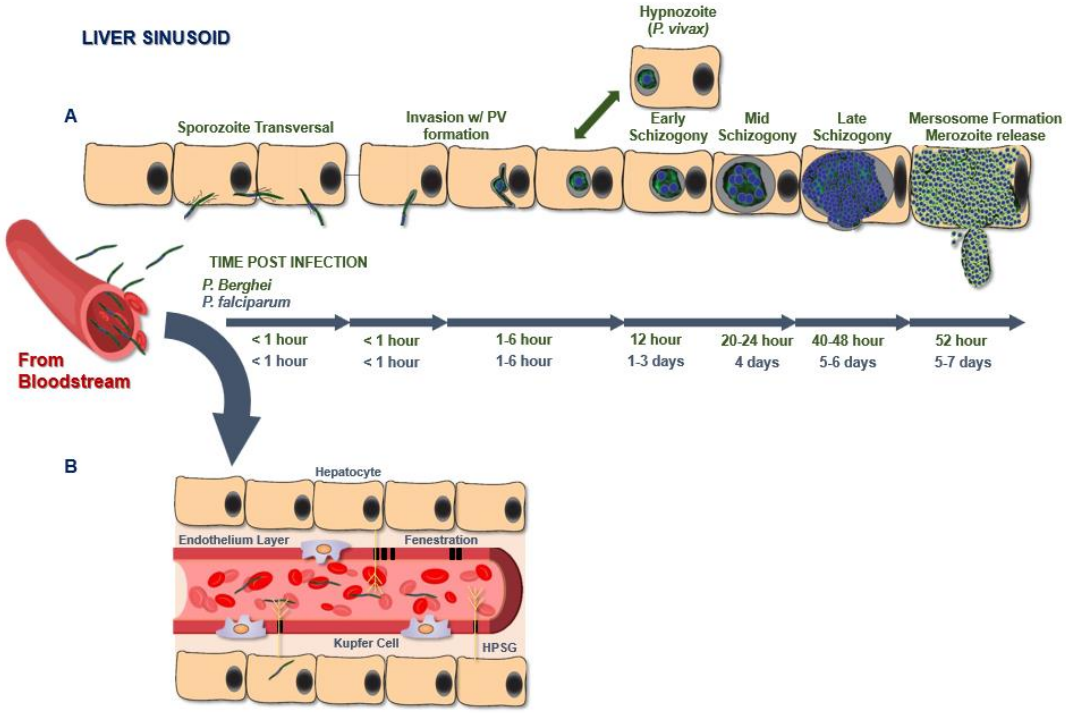


Figure 3 - Invasion process of *Plasmodium* and respective liver development: (A) Multiplication and differentiation in the liver; **(B)** Sporozoite sequestration from bloodstream to the liver. Image adapted[13].

causing the symptoms of malaria[32,33]. Additionally, during the blood stage, some parasites differentiate into the sexual forms, termed gametocytes, which are eventually taken up by an uninfected female *Anopheles* mosquito[31]. However this process of gametogenesis only occurs with a small number of parasites in the blood stage, and allows the continuation of the *Plasmodium* life-cycle[34].

In the mosquito, after ingestion, parasites sense a drop of temperature, a change in pH and a presence of xanthurenic acid, which trigger parasite maturation into gametes in the mosquito midgut. The next steps comprise the fusion of these gametes resulting in a diploid zygote, which develops into an ookinete before burrowed into the midgut epithelium and differentiate into oocysts. Within two weeks the oocysts originate sporozoites that go through the hemolymph and finally reach the salivary glands of the mosquito (**Figure 2 (C)**)[35].

1.1.4 Malaria Vaccine: An Immunological Approach Against the Parasite

Although malaria still represents one of the most pressing public health problems in the world, over the last decade the number of malaria victims has significantly decreased due to the implementation of control intervention measures enhanced by a huge investment effort ($2.6 billion, in 2013)[36]. Nevertheless, mosquitoes and parasites routinely develop resistances against drugs and insecticides and the demand for a safe, effective, and affordable tool to fight malaria is crucial[37]. A vaccine would help close the gaps left by current malaria control interventions and constitute an opportunity to facilitate interruption of malaria transmission and elimination of malaria in previously highly endemic areas[38]. Historically, vaccines have recorded dramatic impacts in many infectious diseases, especially on the currently eradicated smallpox. It is because of these successes that vaccines are considered the most cost effective single intervention for control, prevention, elimination and eradication of infectious diseases[36]. However, the development of a vaccine against malaria has proven to be much more complex than was imagined when 40 years ago, the vaccination against malaria was believed to be within reach[39]. The malaria parasites are very complex in their biology, with large genomes, antigenic diversity and different stages in their life-cycle differing vastly from virus and bacteria for which there are vaccines. Furthermore, a hypothetically harmful immune anti-inflammatory response must be considered, especially because the immune response also contributes to pathogenesis. Due to all these limitations on the development of a vaccine, there is still no effective vaccines available in the market for any parasitic disease[36,40].

1.1.5 Different Types of Malaria Vaccines

A crucial step towards an effective malaria vaccine development would be fully understand the parasite throughout its life-cycle. Malaria vaccines might be categorized regarding their target of the parasite life stage, and also according to the parasite unit used to trigger the immune response[41].

Regarding vaccines that target a specific parasite stage, they can be classified in 3 types: pre-erythrocytic (if they target any parasite form from sporozoite injection until completion of the liver stage); erythrocytic vaccines (target the asexual blood stage parasite forms); transmission-blocking vaccines (where transmission between different hosts is compromised by targeting the gametocyte or sporogonic stages of the parasite.

Malaria Pre-Erythrocyte Vaccines

If effective, malaria pre-erythrocytic vaccines avoid the disease by preventing the infection while also blocking the transmission to mosquitoes[42]. During this initial stage of mammalian infection there are only a hundred or even fewer parasites to be blocked, whereas after the liver stage thousands of merozoites are released into the blood stream that need to be tackled. Since there are no symptoms of malaria associated with the liver stage of infection, this type of vaccines completely prevents the disease[43].

Malaria Erythrocyte Vaccines

In contrast to malaria pre-erythrocytic vaccines, erythrocytic vaccines do not prevent *Plasmodium* infection, and are only capable of reducing the intensity and progression of the disease. This type of vaccine targets the asexual form of parasite, in the blood, by employing antigens presented on the surface of the merozoites that are involved in the process of erythrocyte invasion. However, this type of vaccine as so far shown low efficacy[44].

Transmission-Block Vaccines

Another approach to malaria vaccines relies in the development of a vaccine aimed at blocking transmission by targeting molecules unique to gametocytes or gametes and, subsequently blocking parasite development in the mosquito[45]. In contrast to the other two types of malaria vaccine previously described, this vaccine does not reduce the probability of a person becoming infected, neither does it reduce the severity of disease. For these reasons, it is considered an “altruistic vaccine” whose efficacy depends on the amount of people vaccinated within a community, depending on herd immunity to decrease parasite transmission. Until this moment, there are two vaccines based on this type of approach that have been tested in clinical trials, Pfs25 for *P*. *falciparum* and Pvs25 for *P*. *vivax*, targeting proteins on the surface of zygote and ookinete[46].

1.1.5.1 Pre-Erythrocyte Vaccines Against Malaria

Among the 3 different types of vaccines aiming at different stages in the *Plasmodium* life cycle, pre-erythrocytic vaccines are the most appealing approach to malaria vaccination because they have the potential for complete sterilizing immunity, arresting parasite development early (at the sporozoite or liver stage) and preventing both clinical disease in the human host and infection of mosquitoes[47]. In the early 1970s Clyde *et al*. have shown the potential of a pre-erythrocytic vaccine to produce sterile immunity in naïve volunteers against *P. falciparum*, making the pre-erythrocytic stage the only stage demonstrated to be capable of eliciting sterilizing immunity against malaria[47].

Malaria pre-erythrocytic vaccines are generally divided in two groups according to the unit used to trigger the immune response: sub-unit pre-erythrocytic vaccines and whole organism pre-erythrocytic vaccines. The former is a vaccine that uses specific antigens to be presented to the host immune system such as peptides or small proteins of the parasite, whereas the latter is based on the use of the whole organism to trigger the host immune response[48].

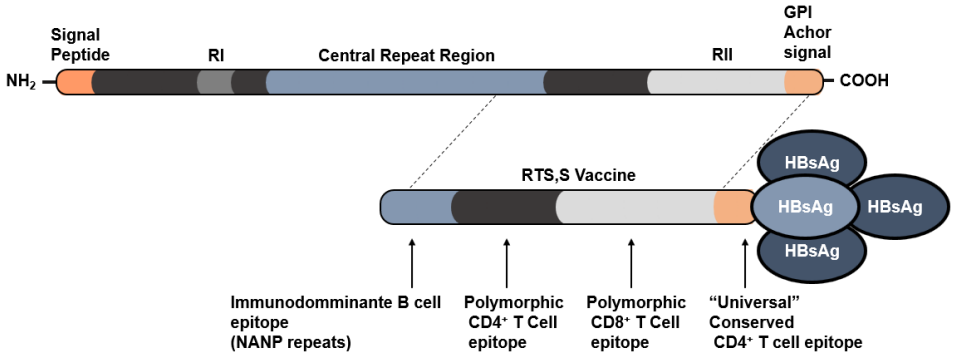


Figure 4 - Schematic representation of the CS protein and the RTS,S vaccine (Image adapted[47])

1.1.5.1.1 Sub-Unit Vaccine: RTS,S

The most advanced malaria vaccine candidate is RTS,S/AS01, also known as Mosquirix™[47]. It was conceived and designed in 1987 by scientists working at GSK laboratories and has become the candidate vaccine furthest along in development globally[47]. In 2014, a large-scale phase 3 efficacy and safety trial of RTS,S was conducted, showing that RTS,S/AS01 could provide meaningful public health benefit by reducing the burden of malaria when used alongside with other control interventions. The final results published on *The Lancet* demonstrated that efficacy against clinical malaria was 39% over four years of follow-up in children receiving four doses[49]. From 2015 until 2016 large-scale pilot implementations of RTS,S in young children in African settings of moderate-to-high parasite transmission, were announced by WHO[1]. The pilot implementation will take place at a sub-national level in Ghana; Kenya and Malawi, being expected to start in 2018.

RTS,S/AS01 uses the pre-erythrocytic CS protein of *P. falciparum*, which presents the particularity of being constituted by a central repeat region (responsible for triggering an immune response), flanked on each side by nonrepetitive regions containing T cells epitopes, as shown in **Figure 4**[50]. Antibodies recognize the central repetitive region of CS protein and bind to it, disabling the protein's capacity of mediate the hepatocyte invasion. Based on the CS protein characteristics exposed above, RTS,S comprises a central repeat region (R), a T cell epitopes (T) of CS protein combined with a hepatitis B surface antigen (S) as carrier matrix (F**igure 4**). As adjuvant, the formulation includes liposomal-based adjuvant system AS01, which contains components that increase the immunological response[43,42,51].

Prospectively, the pilots to be conducted between 2018 and 2022 will assess if the feasibility, safety and vaccine's protective effect previously demonstrated in phase 3 trials remains the same in a real-life setting. They also evaluate: **(1)** the feasibility of providing all four doses of RTS,S through existing health services; **(2)** the vaccine's potential role in reducing childhood deaths; and **(3)** its safety in the context of routine use. The vaccine will be delivered in the areas and regions selected by the routine national immunization programs and a rigorous evaluation will be done[47].

In summary, despite the tremendous efforts for RTS,S development, the results of phase 3 are modest and do not reach the objectives proposed in 2013 WHO malaria vaccine Technology Roadmap, where

was stated that malaria vaccine should be viable for use and licensed by 2030, having at least 75% efficacy[1].

1.1.5.1.2 Whole Organism Pre-Erythrocytic Vaccines

Considering the limitations of a sub-unit malaria vaccines, one of the oldest approaches, which uses the whole organism live and attenuated, has seen an increased interest to become a potential solution to this problem[42].

In the early 1940s, it was shown by Mulligan, Russell and Mohan the first evidence that by using whole organism sporozoite could partially be possible to protect a fowl against the bird parasite, *Plasmodium gallinaceum*[52] (*P. gallinaceum*). During the years thereafter, Freund *et al*. first with ducks immunized with *Plasmodium lophurae* (*P. lophurage*) and then with rhesus monkeys with *P. knowlesi* it was demonstrated that through a whole-organism vaccine is conceivable to achieve a good protective efficacy against the parasite[53]. Studies performed by Nussenzweig *et al*. first showed, in mice, that protective immunity could be induced through the injection of radiation-attenuated *Plasmodium berghei* sporozoites, while latter Clyde *et al*. demonstrated that 90 percent of naïve humans could be immunized against *P. falciparum* after immunization with infected and irradiated mosquitoes[54,55].

The sporozoites that constitute the whole organism pre erythrocytic vaccine may experience 3 types of manipulation in order to be used as vaccine that offers immune protection without disease development and progression. These are outlined below.

Radiation Attenuated Sporozoites (RAS)

Until the moment, Sanaria™ has been one of the companies more focused in the development of a vaccine candidate to target *P. falciparum* using RAS inoculation as a basis for vaccination, as well as in creation of methods for purification and cryopreservation of sporozoites attenuated by irradiation-metabolically active and non-replicative[56]. However, its patented standard cryopreservation method leads to 7.4 times less sporozoite infective capability, which directly affects vaccine efficiency[57]. Initially, the downside of PfSPZ was the route of administration which until very recently was only accomplish by mosquito bite. Based on the manufacturing processes developed for the production of aseptic, purified, vialed PfSPZ vaccine, the first clinical trial Sanaria PfSPZ vaccine was proved to lead to low levels of immunogenicity and protective efficacy when injections were subcutaneous and intradermal[58,6]. Studies in non-human primates indicated the vaccine would be protective if administered by intravenous (IV) injection. In a second clinical trial, 5 doses of PfSPZ vaccine administered by IV injection protected 6 of 6 subjects against controlled human malaria infection (CHMI) with homologous (same as in vaccine) *P. falciparum* parasites 3 weeks after final immunization[59]. In a third trial, 55% protection was achieved at 14 months after a 4-dose immunization regimen with $2{,}7 \times 10^5$ sporozoites[60]. For higher protective levels with such vaccine, both the dose per vaccination and the number of vaccinations needs to be increased[61]. Besides vaccine efficacy requires several doses and high number of cryopreserved PfSPZ, this vaccine only offers protection against one *Plasmodium* species, *P. falciparum*, not meeting the WHO Malaria Vaccine Technology Roadmap guidelines which state the goal for the year 2030 of a malaria vaccine with protective efficacy of at least 75% against clinical malaria by *P. falciparum* and *P. vivax*, and development of malaria vaccines that reduce transmission of the parasite[62].

Genetically Attenuated Parasites (GAP)

Although the feasibility and protective immunity are being obtained by inoculation with radiation attenuated parasites, recent innovations in genetic engineering and the increased knowledge about complete parasite genome sequence has enabled the development of a new kind of attenuated parasites[63]. It was in 2005 that sporozoite attenuation by targeted removal of genes essential for liver development was reported, thereby creating genetically attenuated parasites (GAP)[42]. The genes selected to be knocked out of the parasite are generally linked to its liver stage replicative activity, so that the parasites are arrested in the liver, unable to proceed to the blood stage of infection. The genetic manipulation technique commonly used is double crossover recombination instead of single crossover recombination, therefore avoid a possible reversion by the parasite and subsequent development in the liver[42].

Two examples of pre-erythrocytic stage development-specific genes are the Up-regulated in Infective Sporozoites 3 and 4 genes, (UIS)3 and UIS4. The UIS3 gene was successfully deleted in *P. berghei* and immunizations in rodents with this genetically attenuated parasite have proved to lead to sterile immunity when challenged with wild type sporozoites and no breakthroughs were observed[63]. Immunization with UIS4-Knock out parasites was also shown, capable of sterile immunity. Nevertheless, when higher doses of sporozoites were administered intravenously, mice became infected with blood-stage parasites. Further genotype confirmed that the blood-stage parasites were UIS4 knockout, therefore the parasite reverted to wild-type[64].

Besides *UIS* genes, P36p, a member of a small family of *Plasmodium* surface proteins (P48/45 family) is another promising vaccine candidate[65]. This vaccine candidate based on the double knockout of P36p and demonstrated to be effective in enhancing sterile protection considering in *Plasmodium* rodent models[66]. A few years later double knockout was applied to human parasite *Plasmodium falciparum* orthologous genes[67]**.** These genetic modifications in human malaria parasites were followed by a clinical trial to evaluate the safety and immunogenicity. Two groups of volunteers were formed where one was subjected to 5 mosquito bites and the other to 200 mosquito bites with genetically modified parasite. The results showed the former triggered a not significant immunological response whereas the 200 mosquito bites group manifested a higher immunologic feedback after parasite injection. Additionally, in one of the elements belonging to the latter group was diagnosed as positive 12 days after inoculation with the blood stage parasites genotyped and confirmed as being the doubled knockout parasite[66].

Chloroquine Chemoprophylaxis with Sporozoites (CPS)

More recently, immunological protection not using an attenuated parasite but combining a controlled injection of fully infective sporozoites with chloroquine administration, a drug known to kill asexual blood-stage parasites but not liver-stage parasites, was demonstrated. This approach was firstly tested with *P. berghei* in rodents and also validated years later with humans when drug-sensitive strain of *P. falciparum* under CQ prophylaxis allowed to accomplish sterile immunity[68]. Furthermore, these studies did demonstrate to be more efficient providing immunologic protection when compared to RAS, mainly due to the high number of sporozoites required by the latter to elicit sterile protection[69]. PfSPZ-CVac is a vaccine approach based on immunization with live, aseptic, purified, cryopreserved, non-irradiated

PfSPZ, injected intravenously in healthy adult volunteers taking CQ for antimalarial chemoprophylaxis (vaccine approach denoted as PfSPZ-CVac). Three doses of $5.1x10^4$ PfSPZ were well tolerated and safe, providing 100% protection (9/ 9), which becomes PfSPZ-CVac a highly efficacious vaccine candidate. However, the long-term protection and the regimen of vaccine administration (56-day regimen (100%) to a 10-day regimen (63%)) are two factors that are limiting the success of this vaccine[70]. Hypothetically this can be solved by increasing the numbers of PfSPZ per dose which would also boost the protective efficacy to 100% with 10-day regimen, as suggested by the authors[70].

iMM's Prudêncio Lab: An out of the box approach

Inspired by the old smallpox vaccine concept, the Prudêncio lab proposed an out of the box idea to vaccinate against malaria. Historically, the first vaccine to be developed in the world was the vaccine for smallpox as result of Edward Jenner's work. Smallpox was one of the worst world threats, responsible for thousands of deaths per year until its eradication in 1979. The idea of vaccine arose when Jenner noticed that women who extracted the milk from cows developed a mild version of the disease (cowpox) and were subsequently protected from severe smallpox. Thereby, came up the idea of use cowpox virus to immunize humans against its human counterpart, smallpox. This enhanced the concept of cross-species protection, where immunization with human-harmless pathogen confers protection against infection by its human-infective counterpart[71].

Based on this concept, the Prudêncio lab came up with the idea of genetically modifying *P. berghei* parasites which are non-pathogenic to humans, by introducing surface proteins of *P. falciparum* so that human immune system recognizes the parasite and develops an immune response free of safety risks. The surface protein used was CS protein that was genetically introduced into *P. berghei* in a neutral locus fused with the UIS4 promoter, which led to Pb(PfCS@UIS4). The inserted CS protein presented a similar pattern of expression to the CS protein from *P. berghei* on its surface and Pb(PfCS@UIS4) presented similar levels of infectivity to those seen by *P. berghei*, making it a strong vaccine candidate. Recently published work already proved that these parasites are capable of infecting and develop in human hepatocytes.

The use of *P. berghei* as vehicle to present antigen of *P. falciparum* or *P. vivax* has some advantages regarding to similar genetic approaches:

- Non-pathogenic and non-infectious in humans;
- Capable of develop inside human liver cells;
- Unable to complete the life cycle inside human erythrocytes.
- Amenable to genetic manipulation;
- May express several stage-specific antigens of diverse species triggering a more efficient immune response.

At this moment the vaccine candidate is being subjected to Phase I/IIa clinical trials, where the safety and protective efficacy of the vaccine candidate is evaluated.

1.1.6 From Cryopreservation to Malaria Vaccine

Considering a live whole-organism pre-erythrocyte vaccine as the best option so far to defeat malaria, there are several constraints that must be solved, particularly the development of an effective sporozoite cryopreservation method.

In the malaria research context, sporozoites are the crucial parasite stages used to trigger an infection and to perform several pre-erythrocytic development tests, either *in vivo* or *in vitro,* on researchers' laboratories. Nonetheless, the studies to develop a new vaccine or to find out new antimalarial drugs remain insufficient due to the limited availability of sporozoites for research[72]. This is a consequence of the relatively reduced number of costly facilities with capacity to reproduce the mosquito life-cycle and, subsequently produce sporozoites[73]. Cryopreservation of malaria sporozoites appears as a technique with high interest to be improved since it would alleviate the need of constantly dissect salivary glands from mosquitoes in order to obtain fresh sporozoites as well as to avoid the costs associated to the maintenance of an insectary facility to reproduce the mosquito life-cycle[74]. Besides, the effort putted in the development of whole organism malaria vaccines has allowed to achieve high levels of protective efficacy but only when high number of sporozoites per dose is used[70]. This is the case of PfSPZ and PfSPZ-CVac which already provided very promising results using a high dosage or a 58-day regimen of administration, respectively, of live attenuated (radiation or chemically, respectively) cryopreserved sporozoites for immunizing[70,59]. The standard cryopreservation method currently used in the production of these vaccines is the one developed and patented by Sanaria. Its effectivity is very low, killing the vast majority of sporozoites, which require injecting more cryopreserved sporozoites to achieve sterile protection[57]. This is the main reason why a more effective cryopreservation process is highly desired to warranty sporozoite survival after cryopreservation, increasing the efficiency of whole organism malaria vaccines as well as facilitating the malaria vaccine production and storage[75,70,59].

1.2 Cryopreservation

It was in 1948 that C. Polge, A.U. Smith and A.S. Parkes accidentally discovered that M (GLY) enabled fowl spermatozoa survival during freezing to -70ºC. Since then, was born a new phase of dramatics developments in this technique, currently known as Cryopreservation[76].

Cryopreservation is by definition the process that leads biological material to ultra-low temperature (-196ºC), preserving structurally intact cells and tissues for a long period of time and finally bringing the them back to physiological relevant temperatures[77]. At cryogenic temperatures, biological samples are in a state of "suspended animation" almost without any significant enzymatic and metabolic activity[78]. However, this process that leads cells to a long-term preservation, may also be fatal for some types of cells under specific conditions. There is a great variation in the cryobiological response and cryosurvival depending on the cell type[79]. The great diversity in survival, varying from cell type to cell type, after cryopreservation, does not lie within the ability of cells to withstand storage at ultra-low temperatures but with the transition phase where cells undergo a series of biochemical and physiological changes when the surrounding medium suffers physical and chemical alterations (water-ice transition)[80]. Generally, the cryopreservation procedure is focused on attenuating those damaging physical, chemical and biological alterations being essentially performed by two distinct processes: *conventional*

cryopreservation and *vitrification* (that are explained afterwards)[77]. Besides, any cryopreservation procedure should comprise the following 4 steps: **(1)** Cells should be mixed with the respective freezing mixture previously to be frozen; **(2)** Cooling of the cells to cryogenic/ storage temperatures; **(3)** Thawing/ Warming of the cells; **(4)** Removal of the freezing mixture[80].

These phenomena are amenable to experiment and analysis, and this has made it possible to develop effective methods for the preservation of a very wide range of cells and some tissues; these methods have found widespread applications in biology and medicine[79].

1.2.1 Cryoinjuries

Life relies on a very complex interaction among macromolecules and chemical reactions that happens in water. Water plays an essential role in biology and more importantly for cryobiology, since without this molecule there is no life or at least life processes[77]. However, during freezing process water can also show deleterious effects on living systems and be responsible for injuries[81]. When living systems are cooled to sub-zero temperatures, they experience potentially lethal events as result of their transition from ambient temperatures to low temperatures (Chilling or cold shock injury) or especially during the water-ice transition phase. Besides freezing, another important step that can directly contribute to increase the damage on cells after cryopreservation is the thawing, where cells that have survived to cooling and extended storage are brought from ultra-low temperatures to physiological and biological active temperatures[77,82]. These types of cryoinjuries are explained in more detail below.

1.2.1.1 Water-Ice Phase Transition

The great hurdle for cells at low temperatures is the water-to-ice phase transition that leads to profound intra- and extracellular alterations. When ice starts to grow there are two main mechanisms that may cause damage on cells: osmotic stress, termed as “solution effect” injury and mechanical damage, caused by shear forces acting during ice crystals formation[83].

At the point of ice formation, water freezes as a pure substance that gathers itself up into crystals pushing everything else out. As the ice starts to grow, cells and solutes are excluded to residual fractions of remaining liquid water where residual solute concentration increases and colligative freezing point depresses[79]. Prolonged exposure of cells to these lethal extracellular concentrations have dramatic detrimental effects[77]. The increase in osmotic strength causes an efflux of water from cells, and subsequently leads to full cell dehydration[81]. For the great majority of cell types, this dehydration has been observed as a consequence of relatively slow cooling rates applications[83]. At slow cooling rates, ice fraction takes more time to grow, leaving cells in the hyperosmotic unfrozen zones and giving them sufficient time to efflux water across their membranes in an attempt to balance the osmotic pressure , Independent studies performed by Meryman and Lovelockled led these two cryobiologists to classify “solution effect” as the most damaging factor during freezing of cells[84,85]. However, if the cooling rate is too high another problem may occur to some biological systems: the ice growth is a rapid process whereas the diffusion of water through cell membranes is relatively slow because membranes act as a resistance. This means that during an extremely high cooling rate, intracellular ice crystals are formed as result of the solidification of water and cells suffer from membrane disruption which ends in cell lysis[81].

Based on these considerations, it is well-established that cooling can have a great impact in obtaining optimal survival for most of biological systems during freezing-thawing (**Figure 5**). Mazur conducted very important studies on this field throughout the years where this author could observe that each specific biological system has its own optimal cooling rate, with decreased survival at cooling rates higher or lower[83,86]. Another important aspect that emerged was that slow or fast cooling rates are not the only factors affecting cells survival, being cryopreservation an even more complex process with several factors having an impact on its success.

Another reason for cell injuring during freezing is attributed to the mechanical damage. During freezing, cells are excluded to unfrozen portions among ice structures, as discussed before. However, besides "solution effects", cells are forced to interact among them and against the constantly forming ice crystals. As result of this interaction, the shape of cells is deformed and their membranes destroyed through extracellular shear forces[80].

1.2.1.2 Chilling and Cold-Shock Injury

The vast majority of protocols require a random cooling from the room temperature (~25ºC) to a temperature slightly below nucleation temperature of solution[87]. During this process, cells can be damage by the cooling rate applied (cold shock injury) or by their residence at low temperatures *per se* (Chilling injury)[77]. Most of the times, cold shock injury arisen from very rapid cooling rate whereas chilling injury is more associated to slow cooling rates events, being mitigated if the cooling rate is increased. In fact, several studies have placed chilling injury as one of the limitations to achieve an optimal cryopreservation process. Chilling changes the lipid membrane structures that normally is in a liquid crystalline state, at physiologically temperature, to transform into a solid-gel phase with lateral separation of membrane proteins. Based on these consequences and since that at low temperatures the cells metabolism is reduced, chilling injury and cold shock reveal conduct to biochemical imbalances and reactive oxygen species formation[87].

1.2.1.3 Thawing/ Warming: Recrystallization

After cells overcome all the stresses during freezing, to achieve a successful cryopreservation, cells still need to face some challenges on their thawing[77]. Although thawing is a different part of the story, it is tightly related with the freezing process applied before. The relationship between warming and freezing as well as its effects on cell survival rely on the cooling rates employed during cryopreservation. In the case of slowly frozen cells, the events during the return to ambient temperature are mostly a mirror image of those that occurred during cooling[78]. For these cells, the thawing response is complex, highly variable and very complicated to be predicted *a priori.* On the other hand, several studies demonstrated that cells subjected to a rapid freezing process revealed a much better response to a rapid thawing process than the slowly thawed ones[77,88]. The benefits of a rapid thawing rate are related with the size of ice crystals formed during the initial cooling and the effect of thawing rate on their size[87]. Higher cooling rates form smaller internal ice crystals, and small crystals appear to be less damaging than large ones. However, if subsequent warming is slow, those small crystals can enlarge to damaging size by the process of recrystallization[77]. Thermodynamically, even at sufficiently low cooling rates when cells are cooled to relatively high-end temperatures (-40ºC) and then immediately transported to liquid

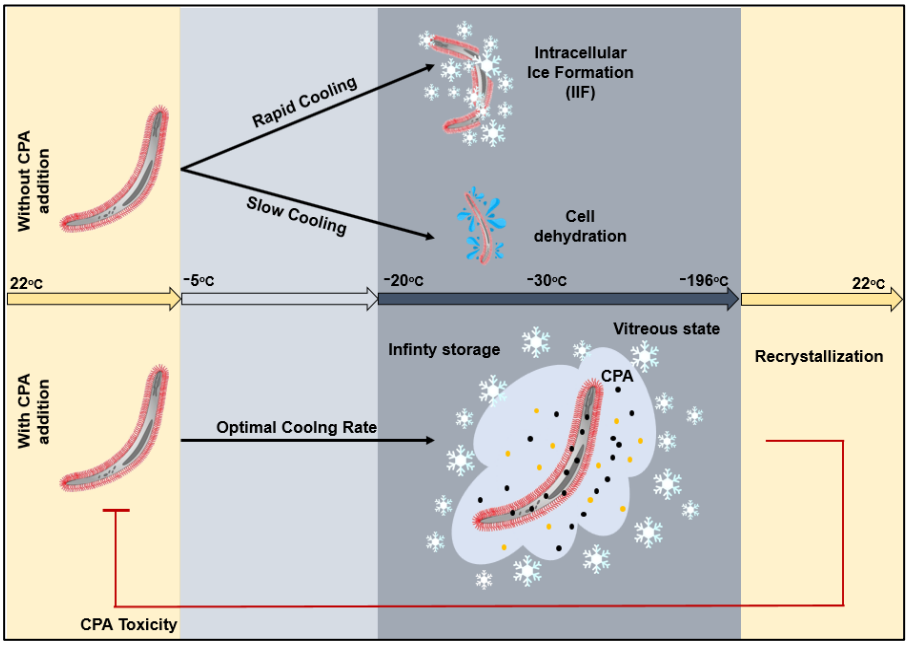


Figure 5 - **Overview of the challenges faced by cells during cryopreservation:** Impact of different cooling rates and mechanisms of protecting cells against freezing and thawing damages.

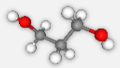
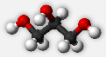
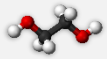
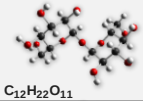
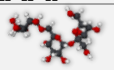
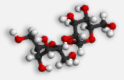
nitrogen, the viscosity increases, the diffusion coefficient is reduced and a glassy matrix is formed. This glassy state is formed when water does not crystalize but undergoes a vitreous state that will be explained in the next topic[86]. Because of this, the thawing process needs to happen as rapidly as possible since as the temperature increases, the water arrested in the glassy matrix suffers a process of devitrification and becomes available to recrystallize[83]. Additionally, if the thawing rate is too slow another problem emerges: cells are exposed during a longer time to a very concentrated solutes which triggers an osmotic stress on them[89]. On the other hand, cells thawed at very high rates are also under considerable differences in solutions and rapid influx of water which may be lethal to very dehydrated cells[77].

Therefore, there are several factors affecting the optimal thawing rate. The thawing condition to be employed on cells frozen, either slowly or rapidly, are closely related with their tolerance to osmotic and solute effect which varies from cell type to cell type.

1.2.2 Cryoprotectants (CPA)

As previously discussed, a wide range of factors affect the effectiveness of cryopreservation of biological systems, particularly in microorganisms[90]. One of the most important conditions is the formulation of the medium used to suspend the organisms for freezing. Although, there are some organisms, such as bacteria and microbial spores, with good survival after freezing in media without any protective additive, normally the presence of cryoprotectants (CPA) is essential for increasing survival considerably[91]. CPAs are chemical additives highly soluble in water that have the ability of depress the melting point of water but more importantly protect cells against the challenges of cryopreservation[92]. The mechanisms used by CPAs to protect cells are still not fully understood but it is well established that they have a great importance by suppressing salt concentration in the unfrozen portion of the solution during slow cooling as well as avoiding ice crystals formation[77,81]. It is presumed that CPAs increase the viscosity of the

Table 1 – Informative table of the most used CPAs in the cryopreservation field. The CPAs are classified regarding their function and molecular features

CATEGORY		CPA DENOMINATION	MW ($g.mol^{-1}$)	MOLECULAR FORMULA	MAJOR APPLICATIONS	APPLICATIONS CRYOPRESERVATION	CONC.	REF.
PENETRATING CPAs	Penetrate cell membranes: - reduction of cell water content; - Less Intracellular ice formation	**1,3-Propanediol (PROH)**	**76,09**	 $C_3H_8O_2$	. Building block in the production of polymers; . Formulated into composites, adhesives, coatings, modlings, etc.; . Electronic cigarette liquid; . Antifreeze compound.	. Tested for cryopreservation of *Leucocytozoon* protozoa; . Applied in canine ovarian cortex cryopreservation;	2-10%	.Solis (1972); . Simione, Frank P. Daggett, Pierre Marc (1977); . C Lopes, A. Alves, *et al.(* (2016)
		Dimethylsulfoxide (DMSO)	**78,13**	 C_2H_6OS	. Useful solvent for NMR spectroscopy; .Topical analgesic; . Drug vehicle; . Important cryoprotectant.	. Originally used to cryoprotect red blood cells (RBC) and spermatozoa. Nowadays, it is widely used for sperm, viruses, protozoan, bacteria, stem cells cryopreservation	1-32%	. J E. Lovelock *et al* (1959) . I. Mitrus *et al.* (2018) . M. Di Santo (2012) . W.E. Collins (1963)
		Glycerol (GLY)	**92,09**	 $C_3H_8O_3$	. Food Industry as preservant, sweetner and thickning agent; . Medical/ pharmaceuticals preparations; . Electronic cigarette liquid; . Antifreezing agent.	. It was the first CPA accidentally discovered by Polge. Besides successful spermetozoa long-term cryopreservations, GLY has also been applied for a wide range of cells types.	1-40%	. Polge C *et al.* (1949); . P. Purdy, (2006) . C. Medeiros (2002)
		Ethylene Glycol (EG)	**62,07**	 $C_2H_4(OH)_2$	. Raw material in the manufacture of polyester fibers; . Antifreeze Formulations.	. Cryopreservation of *Plasmodium chabaud* alongside others bacterial and fungi species; Revealed high tocixity to some protozoans;	2–40%	. H. Chi *et al.* (2002); . M. Kasai *et al.* (2006) . S. Voelkel *et al.* (2002)
NON-PENETRATING CPAs	Sugars Depression on freezing point: - Smaller ice crystals formation; - Energy substrate; - Balacing osmotic pressure	**Trehalose (Trea)**	**378,33**	 $C_{12}H_{22}O_{11}$	. Used as sweetener agent in food industry, however is less soluvel and sweet than sucrose.	. Natural CPA produced by yeasts and insects. Effective CPA for certain viruses, *S. cerevisiae,I ctobacillus bulgaricus* . Lately, Trea has been imensively applied to embryonic stem cells cryopreservation and freeze dry protocols	5–19%	. C. Coutinho *et al.* (1988); . R. STefanello *et al.* (2018) . S. Voelkel *et al.* (1999)
		Raffinose (Raff)	**594,52**	 $C_{18}H_{32}O_{16}\_5H_2O$	. Induce rapid germination of arabidopsis in the dark; . Less used in food industry than sucrose.	. In combination with 10% glycerol cryoprotected algae, *Scenedesmus quadricaud* , S. brasiliensis, and Chlorella vulgaris	~10%	. N. Tada *et al.* (1990); . B. Storey *et al.* (1998)
		Sucrose (Suc)	**342,3**	 $C_{12}H_{22}O_{11}$	. Suc is intensively used by food industry as the main sugar and important source of energy; . Add to icecreams to originate a smooth texture, with small ice crystals.	. Cryoprotection for viruses *E. coli* , *E. aerogenes, Lactococcus lactis ssp. lactis, L. delbrueckii, Methanococcus vannielii, Chlamydia spp, Mycoplasma spp*. *A. Marginale* However, less protective for cryosensitive cells.	1–68%	. R. Dalgliesh *et al.* (1980); . R.S. Rumsey *et al.* (1992)
	Lipids & Proteins (polymers) Depression on freezing point: - Smaller ice crystals formation; - Membrane Stabilization; - Source of Energy	**Fetal Bovine Serum (FBS)**	**N/A**	N/A	. Serum-supplement for the in vitro cell culture of eukaryotic cells.	. Mouse serum used in *Plasmodium* sporozoites (with Hidroxylethyl Starch); . Embrionic and cell lines cryopreservation.	10-90%	. H. Men *et al.* (2005); . J.L.. Leef *et al.* (1979) . M. Barceló-Fimbres *et al.* (2007)
		Egg yolk (EY)	**N/A**	N/A	. Employed as an emulsifier by food industry; . Ingrient for liqueurs; . Its extratct has been used in cosmetic, nutrition, and medicine.	. Important component in spermatozoa cryopreservation extender	2-30%	. F. Marco-Jiménez *et al.* (2004); . S. Layek *et al.* (2016) . E. Aboagla *et al.* (2004)
		Egg Albumin (EA)	**N/A**	N/A	. Used in clarification and stabilization of wine; . Source of proteins.	. Spermatozoa cryopreservation extender (combined with Egg yolk)	2-10%	. D. Huang *et al.* (2006);
		Bovine Serum Albumin (BSA)	**N/A**	N/A	. Biochemical applications.	. Cryopreservation of Rabbit semen and stem cells	2-5%	. Y. Liu *et al.* (2011); . M. Rosata et al. (2013);
		Skimmed milk (SKM)	**N/A**	N/A	. Mostly used for balancing formulas for dairy products.	. Cryopreservation of spermetozoa of several species;	1-10%	. S. Kakar *et al.* (1978); . R. Athurupana et al. (2016);
		Polyvinylpyrrolidone 40 (PVP40)	**N/A**	N/A	. Plasma volume expander; . Adhesive in glue stick; . Emulsifier.	. Cryopreservation of RBCs and organs. Also effective for bacterial, protozoan and algae species cryopreservation	1-30%	. P Madden *et al.* (1993); . J. Bakhach et al. (2016);

media by interacting with water molecules which during the lowering of the temperatures may lead to an inhibition of the mechanical damages caused by ice crystal growth[81]. This occurs because water bound to solutes is termed as osmotically inactive and it is no longer available to participate in ice crystal formation[78]. Furthermore, studies performed by Crowe *et al.* formulated the "water replacement theory", where water molecules are replaced by CPAs in their interaction with biomolecules, explaining that CPAs protection comes also from membrane stabilization as result of interactions between these chemical molecules and the lipids on cell membranes[93,94,95].

Since the discovery of glycerol, a wide range of chemicals have been identified and studied as CPAs. CPAs can be classified according to their molecular weight and function: **(1)** penetrating (pCPAs), those that have low-molecular weight and are able to across cell membrane avoiding intracellular ice formation and reducing cell dehydration; **(2)** Non-Penetrating CPAs (npCPAs) defined as chemicals with high-molecular weight. The npCPA are large molecules that have functions very similar to pCPAs but in the extracellular environment. Moreover, these CPAs, predominantly npCPAs have been also used to achieve the less mechanically harmful vitreous state (discussed later) by depressing the melting point of water (**Table 1**)[91, 81].

The pCPAs such as methanol, ethanol, ethylene glycol, propylene glycol, dimethyl sulfoxide, glycerol and some amides (for example dimethylformamide) are the most used. Although all these CPAs exhibit cell permeability, the rate of penetration is slightly variable according to their molecular weight, chemical characteristics as well as cell type and temperature[77]. Moreover, some pCPAs only penetrate the cell wall not reaching the cytoplasm. One the other hand, Mono-, oligo- and polysaccharides, mannitol, sorbitol, albumin, gelatin, lipids and proteins, polyvylpyrrolidone, polyethylene glycol are all examples of npCPAs. This type of compounds are normally used in concentration ranging from 5% to 40% and are much less toxic than pCPAs (**Table 1**)[91].

Ashwood-Smith listed a series of the most effective CPAs commonly applied by the scientific community[96]. Zdenek Hubálek also published a review that gives some insights about the mechanism of action of 55 CPAs and the relevant experimental findings for microorganism associated to each CPA[91]. However, from those CPAs only around 8 CPA (The same 8 of the Ashwood-Smith list) yield substantial survival increase after freezing-thawing cycles. Over the last years the progression for increase the survival of microorganism has not been done by discovering new CPAs but understanding biophysical factors such as control ice nucleation and/ or crystal growth and formulating new freezing mixtures with combination of different cryoprotectants[81].

Over the last years, the progression towards an increased survival of microorganisms has not been related to the discovery and use of new CPAs, but by understanding the biophysical factors at play, such as controlled ice nucleation and/ or crystal growth and formulating new freezing mixtures with combination of different cryoprotectants[81].

1.2.3 CPA Toxicity

Cryopreservation of living material requires the presence of CPAs to inhibit ice formation. Freezing could occur without ice formation if there were no limits to the amount of CPA used[87]. However, the CPAs can be very toxic for living systems when used in great amount, and its toxicity is considered as one of the

most harmful aspects of cryopreservation[97]. CPA's toxicity follows different rules varying with the type of organism and with others experimental conditions such as time of exposer, temperature and CPA concentration. In 2015, Benjamin *et al.* reviewed several theories of CPA toxicity and discussed several mechanisms to decrease toxicity[98]. Besides, this author also acknowledged that in spite of CPAs being toxic at room temperatures when cooled to sub-zero temperatures they can become non-toxic, as happens with ethylene glycol[99]. Moreover, it is also well described that some CPAs are able to depress the toxicity of other CPAs when they are combined in the same freezing mixture. DNA and protein damage, mitochondrial function, motility systems, enzyme disfunctions, membrane disruption and active oxidative species formation are all possible outcomes of CPA toxicity[90]. In order to improve the utilization of CPAs a better understand of the molecular mechanisms that cause damage on microorganisms should be determined[97].

1.2.4 Vitrification

In 1984, the cryobiologist Gregory Fahy proposed an alternative approach to conventional freezing in cryopreservation that enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice[100]. Vitrification thus appears as the process of "glass formation" where a liquid is transformed into an amorphous solid with a non-crystalline structure[101].

To achieve vitrification, Fahy stated that is only required: **(1)** a much higher concentration of CPAs than used for conventional freezing[79]. It is possible to eliminate the formation of any ice crystals through addition of large quantities of CPAs but CPA's toxicity may be a limiting factor[88]. Moreover, npCPA also have the essential function of increasing viscosity of the media which is one of the key factors to achieve a vitreous state. **(2)** a sufficiently high cooling rate: with a high concentration of solutes in the freezing medium, it is possible to decrease the temperature of water-glass transition without significant ice formation[81,101]. **Figure 5** shows that an optimal cooling rate and a suitable freezing mixture can be sufficient to reach a vitreous state and have a maximum survival rate during cryopreservation.

Based on these considerations and inspired by the need of abolish the damages caused by ice formation, Fahy proposed vitrification as alternative method[79]. The severe cell dehydration, intracellular ice formation and mechanical action of ice on cells are the leading causes of injuries by conventional freezing[102]. In vitrification the intra- and extracellular ice formation is avoided and the cooling rate applied can be very high so that cells are not extensively expose to hyperosmotic environments neither subjected to intracellular ice formation. Additionally, during vitrification the molecular movements of solutes are also arrested which prevent cell of continuing dehydration. The cell distortion caused by mechanical action of ice growth is also decreased since the glassy phase is smoother to cells when compared to crystalline structures[99].

The major advantage of vitrification is the effective protection against cryoinjury. However, the high potential of contamination with pathogenic agents, the toxicity associated to CPAs, the risk of fractures on the vitreous solution, which leads to detrimental effects on cells integrity and, the possibility of devitrification, are barriers to an optimal vitrification[79].

1.2.5 Cryopreservation in the Malaria Context

The first evidence of cryopreservation applied to malaria context is referent to 1945 by Wolfson where *Plasmodium cathemerium* (*P. cathemerium*) inside the erythrocytes were frozen without addition of a CPA, only based on the serum from blood plasma[103]. The technique used to drop the temperature and lead to a frozen state was dry ice/ ethanol (-79ºC) and thereafter warm rapidly at 40ºC. As expected, the results evaluated by parasitemia revealed a much lower level when compared with the controls[103]. In 1955, Rendtorff and Jeffery conducted a study based on sporozoites dissected from infected mosquitoes' salivary glands and frozen in droplets of plasma using slurry of dry ice/ ethanol. After the thaw of the samples, 37 volunteers were infected and only 7 did not develop malaria, thus demonstrating that malaria cryopreservation may be achieved[73]. Later, in 1979 an intense study was performed by using a constant sporozoite concentration and testing different concentrations of dimethyl sulfoxide, glycerol, Polyvinylpyrrolidone and hydroxyethyl starch within a range from 5% to 15% mixed with mouse serum as solvent and varying the cooling rates from 0,2 to 400ºC/ min. The study concluded that the serum is indispensable to improve the efficiency of freezing and also that L offered the lowest effective yielding of preservative infection (9%) at 1ºC/ min. Moreover, the best result was obtained with HES and serum, translating 60% of infectivity[104]. Six years later, Michael R. *et al.* assessed the infectivity of cryopreserved *P. berghei* sporozoites, *in vitro.* Since these first studies, other articles focused on *Plasmodium* cryopreservation have emerged.

More recently, enhanced by the growing demand for availability of sporozoites to studies in malaria context, namely on the development of a whole-organism vaccine against malaria, several tests have been done in the field of malaria cryopreservation[36,105,74,106,75]. Sanaria is a biotechnology company responsible for the production of PfSPZ vaccine and optimization of a manufacturing process with radiation attenuated, aseptic purified, vialed sporozoites (PfSPZ), that inclusively already have been shipped successfully to more than 12 clinical sites in the USA, Europe, and Africa[74,106]. In 2013, data published by this company demonstrated that these cryopreserved PfSPZ demonstrated approximately a 7,4-fold an 6-fold loss of infectivity, in mice and humans, respectively, which means that only around 13,5% of sporozoites are surviving after cryopreservation[107, 57]. In 2017, Singh *et al*, evaluated the performance of several cryoprotective solutions on *P. berghei* sporozoites viability after freeze-store-thaw. The results demonstrated successful cryopreservation of *P. berghei* sporozoites with CryoStor CS2, which is a mixture of 2% of L with modest recovery and *in vitro* infectivity in HC-04 hepatocytes. Singh *et al*.'s cryopreservation protocol retained approximately 24% of sporozoites viability but induced 100% infection in mice[74]. Then, in 2018 by applying the same cryopreservation method, using CryoStor CS2, they tested the efficiency of genetically attenuated cryopreserved sporozoites for immunization of mice in comparison with freshly isolated controls[75]. In this study, only 20% efficiency in liver infection was observed, which greatly impacted their capacity to generate protection of animals in immunization experiments[75].

Although a great evolution on this field has been achieved, there is still opportunities for further improvements and to design a cryopreservation procedure which conserves all or at least the majority of sporozoites infectivity.

1.2.6 Challenges for *Plasmodium* Sporozoites Cryopreservation

Effective methods for malaria sporozoite cryopreservation are progressively emerging, however this parasite still face some limitations during this process. So far, the emphasis on cryopreservation techniques has mostly been given to sperm cells and stem cells[77]. Recent studies on *Plasmodium* cryopreservation have placed this parasite as a very temperature sensitive type of cell but there is still a great lack of knowledge about the mechanisms causing ice damage on sporozoites[72,75,105]. The optimal cryopreservation method is strictly dependent of the cooling and thawing rates that must be appropriate to this specific organism. More importantly, the formulation of a freezing medium with non-toxic concentration of additives such as CPAs used to osmotically balance and protect cells against the challenges of freezing also represents an urgent need[90]. Besides these 2 factors, there are other aspects that if controlled would lead to a higher sporozoite survival after cryopreservation such as ice growth geometry, vial geometry, Ph changes, etc.

1.2.7 SmartFreez Technology: Unidirectional Freezing

SmartFreez is a company specialized in providing highly efficient cryogenic services. Upon the need of effectively cryopreserved malaria sporozoites, iMM's Prudêncio lab established a collaboration with SmartFreez Lda. that allowed the employment of a patented controlled freezing technology (**Figure 6**) to cryopreserve *Plasmodium* sporozoites. Rodrigues *et al.* described the importance of the geometry of freezing direction on the frozen matrix conformation, in terms of local concentration of solutes/ cells and thermal history[108]. Significant improvements were achieved when unidirectional freezing geometry was employed, *i.e*., the freezing front progression was unidirectional from bottom to top. Bottom-up unidirectional freezing revealed to produce a uniform distribution of sugar and cells throughout the frozen solution which potentially increase cell survival during freezing process[108].

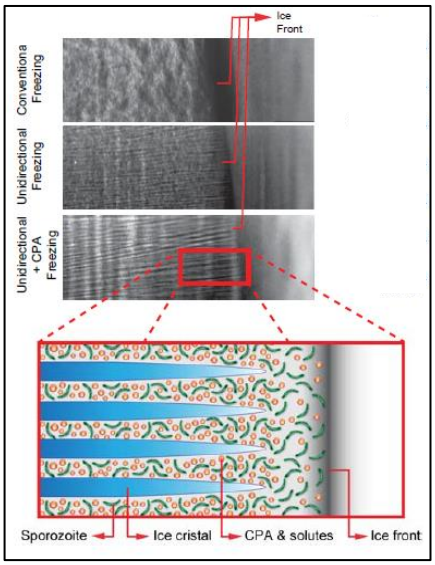


Figure 6 - X-ray radiographs of different freezing regimes recorded during steady-state solidification. Inset: Illustrative model of sporozoite cryopreservation in unidirectional ice matrixes.

1.2.8 Previous results

The Prudêncio lab initiated a series of optimizations on several parameters for selecting the appropriate freezing conditions to malaria sporozoites cryopreservation using the unidirectional freezing technology from SmarFreez. The first challenge for malaria sporozoites after their extraction from mosquitoes' salivary glands is to maintain the infectivity levels so that they are able to be used posteriorly. To extend the longevity of extracellular sporozoites after mosquito dissection, it is important to determine the buffer conditions more closely mimicking the insect microenvironment which allows the preservation of malaria sporozoites, *ex vivo*[109]. Moreover, the dissection buffer medium is also an essential element in the formulation of a freezing mixture, providing protection for sporozoites. RPMI1640 was the only medium able to maintain the infectivity of sporozoites for 4 hours almost with no difference relatively to control. Having established RPMI1640 as buffer medium, the next step was to complement the medium with a cyoprotective additive (CPA). Among all CPAs known, C is the most rational choice considering that it is the predominant source of energy used by *Anopheles* mosquitoes and potentially also very important for *Plasmodium* development. By using a VG2 sporozoite viability was determined upon cryopreservation with several C concentrations ranging from x12i% to z15i%. C at x13i%, already allowed to retain 30% of malaria sporozoites viability. Based on those previous results, Prudêncio lab move forward to identify the cooling rate recommended for a cryopreservation process using C as CPA and aiming to preserve this type of cells. These results paved the way towards achieving an optimal formulation and cryopreservation protocol. Such a methodology can have a significant impact in the malaria research and potentiate the development of numerous new anti-malarial interventions.

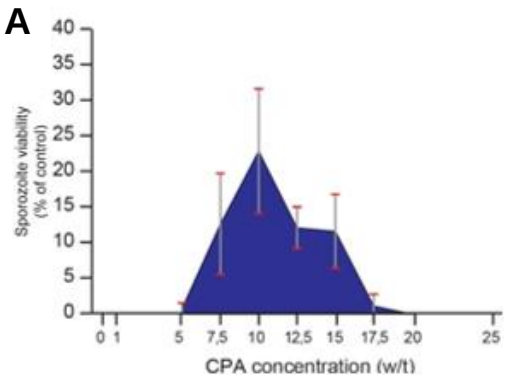


Figure 7 - Freezing parameters previously optimized in the Prudêncio Lab. (A) Sporozoite infectivity after cryopreservation using freezing mixtures with several concentrations of C, in RPMI1640.

1.3 Aims

The Prudêncio lab has been studying several parameters that influence the process of *Plasmodium* sporozoite freezing, towards developing an ideal methodology for cryopreservation of this form of the malaria parasite. This Master's thesis project aims at optimizing this methodology by testing a wide range of CPAs combinations and freezing conditions in order to identify the ideal freezing mixture to cryopreserve *Plasmodium* sporozoites. A comprehensive analysis on the potential impact of these CPAs for sporozoite infectivity was performed either *in vitro* and *in vivo* and their hypothetical mechanism of protection was further discussed. To address these experiments, we also performed a detailed evaluation of different commercially available vial geometries.

To assess the effectiveness of this methodology we investigated sporozoite viability or infectivity after cryopreservation through a variety of well-established assays, such as *in vitro* infection of HC-04 and Huh7 human hepatoma cells with *P. berghei* and *P. falciparum* parasites. Initial *in vitro* results were also validated by *in vivo* experiments using rodent models of *Plasmodium* infection.

2. Chapter - Materials and Methods

2.1 Parasites

Luciferase-expressing *P. berghei* ANKA sporozoites were obtained from the dissection of the salivary glands of infected female *Anopheles stephensi* (*A. stephensi*) mosquitoes, bred at Instituto de Medicina Molecµlar (iMM) (Lisbon, Portugal), prior to being employed on *in vitro* and *in vivo* essays. *P. falciparum* NF54 sporozoites were obtained from the salivary glands of infected *A. stephensi* mosquitoes provided by Radboud UMC in Nijmegen (Netherlands).

Briefly, infected mosquitoes were chilled at -20ºC for 5 min and washed with 70% ethanol before dissection. Mosquito salivary glands were removed by manual dissection and collected in sterile incomplete RPMI1640 (Gibco) supplemented with 1:300 fungizone 0.25 µg/ml (Gibco), 1% penicillin 5 U/ ml (Gibco), 1% streptomycin 5 µg/ml (Gibco), 1:1000 gentamycin 50 mg/ml (Gibco), at day 19-21 post blood meal. Salivary glands collected were then homogenized by using a pestle and the free sporozoites were counted in a Bürker-Türk counting chamber on a phase-contrast microscope.

2.2 Cryoprotectants (CPAs)

Table 2 - Variety of CPAs assessed.

CPAs	Type of CPA
A	Non-Penetrating
B	Non-Penetrating
C	Non-Penetrating
D	Non-Penetrating
E	Non-Penetrating
F	Non-Penetrating
G	Non-Penetrating
H	Non-Penetrating
J	Penetrating
K	Penetrating
L	Penetrating
M	Penetrating

2.3 Freezing-Thawing Process

Sporozoites were frozen employing a steel platform designed by SmartFreez Lda. which led to a unidirectional freezing (from the bottom to the top). The steel platform was incubated in dry ice for 30 min prior to use. Sporozoites resuspended in incomplete RPMI1640 were maintained on ice after their isolation from salivary glands and subsequently mixed and chilled with the respective cryopreservant solutions. At each VG2 (n.a) were added $80x10^3$ sporozoites in v(iii). of cryopreservative solution which

was immediately frozen. An ice thin layer on the bottom of the vial was induced (nucleation) and the vials were readily placed on a y mm polystyrene foam plate over the steel platform so that a freezing rate of approximately X^{o}C/ min was achieved.

Heat transfer during the freezing was promoted by adding absolute ethanol (VWR Chemicals) to eliminate the air layer between the bottom of the vial and the source of heat. The thawing process was performed at 37^{o}C, as rapidly as possible. The thawed sporozoites were resuspended in RPMI 1640 (supplemented as described above) to dilute cryoprotectants before infection.

2.4 Cryogenic Vial Assessment

The different vials geometries evaluated during this thesis are presented in the following table:

Table 3 - Brief characterization of the different vials used in the present work

Vial Denomination	Material	Capacity
VG1	Plastic	$V \leq 1$ml
VG2	Glass	$V \leq 2$ml
VG3	Plastic	$V \leq 1$ml
VG4	Plastic	$V \leq 0.5$ml
VG5	Plastic	$V \leq 0.5$ml

2.5 *In vitro* Essays

2.5.1 Huh7 and HC-04 Cell Maintenance

Human hepatoma cells (Huh7 cells) were cultured in RPMI culture medium supplemented with x13i% FBS (Gibco), 1% of non-essential amino acids (Gibco), 1% of penicillin 5 U/ml and 1% streptomycin 5 µg/ml, 1% glutamine 2mM (Gibco), and 1% Hepes at pH 7 (Gibco). The cells were seeded in 96-well plate (10x10^{3} cells/ well) and incubated at x7iv% CO2 and 37^{o}C, one day before infection with sporozoites.

Hepatocytoma HC-04 cells 1fg were grown in culture flasks in DMEM F12 (Gibco) medium supplemented with x13i% FBS, 1% of non-essential amino acids, 1% of penicillin 5 U/ml and 1% streptomycin 5 µg/ml, 1% glutamine 2mM, incubated in x7iv% CO2 at 37^{o}C. For experimental use cells were seeded in 24-well plates with or without glass coverslips, (100x10^{3}/ well) one day prior to infection with sporozoites.

2.5.2 Characterization of *P. berghei* Hepatic Infection

In order to evaluate hepatic infection, Huh7 cells were seeded in 96-well plates ($10x10^3$ cells/ well) and infected next day with $10x10^3$ firefly luciferase-expressing *Plasmodium berghei* line obtained from the salivary glands of infected *A. stephensis* mosquitoes. The addition of sporozoites was followed by centrifugation at 3000 rpm, for 5 min. The viability of huh7 cells was analyzed 46h after sporozoite addition through AlamarBlue assay (Invitrogen™) following the manufacture´s protocol. Parasite load was measured by luminescence following addition of the luciferin substrate (Biotium) 48 h after infection, as previously described[110].

2.5.3 Characterization of *P. falciparum* Hepatic Invasion

In the conditions described above, HC-O4 cells were seeded in a 24-well plate ($100x10^3$ cells/ well) with or without glass coverslips, 24 h prior to addition of *Plasmodium* sporozoites. At 0 h, $100x10^3$ sporozoites were added to each well previously filled with 800 µl of RPMI supplemented (as described above). Following addition of sporozoites, the plate was centrifuged at 3000 rpm, for 5 min. After 3h of incubation at 37ºC and x7iv% of CO_2, the wells with glass coverslips were rinsed with 1xPBS and fixed for 10 min with 4% of paraformaldehyde to be used for immunofluorescence microscopy.

2.5.4 Immunofluorescence Microscopy Imaging of *Plasmodium* infected HC-04 Cells

Following fixation with 4% of paraformaldehyde for 10 min, coverslips containing HC-04 cells were blocked with a solution of 1% D (VWR Chemicals) in 1xPBS, for 30 min at room temperature (RT). Incubation with an anti-*P. falciparum* CS protein primary antibody (mAb 2A10 1:300 diluted in 1% BSA/ 1xPBS) at RT for 1 h to stain extracellular parasites was followed by two washing steps and incubation with a secondary antibody goat anti-mouse Alexa Fluor® 546 (1:300 diluted in 1% BSA/ 1xPBS) for 1h at RT. Ice-cold methanol was added and incubated for 15 min at RT in order to induce cell permeabilization and subsequently washed-out by 2 rinse steps with 1x PBS. After cell permeabilization and rinse, an additional blocking step with 1% BSA/ 1xPBS was performed prior 1h incubation with the anti-CS protein *P. falciparum* primary antibody for extracellular staining. Incubation of 1h at RT with a secondary antibody goat anti-mouse Alexa Fluor® 488 (1:300 diluted in 1% BSA/ 1xPBS) and Hoechst 33342 (1:900 diluted in 1% FCS/ 1xPBS) was followed by 2 washes with 1xPBS after the second blocking. Samples were mounted using Flourmount G (Southern Biothec) and images were acquired on a Zeiss Axiovert 200M Widefield Fluorescence microscope.

2.5.5 Mice

In vivo experiments were performed using C57BL/6 and Balb/c mice that were purchased from Charles River and housed in IMM's specific pathogen-free rodent facility. All animal experiments were performed in strict compliance to the guidelines of our institution´s animal ethics committee and the Federation of European Laboratory Animal Science Associations (FELASA)).

2.5.6 Liver Infection

Mice were inoculated by injection of infected luciferase-expressing *P. berghei* ANKA sporozoites. . For each inoculation, sporozoites were resuspended either in incomplete RPMI1640 (controls without CPA) or in freezing mixtures (with CPA) and inoculated by retro orbital intravenous injection ($3x10^4$ sporozoites per mice).

2.5.6.1 Assessment of Liver Infection Load by Bioluminescence

Parasite liver loads in live mice after infection were quantified by real time *in vivo* bioluminescence imaging as previously described[111,112,110,113]. The bellies of C57BL/6 mice were shaved prior to imaging in order to minimize the absorption of light by the highly pigmented fur. At 44 h post-infection, animals were anesthetized using the isoflurane (Zoetis), and D-luciferin (Biotium) dissolved in PBS (100 mg/kg; Caliper Life Sciences, USA), was injected subcutaneously. Within 3 to 5 min after injection of D-luciferin, *Plasmodium* liver stages were visualized and liver loads quantified by measuring luciferase activity of parasites in whole bodies of mice through IVIS Lumina II Imaging System (Perkin Elmer Life Sciences).

Quantitative analysis of bioluminescence of whole bodies was performed by measuring the luminescence signal intensity using the region of interest (ROI) settings of the Living Image® 3.0 software. The ROI was set to measure either the abdominal area at the location of the liver for whole body imaging. ROI measurements are expressed in total flux of photons.

2.5.6.2 RNA Isolation, cDNA Synthesis and qRT-PCR Quantification

Table 4 - cDNA Reaction Mixture

cDNA Reaction Components	Volume per reaction (µl)
10x Buffer	2
50 ng/µl Random Hexamer	2
400 U/µl Rnase Inhibitor	0.35
10 mM dNTPs mix	2
200 U/µl Reverse Transcriptase	0,5
H2O	8.15

Table 5 - cDNA Thermo-Cycler Reaction Program

Temperature (ºC)	Time (min)
25	10
55	30
85	5
14	oo

For liver RNA extraction, livers were homogenized in Denaturating Solution (4 M guadine thiocyanate, 15 mM sodium citrate [pH 7], 0.5% *N*-lauroylsarcosine in pyrocarboate [DEPC]-treated water) supplemented with 0,1 M β-mercaptoethanol. RNA was extracted employing TRIzol (Invitrogen™), according to the manufacturer's protocol.

RNA was quantified on a NanoDrop 1000 Spectrophotometer and cDNA was synthetized using NZYTech First-Strand cDNA synthesis kit, according to the manufacturer´s instructions. cDNA was synthetized using a Biometra personal thermocycler, employing the following reaction mix and thermocycling parameters

Table 6 - qRT-PCR Components Mixture

qRT-PCR Component	Volume per Reaction (µl)
SYBRGreen Master Mix	4
Primer Foward	0.2
Primer Reverse	0.2
H2O	1.6

Table 7 - qRT-PCR Reaction Program

Temperature (ºC)	Time
50	2 min
95	10 min
95	15 sec
60	1 min
95	15 sec
60	1 min
95	30 sec
60	15 sec

Following cDNA synthesis, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in ViiA7 applied biosystems real-time thermocycler (Applied Biosystems), using the iTaq™ Universal SYBR® Green kit (BioRad) (**tables 4 and 5**).

For quantification of the parasite liver load, *P. berghei* 18S mRNA gene expression level was normalized against that of the hypoxanthine-guanine phosphoribosytransferase (HPRT) housekeeping gene using the comparative threshold cycle (C_T) (**table 6**).

Table 8 - Primers used in qRT-PCR Analysis

Gene	Primers	Sequence
***Pb* 18S**	F: 33	AAGCATTAAATAAAGCGAATACATCCTTAC
	R: 34	GGAGATTGGTTTTGACGTTTATGTG
HPRT	F: 249	TTTGCTGACCTGCTGGATTAC
	R: 250	CAAGACATTCTTTCCAGTTAAAGTTG

2.6 Statistical Analysis

All data were tested for normality with the Shapiro-Wilk test prior to analysis. The statistical difference between groups was determined using the 1-way analysis of variance for data following a normal distribution and the Kruskal-Wallis for data not following a normal distribution. A Dunett multiple comparison test was used to identify the statistical differences between groups. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad,San Diego, CA, USA).

3. Chapter – Results

3.1 Summary

The work described in this thesis is part of an ongoing effort by the Prudêncio lab to develop an effective cryopreservation method for *Plasmodium* sporozoites. In particular, this thesis describes the experimental work performed to identify an appropriate cryogenic vial and formulation of a cryopreservation freezing mixture for *Plasmodium* sporozoites.

We performed a series of *in vitro* experiments combining the use of a rodent malaria parasite, *P. berghei*, and a standard bioluminescence assay to compare the infectivity of sporozoites cryopreserved in a variety of cryogenic vials and freezing mixtures to that of fresh sporozoites. The formulation of all freezing mixtures tested during this study consisted in supplementing RPMI1640, which is the basal preserving sporozoite suspension (composition in **Supplementary Figure 1**), with several CPAs or combinations of CPAs (**Table 8**). After these primary *in vitro* studies, the same experimental setup was expanded to *in vivo* studies with laboratory mice.

Finally, since the majority of whole organism malaria vaccine candidates currently in development are based on the use of live attenuated *P. falciparum* parasites, we assessed the efficiency of our cryopreservation methodology in preserving *P. falciparum* sporozoites infectivity. To perform these tests, we assessed the capacity of cryopreserved sporozoites invade HC-04 cells in comparison with fresh sporozoites, by immunofluorescence imaging.

3.2 Evaluation of Different Cryogenic Vials

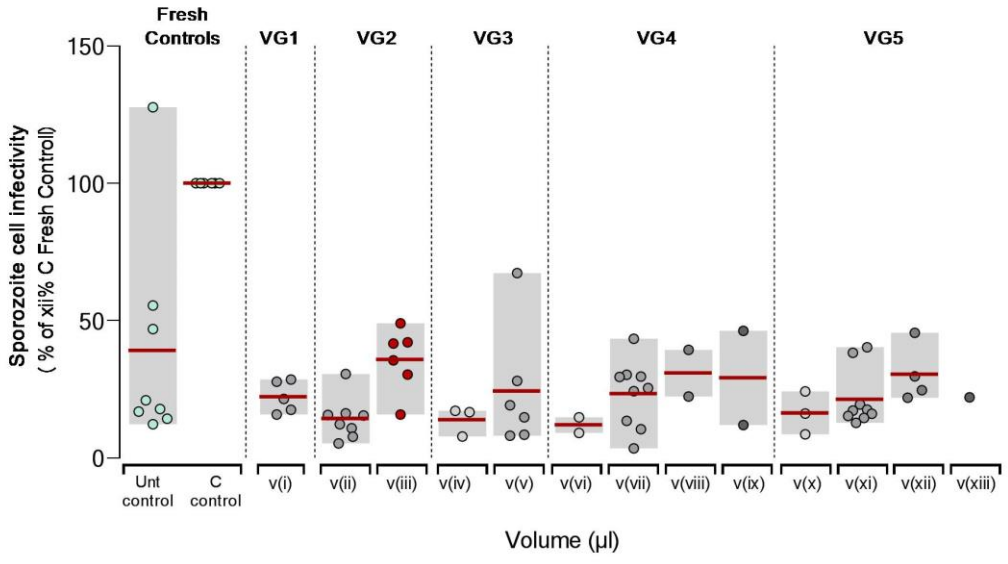


Figure 8 - Performance of different vial geometries for *Plasmodium* sporozoites cryopreservation. Each dot represents an individual technical replicate; Red lines are the average of sporozoite infectivity.

The vial geometry is known to be a key parameter that can affect heat-transfer during cryopreservation and therefore strongly influence the efficiency of cryopreservation[114]. In previous preliminary studies performed at Prudêncio lab, only VG2 vials were used to cryopreserve with sporozoites resuspended in

v(iii) of x13i% C freezing mixture, resulting in approximately 30% of sporozoite survival after cryopreservation. Based on these preliminary results, we extended the vial geometry evaluation to 6 other commercially available cryogenic vials (**Table 2**) made of different materials and with different physical-thermodynamic features, in terms of their performance for sporozoite cryopreservation. Our results confirmed that cryopreservation in VG2 vial using v(iii) of freezing mixture retains sporozoite survival rates above 30% and is the best option among the various vial geometries evaluated. Regarding the freezing mixture volume, our results indicate that decreasing of total volume below v(iii) has a negative impact on sporozoite survival during cryopreservation (**Figure 8**). We hypothesize that lower volumes can results in faster freezing rates, which leads to a decrease in sporozoite survival. Based on this study, we have selected VG2 vial and v(iii) of total volume of freezing mixture as the most appropriate conditions for the subsequent studies.

3.3 In-House *Plasmodium* Freezing Mixture Formulation

Having determined the most appropriate type of vial geometry and the ideal volume of the freezing mixture, and employing some parameters previously determined by the Prudêncio lab, such as an optimal cooling rate of approximately X^{o}C/min and unidirectional freezing technology, the next step was to evaluate various formulations of freezing mixtures for sporozoite cryopreservation. We aimed for a comprehensive analysis of the effect of adding a variety of well-established chemical compounds with known cryoprotectant qualities (CPAs: cryoprotectant agents) to the freezing mixture used for sporozoite cryopreservation[91]. We selected a list of CPAs that can be divided into 3 different categories regarding their function, mechanism of action and molecular features: Non-penetrating sugars; Non-penetrating complex mixtures of lipids & proteins; Penetrating CPAs.

3.3.1 Effect of CPAs on the Infectivity of *P. berghei* Sporozoite

Although the addition of CPAs to freezing mixtures can have an important role in protecting cells against the deleterious effects of freezing, under specific conditions and concentrations, CPAs can also have a toxic effect that should be considered. Therefore, we conducted a detailed study to determine the effect of selective CPAs addition to mixtures used for sporozoite suspension in the absence of cryopreservation. To that end, fresh sporozoites were either incubated, at 4^{o}C, in a mixture including the CPA concentration under evaluation, or without CPA (untreated control), for 2 h, and their relative huh7 cell infectivity was subsequently assessed. We started by evaluating the effect of adding different sugars at various concentrations in the sporozoite suspension. Our results demonstrate that there is no significant decrease in viability associated to A and B in all concentrations tested (**Figure 9 (A)**). The presence of C at concentrations higher than c12i% led to a considerable decrease in sporozoite viability indicating a potential toxicity profile for this CPA.

We then extended our evaluation to another category of non-penetrating CPAs, which includes complex mixtures of Lipids & Proteins (**Figure 9 (B)**). Our results indicate that the addition of I, E, and very high concentrations of H, D and G resulted in an average decrease of over 60% in sporozoite infectivity, relative to fresh untreated control. These CPAs and CPA concentrations were therefore excluded from subsequent studies.

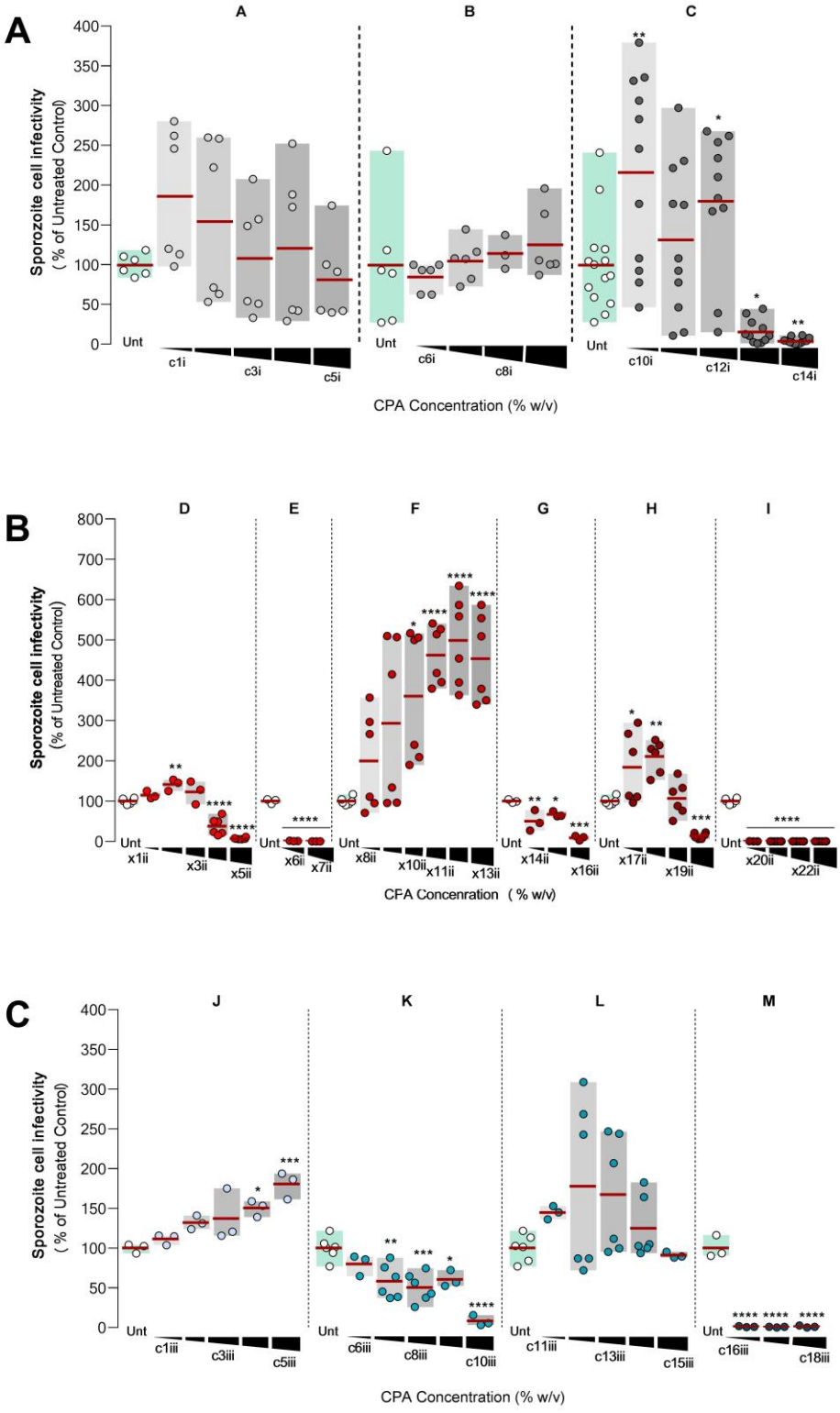


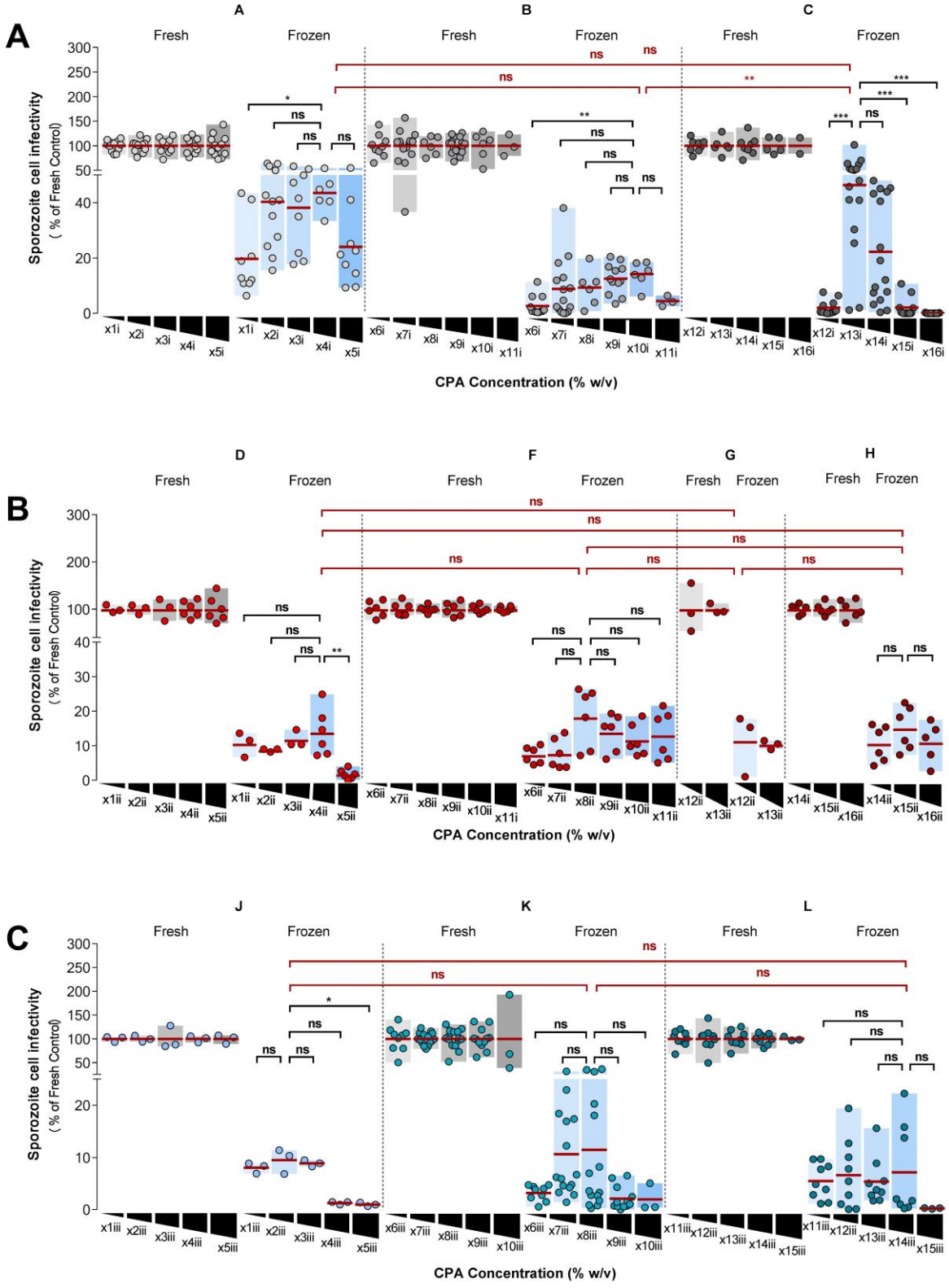
Figure 9 - **Incubation of *P. berghei* sporozoites in freezing mixtures containing the respective CPAs, for 2h, and evaluation of their infectivity for Huh7 cells, 48h post-infection.** Infectivity of fresh sporozoites (10,000 sporozoites/well) was evaluated either in RPMI1640 media without CPAs (Untreated Control) or in medium containing CPAs at several concentrations. Relative sporozoites infectivity was calculated as percent of Huh7 cells infected by sporozoites in freezing mixtures (with CPAs) relative to untreated control. Each dot represents a technical replicate; Red lines are the respective average of relative sporozoite infectivity. **(A)** npCPAs: Sugars; **(B)** npCPAs: Complex mixture of Lipids & Proteins; **(C)** pCPAs. Statistical significance was acquired with one-way ANOVA for normally distributed data and by Kruskal-Wallis for non-normally distributed data.

The next category of CPAs evaluated was that of Penetrating-CPAs, which included compounds well known for their cryoprotective features, such as L[79]. However, some are also known to have a high toxicity profile for some types of cells, which impairs their widespread use. Of the Penetrating CPAs assessed, both K and M resulted in reduced sporozoite survival when included in the sporozoite suspension (**Figure 9 (C)**). However, while a drastic reduction in sporozoite survival could only be observed at K concentrations higher that c8iii%, all the tested concentrations of M presented a high toxicity profile, which excluded its further inclusion in sporozoites freezing mixtures.

3.3.2 Performance of CPAs Included in Freezing Mixtures of *Plasmodium* Sporozoites

Following the evaluation of the impact of exposing sporozoites to various CPA-supplemented mixtures, we sought to analyze the protective effect of selected mixtures against the hostile conditions of sporozoite freezing-thawing processes. To this end, we compared the infectivity of cryopreserved sporozoites suspended in different freezing mixtures with that of fresh sporozoites exposed to the same freezing mixtures. The cryopreservation process employed was based on unidirectional freezing, a technology developed in collaboration with our partners, SmartFreez, which has been proven to provide better survival results than conventional freezing through the effective control of ice formation[108]. A single CPA was employed per freezing mixture so that any effects could be directly correlated to a single parameter. We started by evaluating the performance of 3 sugars, A, B, C. As shown in **Figure 10 (A)**, the addition of x13i% of C to the freezing mixture provided high protection for sporozoites, retaining approximately 46% of sporozoite survival after cryopreservation and thawing. A provided similar protection at a wider range of concentrations (from x2i% to x4i%), showing that it is well tolerated by sporozoites. We next assessed the cryoprotection provided by complex mixtures of lipids and proteins, for which sporozoite survival is generally inferior, to what was observed upon sugar addition to the sporozoite suspension. This is particularly the case of x13ii% SKM and x2ii% BSA, which in the reported **experiments 3.3.1** (**Figure 9 (B)**) already had shown to have a negative impact on sporozoites survival, a profile that was maintained even in terms of cryoprotection (**Figure 10 (B)**). Within this category of CPAs, F and H appear to provide the highest protection among the CPAs evaluated. Lastly, we assessed the effect of adding pCPAs, K, J and L, to the freezing mixtures. **Figure 10 (C)** shows that the addition of very high concentrations of J, K, L leads to a reduction of sporozoite survival, whereas x8iii% K and x2iii% J led to the highest sporozoite survival in this CPA category. Although pCPA conferred lower protection than all other CPAs, a potential combination of CPAs from different categories, including pCPAs, has been described as enhancing the overall protective effect[91,79].

On the basis of that assumption, we selected the best sugar, in this case C, the two best npCPAs of Lipids and proteins, H and F, and K and J from pCPA, to continue our study.



evaluation of their infectivity for Huh7, 48h post-infection. Infectivity of sporozoites (10,000 sporozoites/well) was evaluated after cryopreservation, with respective CPA at several concentrations. Relative sporozoite infectivity was determined as percent of Huh7 cells infected by cryopreserved sporozoites relative to fresh control in the same freezing mixture. Each dot represents an individual technical replicate; Red lines are the respective average of relative sporozoite infectivity. **(A)** npCPAs: Sugars; **(B)** npCPAs: Complex mixture of Lipids & Proteins; **(C)** pCPAs. Statistical significance was acquired with one-way ANOVA for normally distributed data and by Kruskal-Wallis for non-normally distributed data.

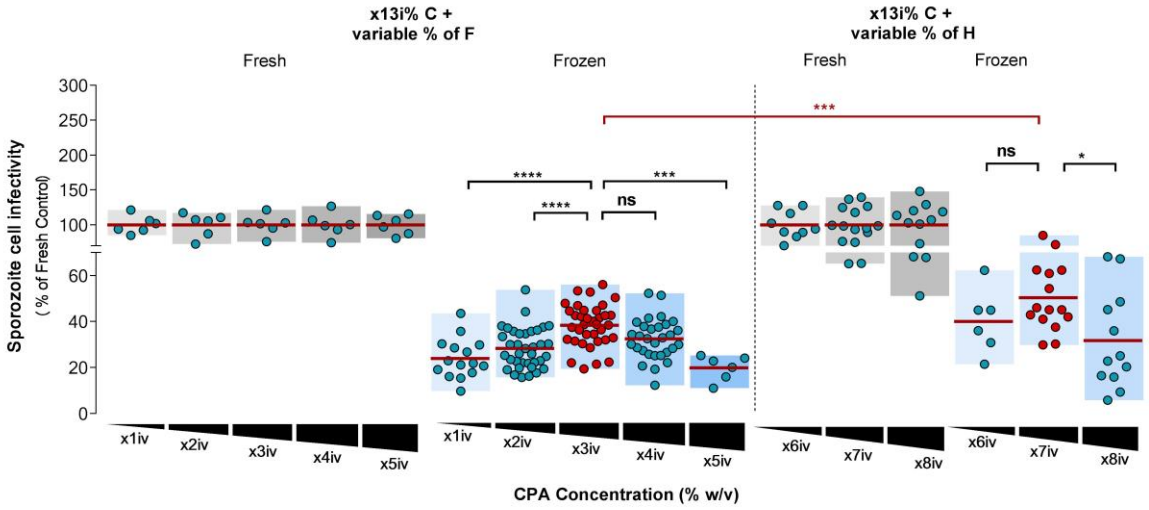


Figure 11 - Cryopreservation of *P. berghei* sporozoites in freezing mixtures containing x13i% C and variable concentrations of H or F and evaluation of their infectivity for Huh7, 48h post-infection. Infectivity of sporozoites (10,000 sporozoites/well) was evaluated after cryopreservation with different CPAs at several concentrations. Relative sporozoite infectivity was determined as percent infected Huh7 cells infected by cryopreserved sporozoites relative to fresh control in the same freezing mixture. Statistical significance was acquired with one-way ANOVA for normally distributed data and by Kruskal-Wallis for non-normally distributed data.

3.3.3 Combined Effect of CPAs in the Same Freezing Mixture

Potentially, a combination of CPAs from different categories, acting in a variety of manners, may boost the protective capacity of a freezing mixture and improve sporozoite survival after cryopreservation. Based on that assumption, we started by selecting x13i% C, the best performing CPA among the Non-Penetrating Sugars tested, and added several concentrations of F or H, which yielded the most promising results among complex mixtures of Lipids & Proteins, to the freezing mixture. Using a freezing mixture containing x13i% C and x3iv% F we obtained a very consistent result of approximately 40% of sporozoite survival. When x7iv% H and x13i% C were employed *Plasmodium* sporozoite survival after cryopreservation reached 50,4% (**Figure 11**). This excellent result represents a substantial increase in comparison to the current standard, in sporozoite cryopreservation.

We next hypothesized that survival rates might be further increased by supplementing the mixture with penetrating CPAs, which are able to penetrate cells and may therefore complement the actions of the other CPAs present. Thus, we supplemented the freezing mixture containing x13i% C and x7iv% H, with K or J, which yielded the most promising results among the pCPAs tested. However, the introduction of pCPA in the mixture did not lead to further improvements, as shown in **Figure 12**.

3.3.4 Analysis of the Impact of Selected Freezing Mixtures on *in vivo* Mouse Models.

Having established that several freezing mixtures are able to provide a substantial protective effect on sporozoites during freezing-thawing cycles, warranting very high sporozoite infectivity after cryopreservation, we decided to evaluate selected mixtures using *in vivo* models of sporozoite infection.

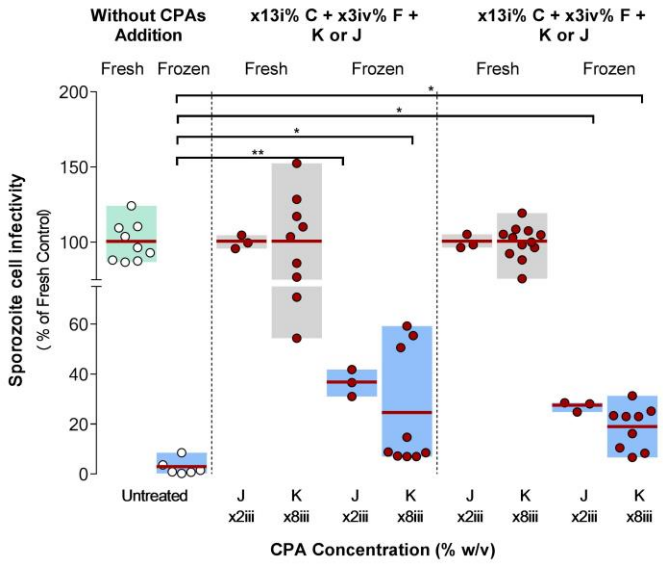


Figure 12 - Cryopreservation of *P. berghei* sporozoites in freezing mixtures supplemented with x13i% C, x7iv% H or x3iv% F and x2ii% J or x8iii% K and evaluation of their infectivity for Huh7, 48h post-infection. Infectivity of sporozoites (10,000 sporozoites/well) was evaluated after cryopreservation with different CPAs at several concentrations. Relative infectivity was determined as percent of infected huh7 cells versus fresh control in the same freezing mixture. Each dot represents an individual experiment; Red lines are the respective average. Statistical significance was acquired with one-way ANOVA for normally distributed data and by Kruskal-Wallis for non-normally distributed data.

Specifically, we employed a freezing mixture supplemented with only x13i% C; a freezing mixture with x13i% C and x7iv% H; and, a freezing mixture supplemented with x13i% C and x3iv% F. We started by analyzing the effect of the selected freezing mixtures on mouse hepatic infections by *Plasmodium*, *in vivo*, in the absence of cryopreservation. To that end, we compared the infectivity of fresh sporozoites injected in the presence of each freezing mixture with that of fresh sporozoites in a freezing mixture without any CPA. Since we observed that an increase in the concentration of injected C led to an enhanced liver infection load (**data not shown**), the concentration of CPA to inject in mice was reduced by diluting each freezing mixture 20 and 40 times so that the percentage of C injected to mice was z1i% and z2i%, respectively. At these dilutions, similar infection loads were observed in the presence and in the absence of C (**Figure 13 (A)**). However, the injection of freezing mixtures containing H enhanced the parasite's liver load in either C57Bl6 or BALB/c mice compared with sporozoites injected with the remaining freezing mixtures or in the absence of CPAs, as shown in **Figure 13 (A), (B)** and **(C)**. Liver infection loads were assessed by both qRT-PCR and bioluminescence, yielding similar results (**Figure 13 (B) and (C)**) and because of that the subsequent experiment was only assessed bioluminescence.

In order to understand the reason behind the increase in liver parasite load observed when freezing mixtures containing H are injected, we performed a study where sporozoites were either resuspended in a freezing mixture supplemented with H either one hour prior to injection or only at the moment of the injection.

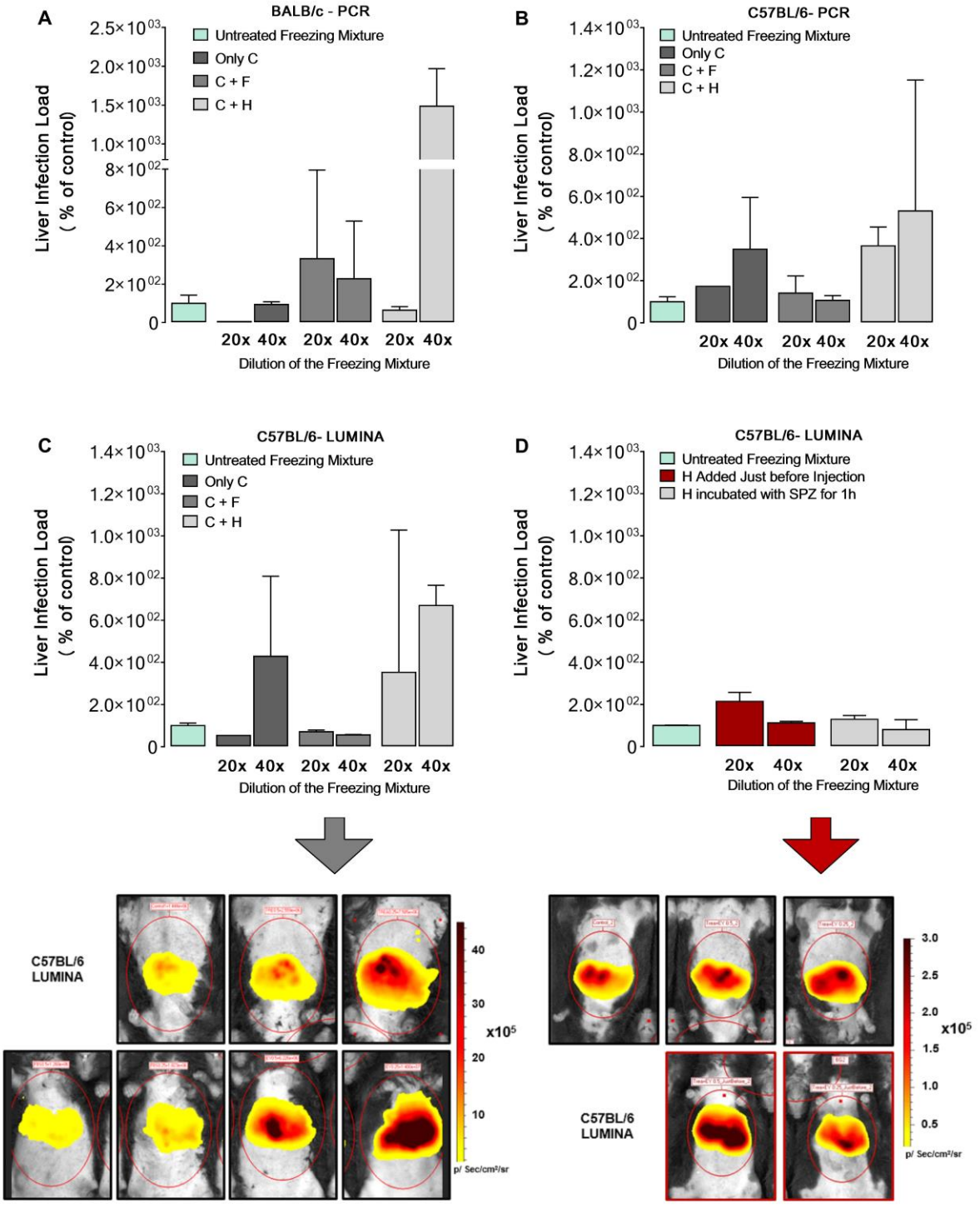


Figure 13 - Effect of injecting different sporozoite freezing mixtures in mice. The sporozoites were incubated in 3 freezing mixtures: x13i%C; x13i%C and x3iv%F; or x13i%C and x7iv%H. Before mice injection, each freezing mixture was diluted 4 or 5 times. **(A)** Effect of injecting sporozoite freezing mixtures in BALB/c evaluated by RT-qPCR. **(B)** Effect of injecting sporozoite freezing mixtures in C57BL/6 assessed by RT-qPCR. **(C)** Effect of injecting sporozoite freezing mixtures in C57BL/6 assessed by RT-Imaging. Images: **(upper – left to right)** Untreated Control; z1i%C; z1i %C; **(Lower – left to right)** z1i%C and z1ii%F; z1ii%C and z2ii%F; z1i%C and z1iii%H; z1i%C and z2iii%H. **(D)** Difference of injecting sporozoites incubated for 1 h in the freezing mixture containing C and H or injecting sporozoites without previous incubation; Images: **(upper – left to right)** Untreated Control; z1i%C and z1iii%H; z2i%C and z2iii %H; **(lower – left to right)** z1i%C and z1iii%H; without incubation z2i%C and z2iii %H without incubation.

Potentially, this would allow us to determine whether the boosted effect of supplementing the freezing mixtures with H rely in providing a more effective medium for maintaining sporozoites viability after their extraction from mosquitoes' salivary glands or only occurs after its injection in mice. The results in **Figure 13 (D)** indicate there is no significant difference between previously incubating sporozoites in freezing mixtures with H compared to adding this medium to sporozoites only at the moment of injection, suggesting that H may act mostly after being injected in mice.

Having achieved these results further studies are required in order to fully understand the mechanisms for which H is boosting liver load infection and posteriorly be possible to assessed the capacity of cryopreserved sporozoites reach the mice liver.

3.3.5 Invasion Capacity of Cryopreserved *P. falciparum* Sporozoites

We then sought to evaluate the efficiency of selected freezing mixtures on the cryopreservation of human-infective *P. falciparum* sporozoites. To this end, we compared the invasion rate of HC04 cells by cryopreserved and fresh *P. falciparum* sporozoites. Additionally, we also quantified the number of sporozoites inside and outside cells as measure of percentage of internalization (**Figure 14 (A)**, **(G)** and **(F)**). As expected, the percentage of invasion in the controls was higher than in the cryopreserved sporozoites (**Figure 14 (B)**), indicating that the cryopreservation process affects the sporozoites' cell invasion capacity. Nevertheless, 45% of the *P. falciparum* sporozoite invasion ability was retained when the parasites were cryopreserved using freezing mixture containing only x13i% C, compared with non-cryopreserved controls (**Figure 14 (C)**). Furthermore, when we introduced x3iv% For x7iv% H in x13i% C freezing mixture, the invasion capacity of cryopreserved sporozoites was increased to approximately 55% (**Figure 14 (C)**). Strikingly, addition of the pCPAs, K, to the freezing mixtures containing x13i% C and x3iv% For x7iv% H led to the retainment of approximately 70% of the HC-04 invasion capacity of cryopreserved *P. falciparum* sporozoites relative to fresh sporozoites incubated in the same freezing mixture. However, this last result needs to be repeated to confirm its reproducibility. Additional normalization between sporozoites cryopreserved in the selected freezing mixtures and fresh sporozoites (**Figure 14 (D)**). Another evaluation was based on comparing the invasion capacity of *P. falciparum* storage during 3 years after cryopreservation with those of fresh sporozoites and of sporozoites cryopreserved for one hour (**Figure 14 (E)**). This long-term assessment showed that there is no substantial loss of invasion capacity during the storage period. Further tests are required to better understand the protection provided by these freezing mixtures in *P. falciparum* and to increase the consistency of our results.

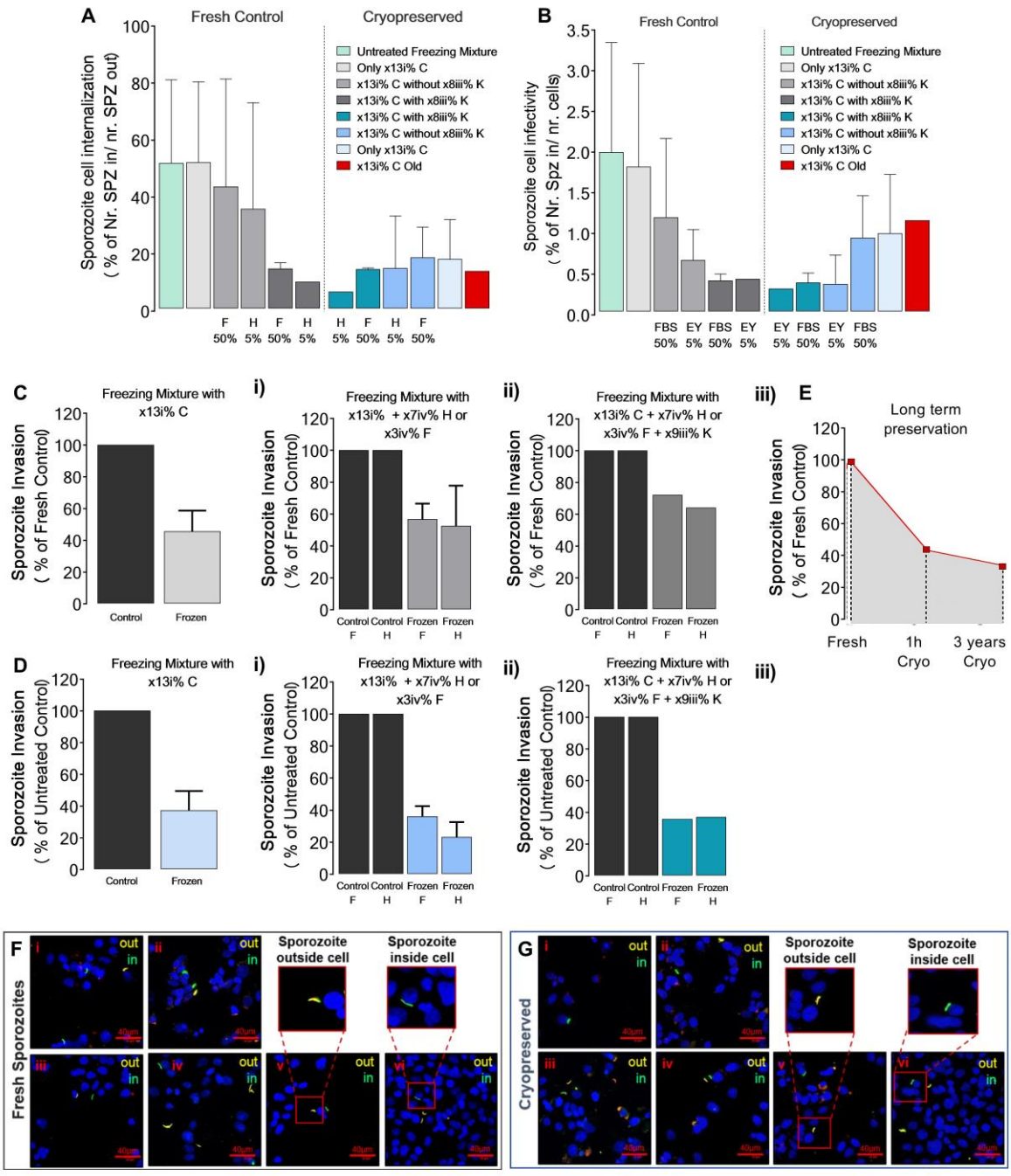


Figure 14 – Invasion capacity of fresh and freezing mixture cryopreserved *P. falciparum* sporozoites in HC-04 cells. 100,000 sporozoites, either cryopreserved or not (Fresh Controls), were added per well. **(A)** % of internalization (nr. of sporozoites intracellularly/ nr. of sporozoites extracellularly); **(B)** Invasion capacity of sporozoites (nr. of sporozoites intracellularly/ total number of cells); **(C)** Cryopreserved sporozoite invasion rate compared with fresh sporozoites exposed to the same freezing mixture: **i)** only x13i% C, **ii)** x13i% C and x7iv% H or x3iv% F, **iii)** with further introduction of x9iii% J. **(D)** Cryopreserved sporozoite invasion rate compared to an untreated fresh control in the same freezing conditions of **(C)**; **(E)** Sporozoite invasion capacity after 3 years of cryopreservation; **(F)** and **(G)** Representative images acquired of fresh and cryopreserved sporozoites in each condition, respectively: **i)** x13i% C + x3iv% F+ x9iii% K; **ii)** x13i% C + x7iv% H + x9iii% K; **iii)** x13i% C; **iv)** In **(F)** is the untreated control and in **(G)** is x13i%C cryopreserved 3 years ago; **v)** x13i% C + x7iv% H ; **vi)** x13i% C + x3iv% F.

4. Chapter – Discussion

Malaria remains one of the most devastating diseases worldwide, with millions of victims every year. One of the main bottlenecks for research on the sporozoite and liver stages of malaria parasites is the difficulty in accessing infective *Plasmodium* sporozoites. Such limitations are a direct consequence of the lack of insect rearing and infection facilities in most laboratories. This is particularly relevant in the case of sporozoites of *P. falciparum* and *P. vivax*, which are the main human malaria parasites, whose production in mosquitoes requires high biosafety containment, severely limiting the number of facilities capable of meeting the required regulatory standards[72,107]. Thus, an effective sporozoite cryopreservation process offers a viable strategy to alleviate these limitations and would constitute a significant scientific advance towards the development of new antimalarial strategies[73]. Furthermore, such a cryopreservation system would be key to improving the efficiency of whole-organism vaccines, as well as to facilitating whole-sporozoite malaria vaccine production and storage. To be effective, a cryopreservation process for *Plasmodium* sporozoites should preserve adequate sporozoite infectivity, maintaining the parasites' ability to complete their liver stage development and produce infective blood-stage parasites. In this work, we were able to identify cryopreservation conditions that reproducibly retain 50% of *P. berghei* sporozoite infectivity (**Figure 11**) and 70% of *P. falciparum* invasion capacity (**Figure 14**) after the freeze-thaw process.

Although *Plasmodium* cryopreservation is still a largely unexplored field for malaria research community, some reports exist in the literature about this subject. In 2002, the company Sanaria was founded with the intention of developing a radiation attenuated sporozoite vaccine for malaria, PfSPZ[115,116]. Since then, scientists at Sanaria established a process of sporozoite isolation and cryopreservation under good manufacturing practice conditions[6,57,117]. Despite initial difficulties regarding the route of administration[57], these cryopreserved sporozoites have been used successfully in several clinical studies where 100% protection against malaria was already accomplished[59,70]. However, the efficacy of Sanaria's PfSPZ vaccine has been compromised mostly by the requirement of high numbers of PfSPZ per subjected to achieve protection[59,70]. These constraints, alongside others[115], limit the production of an efficient whole-organism vaccine meeting the objectives proposed in 2013's WHO Malaria Vaccine Technology Roadmap[62]. Although Sanaria's cryopreservation methodology is patented and therefore unavailable for consulting, there are few articles where the infectivity of cryopreserved PfSPZ was assessed[107,57]. These reports show that Sanaria's cryopreservation process leads to approximately 7.4[57] and 6.4-fold losses[107] in sporozoite infectivity in mice and humans, respectively, compared with fresh sporozoites. On the basis of these studies, Sanaria suggests that the PfSPZ's vaccine protective efficacy may be limited by the efficiency of cryopreservation[107]. Using the cryopreservation method described in this work, cryopreserved *P. berghei* sporozoites exhibited only a 2-fold reduction in comparison with fresh ones.

Other recent studies have also focused on evaluating different cryogenic solutions for resuspension of *Plasmodium* sporozoites for cryopreservation[72,105,74,75]. Initially, Rapatbhorn *et al*. developed a cryopreservation methodology where a freezing mixture containing 50% FBS and 10% Sucrose in

RPMI1640 provided less than 5% of *P. vivax* infectivity[105]. On the contrary, the freezing mixture employed in our work, containing x13i% C and x3iv% F, retained 40% of cryopreserved *P. berghei* sporozoites infectivity. Although the compositions of the two freezing mixtures are similar, the rest of the cryopreservation parameters (vial geometry, cooling rate, etc) are different in the two studies, which might explain the different experimental outcomes[105]. Other research groups have also published several studies assessing the performance of different commercially available cryogenic solutions for *Plasmodium* sporozoites[72,74,75]. These studies highlighted the performance of a cryogenic solution based on 2% of DMSO[74]. Initial studies using this cryogenic solution (CryoStor CS2) provided modest results, of approximately 24% of *P. berghei* sporozoite infectivity of HC-04 cells[74]. Although using Huh7 cells, cryopreserved sporozoites resuspended in the freezing mixture formulated in the present study (x13i%TRE and x7iv% H in RPMI) retained approximately 36% more infectivity of *P. berghei* sporozoites than cryopreservation with CryoStor CS2 (**Figure 11**). Singh *et al*. continued their previous studies[72,75,74] and tested the recently established cryopreservation protocol on experimental vaccine efficiency of a genetically attenuated *P. berghei* parasite[75]. This studied indicates that least 5 times more cryopreserved than fresh sporozoites are required to achieve similar levels of protective efficacy[75]. Singh *et al*.' s cryopreservation protocol provided a slight improvement over Sanaria's, where the efficiency of cryopreserved sporozoites is approximately 7.4 times lower than that of fresh sporozoites[57]. Importantly, Singh *et al.* also measured the invasion capacity of cryopreserved *P. berghei* sporozoites, which was 5 times lower than that fresh sporozoites[75]. By applying the same method to assess sporozoite invasion capacity after cryopreservation we obtained only 1.4-2 times less *P. falciparum* sporozoite's invasion capacity (**Figure 14**). Thus, our methodology presents substantial improvements relatively to the two cryopreservation protocols mentioned above. Additional *in vivo* assessments are necessary to validate the hypothesis that sporozoites upon cryopreservation with our protocol remain able to trigger high levels of liver infection and thereby induce protective efficacy.

The effectiveness of cryopreservation is influenced by several factors, such as cell type, cooling and thawing rate, vial geometry, and the freezing medium used for resuspending biological materials[91]. Nowadays there are a wide range of commercially available cryogenic vials that can potentially be employed for *Plasmodium* sporozoite cryopreservation. Our results identified VG2 vials as most appropriate to cryopreserve v(iii) of sporozoite suspension (**Figure 8**). Glass has a higher thermal conductivity than plastic-based materials[118], a difference that may influence the efficiency of the cryopreservation process. Additionally, we speculate that volumes below v(iii) freeze faster than volumes higher than v(iii), which may explain the drastic decrease in sporozoite survival when a v(ii) is used.

Despite the importance of all parameters evaluated in this work for the efficiency of cryopreservation, the main focus of this thesis was the formulation of an ideal mixture for sporozoite freezing. The composition of the freezing mixture is indeed one of the key factors contributing to optimal cryopreservation, especially because such medium protects sporozoites against freezing and thawing stresses[91]. A wide number of commercially available cryogenic solutions can be employed in the cryopreservation of several types of cells, such as spermatozoa, stem cells and also protozoan

sporozoites[91]. Nevertheless, an in-house formulated freezing mixture enables the inclusion of a variety of well-characterized components that enhance protection and stability of the cell type of interest[79]. The formulation of cryopreservation freezing mixtures normally relies upon 3 main components: a basal carrier solution, several types of CPAs and, occasionally, anti-freezing proteins (ATF)[79,90]. A carrier solution is a component of freezing mixtures that normally holds the rest of the components in suspension. It contains a pH buffer, osmotic agents (balanced salt solution) and sometimes apoptosis inhibitors that provide basic support for cells at near-freezing temperatures[90,119]. RPMI1640 (composition in **Supplementary Figure 1**), was employed as the carrier solution for sporozoite suspension following their extraction from mosquitoes, since it presents a near isotonic salt concentration, preventing sporozoite shrinking or swelling. This carrier solution can be further supplemented with several types of CPAs at different concentrations, towards improving the solution's protective capacity and potentially inducing vitrification under specific freezing conditions[88]. On the basis of these theoretical concepts[91,90,79,77], we formulated an in-house *Plasmodium* sporozoite freezing mixture, including CPAs belonging to different categories, employed either individually or in combination. Sporozoites are very susceptible to freezing mixtures containing high concentrations of specific CPAs, being totally intolerant to M, E and I (**Figure 9**). The presence of these CPAs in sporozoite suspensions showed a toxicity profile that has been described in the literature, which is especially critical when very high concentrations of these CPAs are employed to achieve vitrification[97,99,120,90]. Usually, cryopreservation solutions are not physiological solutions, since the high concentrations of CPAs increases the hypertonicity of the solution[90]. Osmotic and biochemical toxicities are two independent mechanisms of damage that can occur during introduction, incubation, and removal of a cryopreservation solution[90]. Adequate development of a cryopreservation protocol requires the characterization of those mechanisms of damage for a given cell type and solution composition. On the other hand, the sporozoites exposed to some albumin-containing npCPAs exhibited high levels of infection (**Figure 9 (B)**). It has been shown that albumin, present in some of these CPAs, may enhance sporozoite motility *in vitro* and potentially increase their infection rate[121].

Addition of CPAs changes the concentration of salts at a given subzero temperature, an effect that is commonly known as the colligative effect[81]. Other studies also associated the presence of these CPAs to the formation of a vitreous state which is an alternative to cryopreservation[122,79,101]. Besides, sporozoites contain more than just water - the post-thaw function of the sporozoites requires the preservation of the integrity of the cell membrane and of the function of intracellular components (e.g., cytoskeleton, proteins, nucleus)[77].

Several studies have analyzed membrane integrity and motility as measures of cryopreserved sporozoites viability, but no significant differences were observed between cryopreserved and fresh sporozoites[123,106]. Previous studies performed by the Prudêncio Lab (**data not shown**) also confirmed that sporozoite membrane integrity and motility remain largely unaffected by cryopreservation. Singh *et al.* described that the high levels of sporozoite motility are directly correlated with high levels of infectivity/ invasion[75]. In this work, we gave special emphasis to sporozoite infectivity after cryopreservation as it is a more accurate method of analyzing the fitness of the parasite following the freeze-thaw process. Our

results identified non-penetrating sugars, particularly C and A, as the class of CPA preserving the highest sporozoite infectivity upon cryopreservation and providing the highest protection during freezing and thawing. C has been used in the cryopreservation of *S. cerevisiae,* psychrophilic yeasts, *Lactobacillus bulgaricus* and a mycorrhizal fungus as well as in stem cells[91]. This sugar is also an important component of different freeze-drying protocols because of its essential features in recovering biological materials upon thawing[79]. Interestingly, C is the most abundant sugar in the hemolymph of *Anopheles* mosquitoes, where it serves not only as a source of energy but also as protection of the mosquito against desiccation and heat stresses[124]. On the basis of the importance of C for *Anopheles* mosquitoes, Lui K. *et al.* suggested that C is also a likely energy source for *Plasmodium* parasites[124]. For that reason, we hypothesize that its addition to sporozoite suspension contributes to the recovery of the fitness of the thawed sporozoites. Additional mechanisms of action of CPAs have been proposed for C besides preventing extracellular ice crystals formation by inducing a glassy state, such as stabilizing cell membranes, reducing alterations in membrane morphology and stability during freezing[94].

H and Fare examples of npCPAs of lipids and proteins that are also essential for preserving the cell membrane integrity and maintaining the physiological viscosity during cryopreservation. H is a very common component of sperm freezing extenders, where low density lipoproteins, LDL, are believed to be the main responsible for H success during cryopreservation[125]. The major functions associated to this molecule are its interaction with cell membranes either to stabilize phospholipidic layer, replacement of damaged phospholipids or binding to cell membrane proteins, leading to the efflux of phospholipids and cholesterol[126]. F is another complex mixture of proteins and lipids widely employed in the cryopreservation of several types of cells and organisms[91]. Indeed, one of the first studies involving sporozoite cryopreservation of *Plasmodium* parasites used a solution containing blood serum or plasma, whose composition is similar to that of FBS[104]. A solution containing 50% FBS was recently described for *P. vivax* cryopreservation, but only afforded very modest protection[105]. Its inclusion in different freezing mixtures is advised to be combined with other CPAs, whose toxicity can be reduced by the presence of FBS[97]. Furthermore, FBS includes several effector proteins and lipids, which can activate sporozoites, enhancing the infection process[127]. We also analyzed pCPAs, a class of agents that have historically been employed in the cryopreservation field[79,77]. pCPAs are able to cross cell membranes, which reduces the intracellular ice formation and thus prevents cell lysis[97]. However, under certain conditions (temperature, cell type, cooling rate, etc.), its intracellular presence may also trigger a toxic response for cells[97]. The molecular weight of water is 18 Da, while that of pCPAs is, on average, 70 Da[78]. This difference in molecular weights leads to water moving more rapidly than pCPAs into and out of the cell, resulting in significant changes in cell[90]. However, some of these Penetrating CPAs are only toxic at a specific temperature and after a given period of time, which is the case of EG[91,97]. At room temperature, J is very toxic, especially when metabolized by the liver, but since it is used for cryopreservation at cryogenic temperatures, toxicity should not be impacts cell survival since J is removed after thawing[97,79].

CPAs can interact with each other in mixtures, or with crucial cell molecules, thereby producing effects other than those that would occur with individual CPAs. In a freezing mixture one of the components

might have a dominant role or they may combine to produce additive or synergistic effects. For this reason, it is advisable to combine CPAs from different categories in the cryopreservation of microorganisms[91]. Our results validate this assumption with the inclusion of two different types of npCPAs, H and C, in the sporozoite suspension.

Using a RPMI1640 sporozoite suspension containing x13i% C and x7iv% H, a x3iv% preservation of sporozoite infectivity upon cryopreservation and thawing was achieved, a 4-fold increase from the current standard (**Figure 11**). This result suggests that the protection of the cell membrane provided in the extracellular environment by H and C is essential during cryopreservation for maintaining sporozoite survival and infectivity. Furthermore, pCPAs present in the freezing mixture containing npCPAs could act intracellularly, balancing osmotic pressures and reducing intracellular ice formation. However, contrary to what was observed in several studies in other microorganisms[79,128], the introduction of pCPA in the freezing mixture led to a decrease in sporozoite survival **Figure 12**. This might be due to an increase in the osmolarity of solution as consequence of the amount of CPAs present in the mixture. The osmolarity of a physiological solution is 270–300 mOsm. A freezing mixture like the one tested in this work contains 3 different types of CPAs and should result in a very high osmolarity that leads to detrimental effects on cells. **Figure 12** also shows the importance of having CPAs in sporozoite freezing mixtures, since sporozoites cryopreserved only in RPMI1640 were unable to survival during cryopreservation.

Additional validation of the present cryopreservation process in *in vivo* models is required. To this end, before initiating the cryopreservation tests we started by analyzing the impact of injecting fresh sporozoites in freezing mixtures containing CPAs. In fact, CPAs such as H, F or C contain several factors and proteins that may behave as foreign molecules in the mice, triggering an immune response that can influence infection, *in vivo.* Since there are no studies describing the impact of injecting CPAs, in mouse models, this is a most important study to standardize our tests. Our results showed that H injection, even at very low concentrations, leads to an increase in the parasite liver load in mice. A possible explanation could be the improved ability (in comparison with RPMI without any CPA) of solutions containing H to preserve sporozoites when they are isolated from mosquito's salivary glands. However, that hypothesis was excluded by the results in **Figures 13 (D)**, which showed that there is no difference, in terms of liver infection load, between incubating sporozoites in a freezing mixture containing H 1 h prior injection or add this freezing mixture only at the moment of injection.

The results discussed so far were obtained with a rodent malaria parasite, *P. berghei.* Although the Prudêncio lab has been developing a whole organism pre-erythrocytic vaccine based on the use of *P. berghei* sporozoites, most other vaccine candidates of this type employ an attenuated form of the human-infective parasite, *P. falciparum*. Thus, it is essential to translate the achievements made with the *P. berghei* model to the *P. falciparum* parasite. Our results show that freezing mixtures containing x13i% C and x7iv% H (or using x3iv% F instead of x7iv% H) are able to preserve approximately 55% of *P. falciparum* sporozoite invasion rates after freezing and thawing, a result that was further improved by introducing the pCPA, K (**Figure 14**). Despite these excellent preliminary results, it is crucial to understand whether cryopreserved sporozoites are able to invade and continue their life-cycle or fail in

subsequent phases of their development. The damages resulting from cryopreservation may have a cumulative effect, allowing a normal invasion but blocking the subsequent parasite development due to injuries in parasite replication machinery. Thereby, further studies are necessary to establish whether this freezing mixture can be employed for *P. falciparum* cryopreservation. A long-term preservation in liquid nitrogen for a period of 3 years after freezing showed only a slightly decrease comparatively with sporozoites cryopreserved only for 1h (**Figure 14 (E)**). This validates that at -196ºC sporozoites are being well preserved with almost total absent of metabolic activity.

Our data suggest that a better understanding of the mechanism of action of CPAs may lead to the identification of even better performing freezing mixtures that further retain sporozoite survival after cryopreservation. Thus, we speculate that the high doses of *P. falciparum* sporozoites currently required to achieve sterile protection against malaria[70] may be decreased if cryopreserved sporozoites with enhanced invasion capacity/ infectivity are employed.

5. Chapter – Conclusions & Future Perspectives

The work performed during this thesis contributes significantly to the goal of developing an ideal method for cryopreservation of *Plasmodium* sporozoites. Several cryopreservation parameters were optimized, particularly vial geometry and formulation of the freezing mixture, that directly influence the efficiency of cryopreservation. Towards the formulation of an ideal cryopreservation mixture, we performed a detailed analysis of several CPAs and attempted to explain the potential mechanisms of action of these CPAs.

A freezing mixture of RPMI1640 containing x13i% C and x7iv% H warranted the preservation of ~50% *P. berghei* sporozoite viability, which constitutes a substantial improvement over the current standard. Alternatively, a freezing mixture containing x13i% C and x3iv% F also consistently led to the preservation of ~40% of *P. berghei* sporozoite viability. Both these freezing mixtures further warranted the preservation of ~55% of *P. falciparum* sporozoite invasion capacity after cryopreservation, a result that was further improved with the addition of a pCPA, K, to the freezing mixture. Preliminary *in vivo* assays indicated that the administration of CPAs to mice has a severe impact on infection by sporozoites, which needs to be taken into account in subsequent cryopreservation experiments.

Importantly, this method for *Plasmodium* sporozoite cryopreservation employs inexpensive, commercially available CPAs, with no need for specialized equipment. It is expected that most malaria research laboratories currently employing infected mosquitoes can adopt the procedures developed in this study. Thereby, we speculate that this reproducible cryopreservation method has the potential to contribute to the development of novel anti-malarial strategies and impact the development and evaluation of whole-sporozoite malaria vaccine candidates.

Despite the objectives accomplished during this thesis, there is still ample room for further improvements. We propose to continue the optimization of the freezing mixture, taking into account its osmolarity and thus including other CPAs as well as different procedures of addition of the freezing mixture to the sporozoite suspension. Additional *P. falciparum* cryopreservation experiments are also required to evaluate the reproducibility of our results. Another aspect that remains to be optimized is the incubation time required to maximize the mechanism of protection of different CPAs, since this can vary with the molecular features of CPAs. The thawing process also remains largely unexplored. As such, it would be interesting to study different thawing rates at different temperatures. Additionally, other vial geometries can be evaluated, and the possibility of designing and printing dedicated vials can also be explored. Once the cryopreservation process has been optimized using an *in vitro* method to assess sporozoite viability, *in vivo* evaluation of the capacity of cryopreserved sporozoites to reach and develop in mouse livers should be evaluated. Finally, in collaboration with SmartFreez we are developing an automated prototype system that will allow us streamline the process and to test various cooling rates, for optimal recovery of parasite viability after cryopreservation.

6. References

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7. Annexes

RPMI-1640 Media continued

COMPONENT	R 1383 g/L	R 7130 g/L	R 7509 R 8632 [1X] g/L	R 5886 R 5632 [1X] g/L	R 1145 [10X] g/L
INORGANIC SALTS					
$Ca(NO_3)_2 \cdot 4H_2O$	0.1	0.1	0.1	0.1	1.0
$MgSO_4$ (anhyd)	0.04884	0.04884	0.04884	0.04884	0.4884
KCl	0.4	0.4	0.4	0.4	4.0
$NaHCO_3$			2.0	2.0	—
NaCl	6.0	6.0	6.0	6.0	60.0
Na_2HPO_4 (Anhyd)	0.8	0.8	0.8	0.8	8.0
AMINO ACIDS					
L-Arginine	0.2	0.2	0.2	0.2	2.0
L-Asparagine (anhydrous)	0.05	0.05	0.05	0.05	0.5
L-Aspartic Acid	0.02	0.02	0.02	0.02	0.2
L-Cystine•2HCl	0.0652	0.0652	0.0652	0.0652	0.652
L-Glutamic Acid	0.02	0.02	0.02	0.02	0.2
L-Glutamine	0.3	—	—	—	—
Glycine	0.01	0.01	0.01	0.01	0.1
L-Histidine	0.015	0.015	0.015	0.015	0.15
Hydroxy-L-Proline	0.02	0.02	0.02	0.02	0.2
L-Isoleucine	0.05	0.05	0.05	0.05	0.5
L-Leucine	0.05	—	0.05	0.05	0.5
L-Lysine•HCl	0.04	—	0.04	0.04	0.4
L-Methionine	0.015	—	0.015	0.015	0.15
L-Phenylalanine	0.015	0.015	0.015	0.015	0.15
L-Proline	0.02	0.02	0.02	0.02	0.2
L-Serine	0.03	0.03	0.03	0.03	0.3
L-Threonine	0.02	0.02	0.02	0.02	0.2
L-Tryptophan	0.005	0.005	0.005	0.005	0.05
L-Tyrosine•2Na•$2H_2O$	0.02883	0.02883	0.02883	0.02883	0.2883
L-Valine	0.02	0.02	0.02	0.02	0.2
VITAMINS					
D-Biotin	0.0002	0.0002	0.0002	0.0002	0.002
Choline Chloride	0.003	0.003	0.003	0.003	0.03
Folic Acid	0.001	0.001	0.001	0.001	—
myo-Inositol	0.035	0.035	0.035	0.035	0.35
Niacinamide	0.001	0.001	0.001	0.001	0.01
p-Amino Benzoic Acid	0.001	0.001	0.001	0.001	0.01
D-Pantothenic Acid•½Ca	0.00025	0.00025	0.00025	0.00025	0.0025
Pyridoxine•HCl	0.001	0.001	0.001	0.001	0.01
Riboflavin	0.0002	0.0002	0.0002	0.0002	0.002
Thiamine•HCl	0.001	0.001	0.001	0.001	0.01
Vitamin B-12	0.000005	0.000005	0.000005	0.000005	0.00005
OTHER					
D-Glucose	—	2.0	2.0	2.0	20.0
Glutathione (reduced)	0.001	0.001	0.001	0.001	0.01
HEPES			—	5.96	—
Phenol Red (sodium)	0.0053	0.0053	—	0.0053	0.053
ADD					
$NaHCO_3$	2.0	2.0	—	—	2.0 at 1X
L-Glutamine	—	0.3	0.3	0.3	0.3 at 1X
L-Leucine	—	0.05			
L-Lysine•HCl	—	0.04			
L-Methionine	—	0.015			
Grams of powder required to prepare 1 L	8.4	10.0	8.4	10.0	N/A

Supplementary Figure 1 – Detailed formulation of sporozoite suspension media, RPMI1640