Malaria vaccinology: from the development of a new malaria vaccine to the ethics of clinical trial participation

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Cover design: Olivia Aurelia Catherina Lamers and Sigmund Dollinar
Lay-out: Parntawan Kidtam I www.ridderprint.nl
Print: Ridderprint I www.ridderprint.nl

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Proefschrift

ter verkrijging van

de graad van doctor aan de Universiteit Leiden,

op gezag van rector magnificus prof.dr. S. de Rijcke,

volgens besluit van het college voor promoties

te verdedigen op donderdag 10 september 2026

klokke 10 uur

door

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geboren te Freiburg im Breisgau, Duitsland

in 1994

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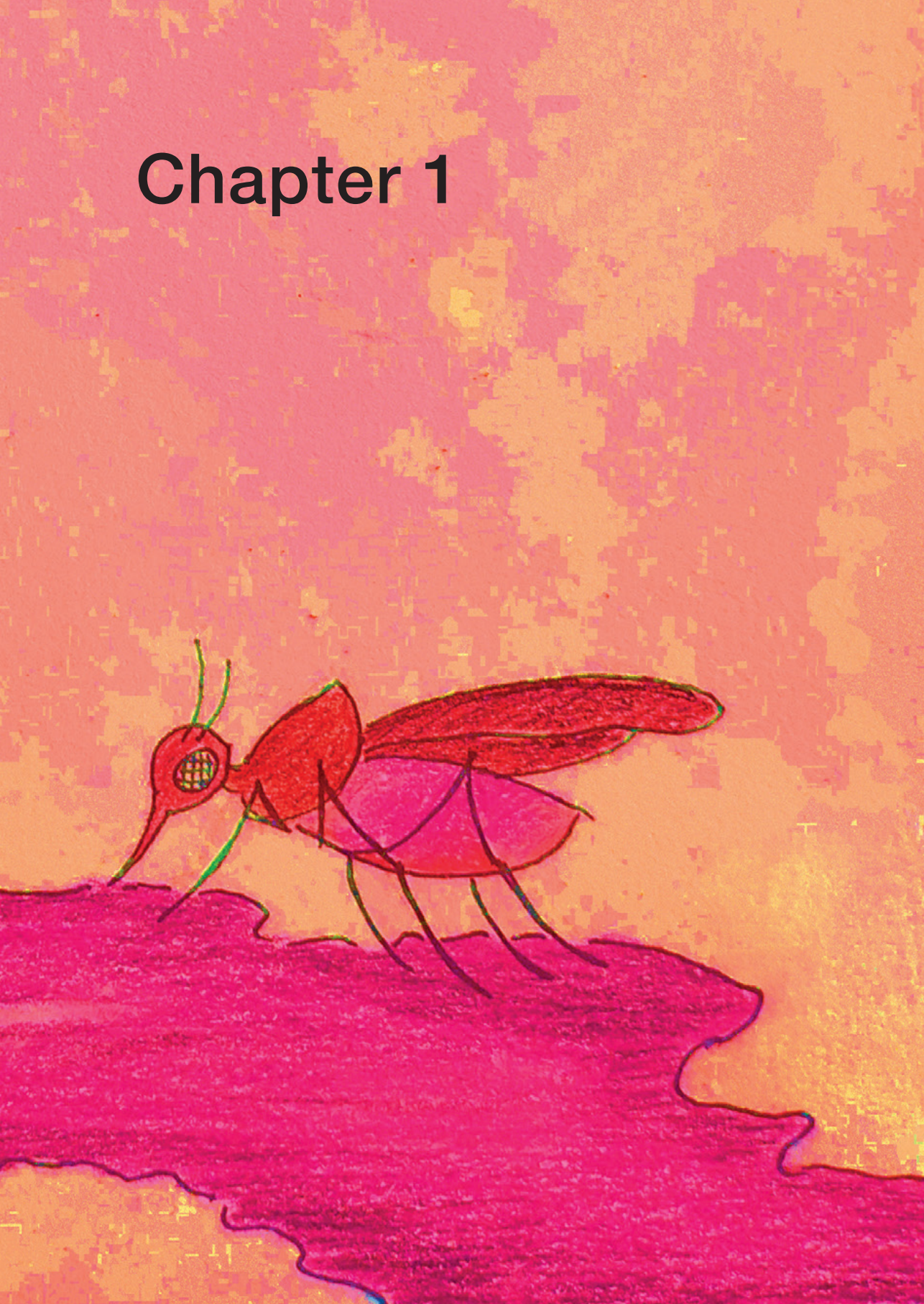
Chapter 1



Introduction

Malaria

What is malaria?

Malaria is a vector-borne disease caused by the protozoan parasite *Plasmodium* and transmitted by female *Anopheles* mosquitoes. There are five species of *Plasmodia* that can cause disease in humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi*. *Plasmodium falciparum* (*Pf*) is by far the most common and virulent species[1] and will be the focus of this thesis.

Malaria life cycle and symptomatology

Sporozoites are injected into humans by female *Anopheles* mosquitoes during a blood meal and then travel to the liver via the circulation. Schizonts develop inside infected hepatocytes and release merozoites into the blood. Merozoites then infect erythrocytes, multiply asexually and eventually cause their host cells to burst, perpetuating the cycle of re-infection[2]. Gametogenesis, that is the production of sexual forms of the parasite, occurs in response to environmental stress in the host. Gametocytes are taken up by *Anopheles* mosquitoes during a blood meal and develop into ookinetes and then oocysts in the mosquito midgut and finally into sporozoites in the salivary glands[3,4]. From here, the life-cycle begins anew (**Fig. 1**). Even though the pre-erythrocytic stage of the *Pf* life cycle is asymptomatic, immunologically it is anything but silent: liver-resident cellular immune responses are thought to contribute to protection against the disease[5]. The cyclic rupture of erythrocytes causes a strong inflammatory response and the typical symptoms of malaria including fever and malaise. Serious complications, such as cerebral malaria and multiorgan failure, may arise in response to mechanical sequestration of infected erythrocytes to capillaries and widespread inflammation[1,6].

Malaria epidemiology and control

In 2023 alone malaria caused 263 million cases of disease and 597.000 deaths. The WHO African region is disproportionally affected, the most vulnerable populations being children under the age of five and pregnant women[7]. While malaria incidence had been steadily declining since 2000, it experienced a resurgence in 2019, most likely due to the negative effects of the SARS-CoV-2 pandemic on preventative strategies[8].

Resistance against antimalarial drugs is another factor that may compromise efficacy of both clinical and preventative treatment. Resistance to artemisinin, the most efficacious antimalarial drug currently available, is especially worrisome: first reported in Asia in

2008[9], it has since spread to the African continent as well. It is caused by several different mutations in the *k13* gene, which codes for a putative E3 ligase adaptor[10,11] and results in delayed clearance of parasitemia. The projection is that, combined with resistance to partner drugs, decreased sensitivity to artemisinin will eventually lead to treatment failure[11].

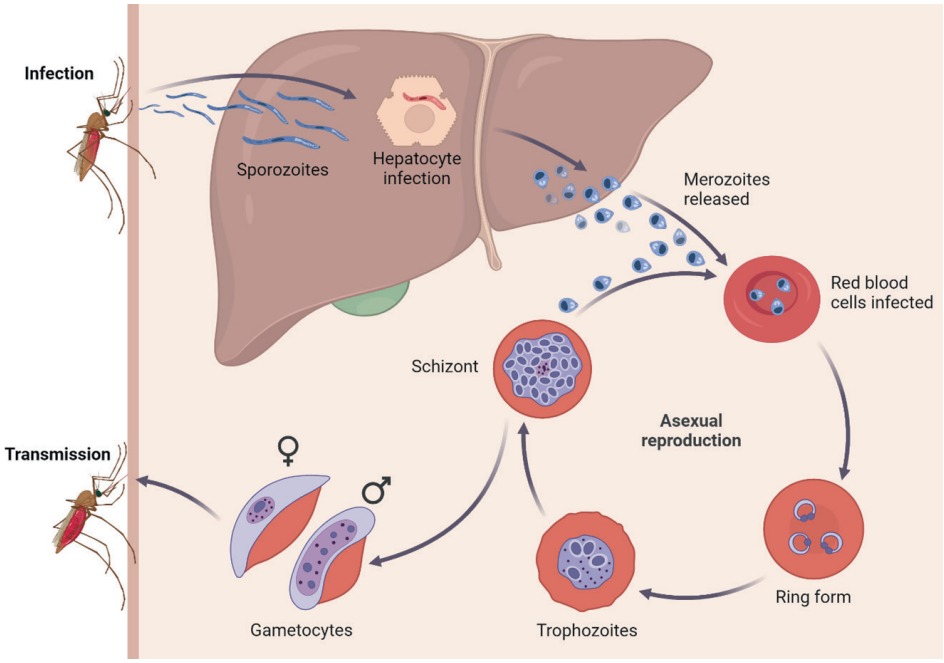


Figure 1. *Plasmodium* life-cycle
Plasmodium enters its human host when a mosquito takes a blood meal. Sporozoites move through the skin, into the circulation and then into the liver. Schizonts are formed inside infected hepatocytes which, upon exponential replication, release merozoites into the blood. Merozoites grow and multiply asexually inside erythrocytes until the host cells burst, releasing even more merozoites. Gametocytes are the sexual forms of the parasite and can be picked up by a mosquito during a new blood meal. In the mosquito gut, gametocytes form ookinetes and oocysts, which hatch sporozoites. Sporozoites then migrate to salivary glands from where they are injected into their human hosts during the mosquito's next blood meal.

Historically, vector control has been one of the most effective ways of eradicating and controlling malaria. After the ban of dichlorodiphenyltrichloroethane (DDT) due to environmental and health concerns, insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) remain the two most important tools for vector control[12]. ITNs serve the dual function of physical barrier against biting and of chemical repellent killing mosquitoes that come in contact with them. IRS is very similar to ITNs with the only difference that insecticides are applied directly to indoor surfaces[13]. However, resistance

to insecticides is increasing and, in combination with climate change, may compromise established control over vector populations[14,15].

Hence, the rising resistance of both parasites against drugs and mosquitoes against insecticides risk to make pharmacological and chemical interventions obsolete in the not-so-distant future[9,16-18]. If the ambitious goal set forward by the WHO Global Technical Strategy[19] of reducing malaria mortality by 90% in 2030 is to be met, new and more effective tools of eradication are needed. Availability of an effective vaccine could greatly reduce the incidence and transmission of malaria and be the decisive factor finally leading to disease eradication[20,21].

Natural immunity to malaria

No such thing as sterile immunity to malaria exists in nature, as continuous re-exposure is required for the development of both tolerance against severe symptoms and resistance to parasites[22].

Natural protection against malaria is thought to rely on humoral immune responses against erythrocytic antigens, as neither parasites nor erythrocytes have HLA class I or II molecules that could be recognized by T cells[23,24]. The specificity of the antibody response is limited by the strong pro-inflammatory environment of the blood-stage infection, which impairs germinal centre formation and effective B cell maturation through excessive interferon-γ (IFN-γ) production[23,25]. Antibodies that protect against severe malaria, such as those directed at the erythrocyte membrane protein 1 (PfEMP1), develop early after the first exposure while prevention of symptoms and inhibition of erythrocyte invasion ensues later in life with increasing titres and broadening of the immune response[23].

However, despite clinical tolerance, infected individuals may still carry asexual and sexual forms of the parasite, thereby perpetuating the cycle of transmission[22].

Vaccine induced immunity to malaria

In contrast to naturally acquired immunity, which elicits a broad immune response, most vaccines are designed to generate a limited yet highly specific response. Ideally, any vaccine against malaria would not only mitigate symptoms, but also prevent their onset and block transmission. It is for this reason that much research has been directed at inducing pre-erythrocytic immunity, which could not only stop merozoites egression from the liver, but also formation of gametocytes[26]. Hence, this is where the focus of this thesis will lie.

Mechanisms of vaccine-induced immunity

The mechanisms underlying vaccine-induced pre-erythrocytic immunity are complex and incompletely understood.

Cellular immunity

Cellular immune responses play a central role in protection against the liver stages of the parasite. Immune patrolling by T cells is dependent on cell recirculation in lymphoid tissues (central effector (T_{CM}) cells that are CCR7$^+$), in non-lymphoid tissues (effector cells (T_{EM}) that are CCR7$^-$), and by tissue residency (characterized by the expression of CD103$^+$)[27].

In malaria, CD8$^+$ T cells are thought to be primed in the lymph nodes by dendritic cells (DCs) and then migrate to the liver where they become tissue-resident effector (CCR7$^-$ CD103$^+$ CD8$^+$) cells and directly kill infected hepatocytes through perforin and granzyme. A proportion of these T cells may then acquire a memory phenotype and persist even after infection clearance[28,29].

CD4$^+$ T cells are another subset that has been intensively studied in malaria. They contribute to CD8$^+$ T cell activation and persistent proliferation by promoting antigen presentation and suppressing apoptosis[30]. Additionally, CD4$^+$ T cells are thought to have a direct anti-parasitic effect through the production of pro-inflammatory cytokines and cytotoxic granules, possibly even killing infected hepatocytes[31].

Humoral immunity

While antibodies are an important component of naturally acquired immunity, their role in pre-erythrocytic immunity is unclear[32]. Most research has focussed on the circumsporozoite protein (CSP), as this is the major protein on the sporozoite surface. Subunit vaccines, designed to induce a strong humoral immune response against CSP, have limited efficacy which diminishes further with decreasing antibody titres[33]. The use of anti-CSP antibodies as correlates of protection also has limited value[34-37]. While it has been shown that sufficient amounts of monoclonal antibodies with high affinity for CSP can protect against infection[38,39], both the quality and quantity of antibodies produced after immunization does not always reach the protective threshold[34-37].

Pre-erythrocytic malaria vaccine candidates

The quest for developing an effective vaccine against malaria has been ongoing for decades, hampered by the complex biology of *Plasmodium* and by a relative scarcity

of funds[22]. There are currently two types of pre-erythrocytic vaccine candidates, namely subunit vaccines and vaccines consisting of live, attenuated parasites[26]. The efficacy of such vaccines can be tested by controlled human malaria infection (CHMI) studies, where healthy participants are exposed to live unattenuated parasites in a controlled setting[40].

Subunit vaccines

The first licensed malaria vaccine was RTS,S/A01 (marketed as Mosquirix), a subunit vaccine based on CSP, in combination with hepatitis B surface antigen and the AS01 adjuvant. It was developed by scientists at GlaxoSmithKline (GSK) and the Walter Reed Army Institute and has recently been approved by the WHO for widespread administration in malaria-endemic countries: protection varies between 30% and 50% in infants and children and wanes over time[41-43]. Similar results were reported for adults[44].

Attempts at improving the efficacy of RTS,S have brough forward R21, yet another subunit vaccine consisting of a virus-like particle containing CSP fused to hepatitis B surface antigen and formulated with the adjuvant Martrix-M. In a recent trial conducted across four African sites and involving children aged 5-36 months, vaccine efficacy amounted to between 68% and 75% after one year[45]. However, despite these promising results, there is currently insufficient evidence to conclude that, on the long term, the efficacy of R21 will be superior to that of RTS,S/A01[46].

Live attenuated vaccines

An alternative to subunit vaccines are whole SPZ (WSp) vaccines, which have shown up to 100% efficacy in malaria naïve populations. WSp vaccines are live malaria parasites, attenuated by radiation, pharmacological intervention or genetic modification[47,48].

The first experiments with radiation attenuated sporozoites (RAS) date back to the sixties of the previous century: after protection had been achieved in mice [49], findings were quickly translated to humans[50-52]. RAS can be administered either by mosquito bites or as an injectable product (PfSPZ Vaccine). Irradiated sporozoites reach the liver, but arrest development after 24 to 48 hours. While RAS are safe and well-tolerated, protection requires high doses (up to 2000 mosquito bites) in malaria-naïve individuals[48] and in pre-exposed populations protection remains low despite increasing doses[53]. One study in Tanzania found that administration of cryopreserved sporozoites in excess of 9×10^5 was actually associated with a decrease in protection[54].

Another possibility is the administration of live sporozoites in combination with chemoprophylaxis (CPS). This has, for a long time, been the most efficient WSp approach,

as three immunizations with a total of 45 mosquito bites were sufficient to induce sterile immunity in malaria-naïve participants[55]. The superior efficacy of this method has been attributed to the prolonged exposure and increased antigenic load presented to the immune system. However, CPS is less suitable for large-scale implementation because it bears the intrinsic risk of un-attenuated parasite administration to healthy individuals.

More recently, genetically attenuated parasites (GAPs) have been created[56-58]. Since GAPs could be made to arrest at virtually any timepoint in the developmental cycle, they could be more immunogenic than RAS without sacrificing safety[59].

The first *Pf* GAP lacked the and *p36* and *p52* genes, which code for a putative GPI-anchored protein and a putative secreted protein, respectively. Despite displaying normal infectivity, this double knock-out (*Pf* GAP2KO) showed an attenuated phenotype *in vitro* and *in vivo*. Parasites halted development in hepatocytes after 4-6 days and were absent in the livers of humanized mice after four days, as measured by microscopy and quantitative polymerase chain reaction (qPCR)[60]. However, when the *Pf* GAP2KO was tested in humans, it was incompletely attenuated and caused a breakthrough infection in one out of six exposed participants[61].

To reduce the likelihood of breakthrough, a new GAP, lacking sporozoite asparagine-rich protein 1 (*sap1*) in addition to *p52* and *p36,* was generated and termed *Pf* GAP3KO due to the triple gene deletion. Gametogenesis and sporogenesis, as well as gliding ability, hepatocyte transversal and infection *in vitro* were comparable to WT[62].

In a trial assessing *Pf* GAP3KO's efficacy, healthy participants received 200 mosquito bites per exposure: ten of them were immunized five times and six of them three times. Upon CHMI four weeks later, protection rate was 50% in both groups[63].

We and others generated one of the first GAPs, lacking the genes *b9* and *slarp (PfΔb9Δslarp*) and henceforth referred to as GA1[57]. B9 is a member of the 6-cysteine domain protein family which is fundamental for liver invasion[64], while sporozoite and liver stage asparagine-rich protein (SLARP) is an asparagine-rich protein that regulates gene expression in the liver stage[65]. GA1 parasites infected primary human hepatocytes *in vitro* but then became undetectable on days 2-7 post-infection. Similarly, when the presence of the parasite was assessed by qPCR in the livers of humanized mice, it was detected at 24 hours after exposure, but was absent four days later[57]. In the subsequent clinical trial, two different doses were tested: 4.5×10^5 or 9×10^5 cryopreserved sporozoites were administered three times at 56-day-intervals to 13 individuals each. After CHMI three weeks later, three participants were protected in total, one in the low dose and two in the high dose group. Hence, albeit safe and well-tolerated, the efficacy of GA1 was lower than desired[34].

The discovery of late-liver stage genes has opened the way to the creation of late-arresting GAPs[66]. The idea is that, similar to CPS, the longer developmental timeframe in combination with the increased antigenic repertoire and load, could elicit a stronger immune response and therefore lead to improved protection[59] (**Fig. 2**). If these GAPs were confirmed to have higher efficacy than their predecessors, they could be a new generation of malaria vaccine candidates.

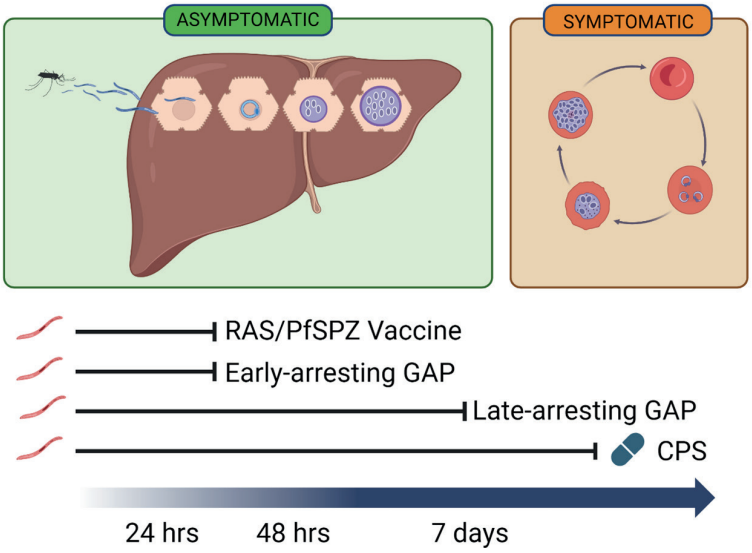


Figure 2. Development of GA2
Comparison between the development of radiation attenuated sporozoites (RAS/PfSPZ Vaccine), genetically attenuated parasites (GAPs) that arrest early or late in the liver stage and sporozoites administered in combination with chemoprophylaxis (CPS).

Clinical trials for vaccine development

The importance of human volunteers

The impact of clinical trials for scientific advancement is undeniable. Clinical research is essential to the approval of new drugs and vaccines and would not be possible without medical volunteers. However, clinical research involving humans has been a long matter of discussion. The horrors of the Second World War, characterized by coerced experimentation on prisoners, resulted in the Nürnberg Code (1947), which was later expanded and revised in the Declaration of Helsinki (1964)[67,68]. Prompted by the Tuskegee Syphilis Study[69], the Belmont Report (1978) re-defined the guidelines for safe research in human subjects[70].

The basic principles underlaying all of these documents is that participation in research should always be voluntary and that potential side-effects for the individual need to be

carefully weighed against the benefits for society. When in doubt, the welfare of the individual should always outweigh other considerations[67,68,70].

Despite multiple attempts at regulation, research involving human subjects remains problematic, especially if it is conducted on healthy individuals. In contrast to phase 2 and 3 studies, phase 1 participants experience no personal advantage and may even suffer health-related complications as a consequence of their participation. In order to make participation more attractive, medical volunteers are financially rewarded. The principal concern then, is that economically vulnerable individuals might feel pressured to participate because they receive a financial compensation[71,72]. How then can we make sure that the principle of self-determination is fulfilled in these cases?

A number of studies have tried to dispel fears by showing that clinical trial participants have mixed motivations, including, but not limited to financial compensation. The desire to help others and to aid scientific advancement are also important factors influencing the decision to participate in a clinical trial[73,74].

Much research has tried to unravel the motivations of medical volunteers, but much discussion still abides. In order to safeguard the ethics of clinical trials involving humans, it is important to understand the underlaying motivations of participants and to keep the discussion alive. It is the awareness of the question and not so much the answer that helps protect the ethical norm and the rights of medical volunteers.

Controlled human infection studies

Controlled human infection (CHI) studies are a form of clinical research which consist of the deliberate inoculation of healthy participants with infectious agents in a controlled setting, where the combination of early detection through good diagnostics and the existence of an effective therapy minimizes side effects and prevents long-term sequelae[75]. CHI studies are a research model that allows for early efficacy assessment of new experimental treatments and vaccines, saving both economic and time resources before proceeding to more complex stage 3 trials[76]. Additionally, CHI studies can provide insights into the pathophysiology of diseases in a way that other research could not[77]. Human challenges with unattenuated pathogens may be especially useful when a rapid response to new infectious threat is required, such as in the context of a pandemic[77,78].

However, despite their many advantages, CHI studies fall under intensive scrutiny as some may suggest that they are more dangerous than traditional phase 1 clinical trials with higher rates of negative side effects and exposure to diseases for which there may not be a cure (yet)[79].

Given these polarizing views on the subject and the extraordinary potential of CHI studies in understanding the pathophysiology of new emerging disease and developing effective vaccines and treatments against them, it is worth exploring whether CHI studies really differ from phase 1 clinical trials and if so, how.

Thesis outline

The scientific part of this thesis is divided into two parts: the first part consists of articles in which I was the main investigator, whereas the second one contains collaborative projects. I chose these particular collaborative projects to be included in the thesis above others because I consider them part of my formative development as a scientist and/or because I deem them important to understand the contents of this thesis.

Chapter 1 describes the first administration of GA2 to humans. The novel GAP was not only safe and well-tolerated but also had a protective efficacy of 89% against challenge with WT parasites. Moreover, we found a correlation between $CD4^+$ T cells and $\gamma\delta$ T cells and protection.

Chapter 2 puts the GA2 in the broader context of other existing GAPs, provides a comprehensive overview of the field and includes recommendations for the future.

Chapter 3 focuses on the motivations and the decision-making process of SARS-CoV2 vaccine study participants. We found that this subgroup of medical volunteers was mostly motivated by the desire to help others. We propose a theoretical framework to explain how this reasoning shift came about.

Chapter 4 is an opinion piece concerning the (perceived) difference in risk between phase 1 clinical trials and CHI studies. It provides general guidelines to attempt to mitigate risks when carrying out the latter.

Chapter 5 and **Chapter 6** are collaborative projects. **Chapter 5** describes the creation of GA2 as a new GAP and is the preclinical research that directly preceded and made the testing of GA2 in healthy human participants possible. **Chapter 6** contains the results of the research that followed the first testing of GA2 as described in Chapter 1. The safety of the parasite was confirmed once more and it was shown that a single dose of 50-GA2 infected mosquito bites is sufficient to achieve 90% protection against challenge with WT parasites.

Finally **Chapter 7** contains a brief summary and extensive discussion on the subjects of the preceding chapters.

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Chapter 2

Safety and efficacy of immunization with a late-liver-stage attenuated malaria parasite

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Published: New England Journal of Medicine, 2024
DOI: 10.1056/NEJMoa2313892

Abstract

Background: Immunization with live-attenuated *Plasmodium falciparum* (*Pf*) malaria parasites is an alternative vaccination strategy to existing subunit vaccines. We report the safety and efficacy of GA2, a *mei2* single knock out *Pf*NF54 parasite (sporozoite form) with extended development into the liver-stage.

Methods: We conducted a comparator-controlled double-blind clinical trial, assessing safety, tolerability and efficacy of mosquito bite (MB) immunization with GA2 in healthy malaria-naïve adults, dose-escalated from 15 to 50 MB per immunization. Following 3 immunizations with 50 MB, protective efficacy against homologous *Pf* controlled human malaria infection (CHMI) was compared to that of an early-arresting parasite (GA1) and placebo.

Results: Adverse events were comparable across dose groups. GA2 resulted in protection in 8/9 participants (89%) against subsequent CHMI, compared to 1/8 (13%) for GA1 and protection in 0/3 amongst placebo recipients. A significantly higher frequency of *Pf*-specific polyfunctional $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells was observed in GA2 compared to GA1 recipients, whereas both GA2 and GA1 induced comparable anti-*Pf* circumsporozoite protein antibody titers.

Conclusion: In this small study, the favorable immune induction profile and protective efficacy of GA2 warrant further evaluation.
Trial registration number: NCT04577066

Introduction

The progress in the eradication of malaria has stalled, creating a need for novel tools[1]. The currently licensed and WHO-approved malaria vaccines, Mosquirix (RTS,S/AS01) and R21, are subunit vaccines based on the major sporozoite surface antigen, circumsporozoite protein (CSP). The resulting protection is modest (50-80%), short-lived (approximately 1 year) and in high endemic areas lower in infants compared to children[2,3]. Alternate vaccine strategies based on whole attenuated *Plasmodium* parasites have the potential to improve protection[4]. Metabolically active attenuated sporozoites invade hepatocytes, the first (asymptomatic) stage of the parasite's lifecycle, but fail to develop into a patent blood-stage infection. Inoculation with attenuated whole sporozoites safely exposes the immune system to a broad array of parasite antigens, eliciting both humoral and cellular immune responses[5-8].

The most advanced whole sporozoite vaccine candidate, PfSPZ Vaccine, consists of *Plasmodium falciparum (Pf)* radiation-attenuated sporozoites, which arrest development early after invading hepatocytes. Albeit safe (i.e., no breakthrough blood-stage infections), radiation attenuated sporozoites require high doses to induce protection, which remains suboptimal in pre-exposed populations[9-14]. In an experimental medicine setting, repeated *Pf* inoculations attenuated through concomitant administration of chloroquine chemoprophylaxis, which kills the parasites only during the blood-stage, induced 100% protection with low numbers of sporozoites[15,16]. The late arrest of parasites in this model exposes the immune system to a broad array of antigens expressed in sporozoites, intrahepatic stage and a small number of asexual blood-stage parasites, thereby improving protective efficacy.

Identification of genes essential to the parasite's intrahepatic development facilitated the creation of genetically attenuated malaria parasites which can be produced and are amenable to vaccine applications[17]. We previously tested *PfΔb9Δslarp* (GA1), with short intrahepatic development (24hrs, similar to radiation attenuated sporozoites)[18,19] and reported low protective efficacy (12%; 3/25) against homologous controlled human malaria infection (CHMI) in malaria-naïve participants[18].

We recently generated *PfΔmei2* (GA2), a parasite showing complete growth-arrest late during the liver-stage (day 6 post-invasion) in humanized mouse models, thus resembling parasites co-administered with chemoprophylaxis but without any blood-stage exposure[20,21]. Here we describe a clinical trial designed to test the concept that extended liver-stage antigen exposure can induce better protection. To that end, we compared safety, tolerability and preliminary efficacy of late-arresting GA2, early-arresting GA1

and placebo in healthy malaria-naive participants by mosquito bite (MB) immunization followed by CHMI.

Materials and methods

Trial design and oversight

This multi-stage study was performed between September 2021 and December 2022 at Leiden University Medical Center (LUMC) and Radboud University Medical Center (Radboudumc). The trial consisted of two stages: stage A was an open-label, dose-escalation safety stage with participants exposed to either 15 (n=5) or 50 (n=15) GA2-infected MB; Stage B was double-blind, placebo-controlled and compared protective efficacy against homologous CHMI in GA1-MB (n=10), GA2-MB (n=10) and placebo groups (n=3) after 3 immunizations, administered at 28-day intervals, with 50 MB each. Because of COVID-19 lockdowns, the study was conducted with fewer participants than originally envisaged (refer to study protocol), with approval of the safety monitoring committee (SMC).

All participants in stage B underwent CHMI with 5 *Pf*3D7-infected MB (**Fig. 1**). *Pf*3D7 is an unattenuated clone of the parental parasite strain *Pf*NF54[22], which was used to generate GA2 and GA1.

Primary and secondary endpoints

The primary endpoint was the number and severity of adverse events (AEs) and blood-stage parasitemia >100 Pf/ml by quantitative polymerase chain reaction (qPCR) after GA2-MB. Efficacy after CHMI was assessed by measuring blood-stage parasitemia by qPCR .

The secondary outcomes consisted of measuring plasma antibodies targeting key *Pf*-antigens using enzyme linked immunosorbent assay (ELISA) and cellular responses in peripheral blood mononuclear cells (PBMCs) stimulated with *Pf*-infected and uninfected erythrocytes *in vitro* and subsequently phenotyped and profiled for cytokine expression by spectral-flowcytometry.

Methods are described in the accompanying **Supplementary Appendix**.

Recruitment of participants

Malaria-naïve adults, aged 18 to 35, in good health as assessed by medical history, physical examination, general laboratory evaluation, including biochemistry, hematology, a toxicological screening for illicit drugs, and electrocardiography (ECG) were included. Female participants were required to use adequate birth control throughout the study. All

participants provided written informed consent prior to inclusion. A list of inclusion and exclusion criteria is provided in the **Supplementary Appendix**.

Ethics

The trial protocol was approved by the Dutch Central Committee for Research involving Human Subjects (CCMO) (identifier NL75555.000.21), registered in ClinicalTrials.gov (identifier NCT04577066), and EudraCT (identifier 2020-005646-41), performed under a license from the Dutch Gene Therapy Office (identifier IM-MV 20-018) and overseen by an SMC.

Recruitment and follow-up of participants were carried out at LUMC, whereas GA2, GA1 and unattenuated *Pf3D7* parasite culturing, *Anopheles stephensi* mosquito rearing and exposure of participants to MBs were performed at Radboudumc. Data analyses and drafting of the manuscript were done by both teams. All authors take responsibility for the integrity, completeness, accuracy of the data and for the fidelity of the trial to the protocol and statistical analysis plan.

Randomization and statistical analysis

Randomization was carried out by an independent statistician. All staff dealing with participants were blinded to randomization throughout the trial.

No formal sample size calculations were performed for stage A of the trial. For stage B, we aimed for a total of 10 participants to undergo CHMI in the GA2-MB group. This would provide 80% power and a margin of error of 5% (2-tailed α) to detect 70% protective efficacy of GA2-MB compared to placebo.

Adverse events (AE) were evaluated according to intention to treat (ITT) analysis (n=43), efficacy and immunological analyses were per protocol (PP, n=40). The proportion of participants in each cohort or group reporting mild, moderate or severe AEs was calculated. The presence of parasitemia was analyzed by χ^2 and Fischer's exact test.

Analyses of humoral and cellular immune responses were prespecified in the protocol. Serological data were log transformed to achieve a normal distribution. A paired t-test was used when comparing pre- and post-exposure values, and an unpaired t-test was used for comparisons between groups. For discrete variables, the χ^2 test or Fisher's exact test was performed (two-tailed). Cellular responses between the groups were compared using the two-tailed Mann-Whitney test. All p-values were considered significant if <0.05. Graphs were produced in GraphPad Prism (version 9.3.1) and R (version 4.2.1).

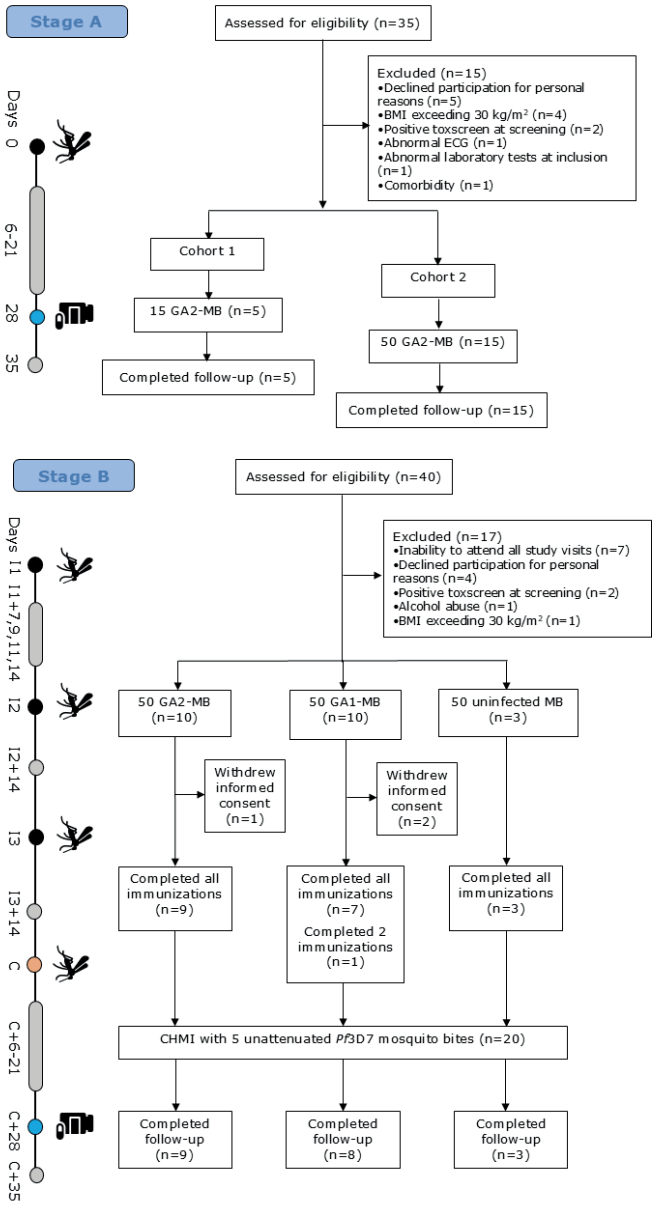

Figure 1. CONSORT diagram and clinical trial design
Stage A: Stage A was open label and eligible participants were recruited sequentially into cohort 1 and then cohort 2. In the dose-escalation stage A, participants were exposed once to GA2-MB (black circle): the first cohort received 15 MB, the second 50 MB.
Stage B: Allocation in stage B was randomized and double-blind. Three groups of participants were exposed thrice to either 50 GA2-MB, GA1-MB or uninfected MB (placebo) respectively, at 28-day intervals (black circles). Three weeks after the last immunizing exposure, participants underwent controlled human malaria infection (CHMI) by bites of 5 unattenuated wild-type (WT) *Pf*3D7-infected mosquitoes (orange circle). All participants were treated with a curative regimen of atovaquone/proguanil at latest day 28 after their final exposure (blue circles).
BMI: body mass index, ECG: electrocardiogram.

Results

Study population

Seventy-five malaria-naïve adults were screened from the 13th of September 2021 to the 28th of January 2022, of which 43 were included. No participants were lost during stage A, and 3 participants in stage B withdrew consent prior to CHMI (**Fig. 1).** In total, 22 (51%) participants were female. Median age was 23.0 years (range 19-35) and median BMI was 24.1 kg/m^2 (range 18.0-29.9) (**Table 1**).

Table 1. Participant demographics.

		Stage A		Stage B			
		Cohort 1	**Cohort 2**	**GA2**	**GA1**	**Placebo**	**Total**
Number of participants		5	15	10	10	3	43
Age (years)	Median	22	23	23.5	23.5	22	23
	Min, max	20-27	19-30	19-31	19-35	21-24	19-35
Sex	Female	4	8	5	3	2	22
	Male	1	7	5	7	1	21
BMI (kg/m^2)	Median	24.8	24.1	22.4	22.6	27.5	24.1
	Min, max	21.9-26.9	20.3-29.1	18.0-29.9	19.9 -28.6	24.4-28.7	18.0-29.9

BMI: body mass index.

Safety results

No serious adverse events occurred during the trial. There were no breakthrough infections after exposure to either 15 or 50 GA2-MB, as assessed by qPCR. There was no significant difference in the incidence and severity of AEs after administration of GA2-MB, GA1-MB or placebo (ITT).

The most common related local AEs were erythema and pruritus after MB, reported by 19 (95%) and 17 (85%) participants, respectively, in stage A (**Supplementary Fig. 1 A, Supplementary Table 1**) and by 23 (100%) and 22 (96%) participants, respectively, in stage B (**Supplementary Fig. 1 B-D, Supplementary Table 3)**, some of which were treated with antihistamines or topical steroids. Myalgia was the most common related systemic AE in 4 (20%) participants in stage A (**Supplementary Fig. 1 A, Supplementary Table 1**). Headache, reported twice (9%), was the most frequently related systemic AE in the immunization phase of stage B (**Supplementary Fig. 1 B-D, Supplementary Table 3)**. Unrelated AEs are summarized in **Supplementary Table 2** and **Supplementary Table 4** in the **Supplementary Appendix**.

In two cases, troponin T levels were elevated to 18 and 19 ng/mL (reference 14 ng/ml) for reasons unrelated to the trial. Antihistamine use led to elevated liver function tests in a third participant.

The clinical course following CHMI in stage B was consistent with prior CHMI trials at our centre[23] (**Supplementary Fig. 2, Supplementary Table 5 and Supplementary Table 6**).

Protective efficacy against CHMI

All participants who completed the immunization phase in stage B (9, 8 and 3 participants immunized with GA2-MB, GA1-MB and uninfected-MB, respectively) underwent CHMI with 5 unattenuated *Pf3D7*-infected MB, 3 weeks after the last immunization.

Immunization with GA2-MB resulted in 89% (95% CI: 31%-98%) protection, with all participants remaining qPCR negative until the end of the trial except one (1/9) who developed a positive *Pf* qPCR on day 11 post-CHMI, whereas 0/3 placebo recipients were protected with a median time to parasitemia of 12 days (IQR=3.0days) post CHMI (χ^2 *P*=0.005 and Fischer's exact *P*=0.02 compared to placebo, PP). In contrast, 1/8 GA1-MB was protected, with others developing parasitemia by a median of 11 days (IQR=2.5 days). In a post hoc comparison, protection against CHMI was significantly different between GA2-MB and GA1-MB groups (χ^2 *P*=0.002 and Fischer's exact *P*=0.003, log-rank *P*=0.002, PP) (**Fig. 2**).

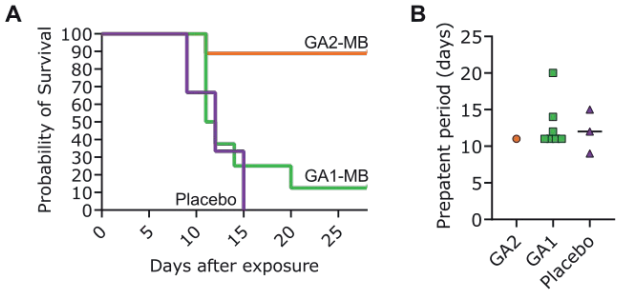

Figure 2. Parasitemia after controlled human malaria infection (CHMI)
A. Kaplan-Meier displaying the percentage of participants remaining free of blood-stage parasitemia over time after CHMI.
B. Prepatent period for participants who developed a positive *Pf* qPCR, measured in days post-CHMI. Lines indicate median.

Immunogenicity

In both GA2-MB and GA1-MB participants, we detected higher anti-*Pf*CSP antibodies on the day before challenge (C-1: 1.33 ± 0.35 µg/ml) compared to baseline (baseline: 0.07 ± 0.35 µg/ml) (paired t-test, p<0.001, CI: 1.07 to 1.44) and placebo (C-1: 0.01 ± 0.16 µg/ml) (independent samples t-test, p<0.001, CI: 0.89 to 1.77) (**Fig. 3 A**). However, titers did not differ between GA2-MB (1.2 ± 0.3 µg/ml) or GA1-MB (1.5 ± 0.4 µg/ml) immunized participants or protected (1.2 ± 0.3 µg/ml) and non-protected (1.4 ± 0.4 µg/ml) participants (**Fig. 3 D**). We did not detect changes in antibodies against apical membrane antigen 1 (*Pf*AMA1; baseline:

0.63 ± 0.52 µg/ml; C-1: 0.57 ± 0.52 µg/ml) (**Fig. 3 B, E**) or a fragment of the merozoite surface protein 1 (*Pf*MSP1; baseline: -0.27 ± 0.2 µg/ml; C-1: -0.23 ± 0.3 µg/ml) (**Fig. 3 C, F**).

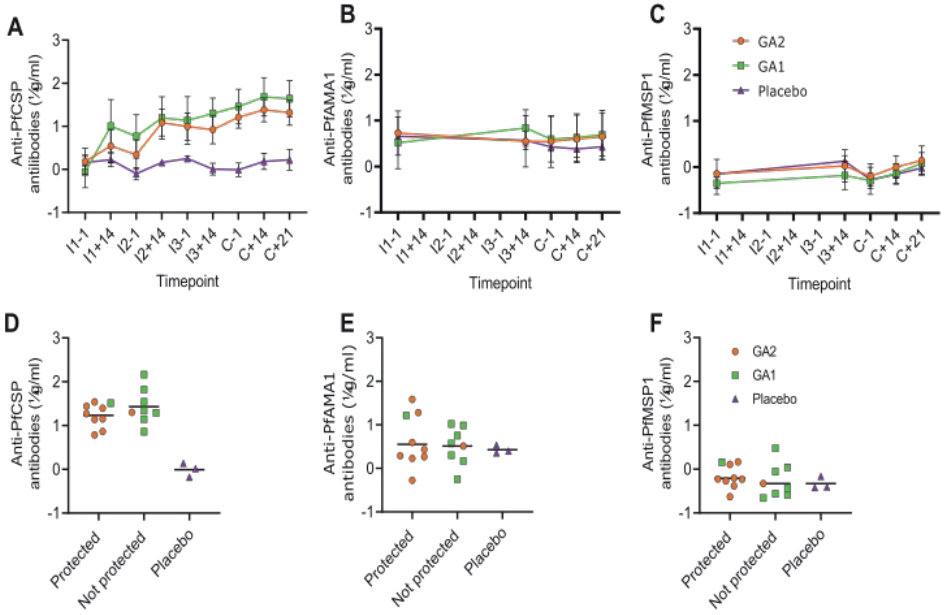

Figure 3. Humoral immune responses
A-C Plasma anti-*Pf*CSP, anti-*Pf*AMA1 and anti-*Pf*MSP1 antibodies measured at the indicated time points for the GA2-MB (orange circles), GA1-MB (green squares) and uninfected-MB placebo (purple triangles) groups. Symbols and error bars indicate mean and standard deviation. Days corresponding to first (I1), second (I2), third (I3) immunization and challenge (C) are indicated as timepoints on x-axes.
D-F Plasma anti-*Pf*CSP, anti-*Pf*AMA1 and anti-*Pf*MSP1 antibodies on the day before challenge for individual participants exposed to GA2-MB (orange circles), GA1-MB (green squares) and uninfected-MB placebo (purple triangles). Black lines indicate mean.
All values are log transformed.

To study *Pf*-specific cellular immunity, participants' PBMCs were stimulated with *Pf*-infected RBC (*Pf*RBC) and uninfected RBC (unRBC) and then assessed by flow cytometry (**Supplementary Fig. 3-5**). While the overall cellular frequency of all studied T cell lineages remained comparable, we observed an increase in γδ T cells expressing the Vδ2 chain in GA2-MB but not GA1-MB participants (**Supplementary Fig. 6 A-E**). From a functional perspective, immunization induced *Pf*-specific $CD4^+$ T cells with a strong type 1 inflammatory (IFN−γ, TNF−α and IL−2) but not type 2 or regulatory (IL−10 , IL−4, IL−5 and IL−13) signature (**Fig. 4 A**). However, we observed higher frequency of *Pf*-specific $CD4^+$ T cells in GA2-MB compared to GA1-MB participants. Similarly, *Pf*-specific $V\delta2^+$ γδ T cells expressing type 1 cytokines were induced only in GA2-MB but not GA1-MB participants (**Fig. 4 B**). *Pf*-specific responses were not detected in $CD8^+$, $V\delta2^-\gamma\delta$ and NK T cell compartments (**Supplementary Fig. 6 F-H**).

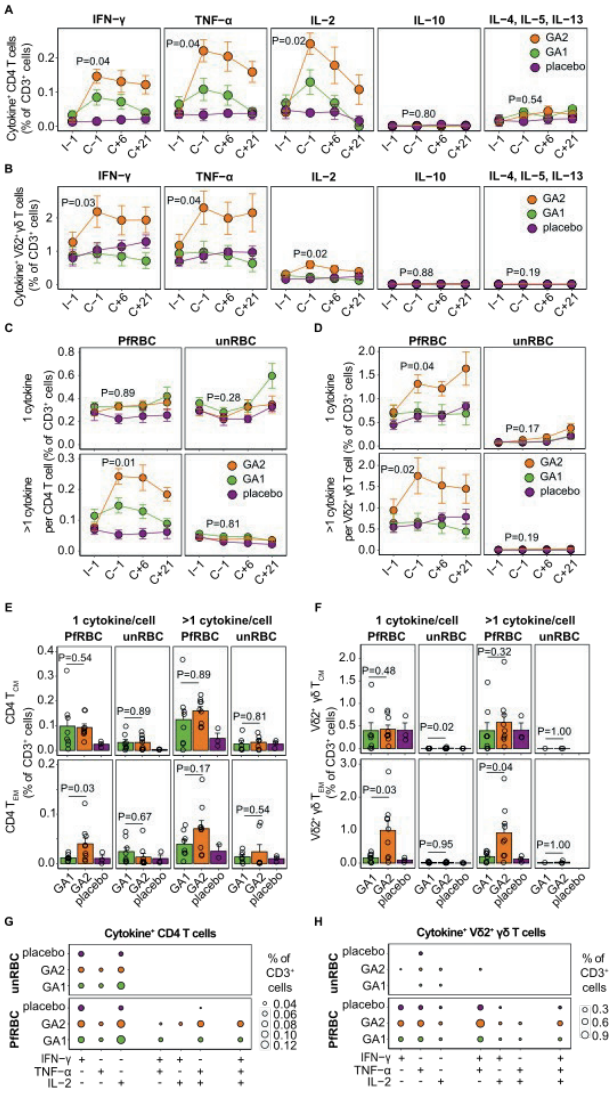

Figure 4. GA2-MB induces stronger pro-inflammatory CD4⁺ and Vδ2⁺ γδ T cell responses compared to GA1-MB.

A, B. Percentage of CD4⁺ (A) and Vδ2⁺ γδ (B) T cells secreting indicated cytokines at baseline (I-1), one day before challenge (C-1), 6 and 21 days post challenge (C+6 and C+21).

C, D. Percentage of CD4⁺ (C) and Vδ2⁺ γδ (D) T cells secreting either single (top) or more than one cytokine (bottom) upon stimulation with *Pf* infected (PfRBC; left) or uninfected RBC (unRBC; right) at the indicated time points.

E, F. Mean percentage of CD4⁺ (E) and Vδ2⁺ γδ (F) T cells secreting a single (left) or more than one cytokine (right) adopting a central (T_{CM}; top) or effector (T_{EM}; bottom) memory phenotype upon PfRBC or unRBC stimulation at C-1. Open circles indicate individual participants.

G, H. Mean percentage of CD4⁺ (G) and Vδ2⁺ γδ (H) T cells secreting cytokines in the indicated combination upon PfRBC (bottom) or unRBC (top) stimulation at C-1.

Data corresponding to GA1-MB, GA2-MB and uninfected-MB (placebo) immunization groups are indicated in green, orange and purple colour, respectively. A-D: Filled circle indicates arithmetic mean. A-F error bars indicate standard error mean. $p<0.05$ was considered significant, two tailed Mann-Whitney test.

Profiling the cytokine expression at a single T cell level showed that higher frequencies of polyfunctional $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells expressing more than one cytokine acquiring an effector memory phenotype were measured in GA2-MB compared to GA1-MB participants (**Fig. 4 C-F**). These polyfunctional T cells expressed combinations of IFN−γ, TNF−α and IL−2, with the preferential expression of TNF−α and IL−2 in $CD4^+$ T cells and IFN−γ and TNF−α in $V\delta2^+$ $\gamma\delta$ T cells (**Fig.4 G, H**).

Discussion

With this study we show evidence for higher cellular immunogenicity and protective efficacy of a late-arresting genetically attenuated parasite (GA2-MB) compared to an early-arresting one or (GA1-MB), indicating a potential role for late-liver-stage antigens in eliciting protective immunity to malaria. Protection did not associate with antibodies to *Pf*CSP, *Pf*AMA1 or *Pf*MSP1, but was related to the induction of *Pf*-specific polyfunctional $CD4^+$ T cells and $V\delta2^+$ $\gamma\delta$ T cells.

The protection induced by late-arresting GA2-MB in this model is higher than that seen in previous replication-incompetent, early-arresting attenuated sporozoites. Early-arresting genetically attenuated parasites, delivered by either intravenous injection or MB provided lower protective efficacy (12% by GA1 and 50% by PfGAP3KO[18,24]). Protection achieved by radiation attenuated sporozoites has varied between 50% to >90% when administered intravenously (dose range 1.35×10^5 to 2.7×10^6 PfSPZ Vaccine) in homologous CHMI[4,25-29]. However, administration of radiation attenuated sporozoites by MB requires higher doses (approximately 1000 MB) to reach similar levels of protection offered by GA2-MB[13]. In contrast, protection induced by GA2-MB in this study is reminiscent of parasites co-administered with chemoprophylaxis where 3 exposures to bites of 15 infected mosquitoes resulted in 100% efficacy against homologous CHMI,[15,30] thus further supporting the hypothesis that extended hepatic antigenic presentation may have a role for the induction of protective immunity.

To elucidate the correlates of protection, we measured both circulating antibodies and cellular responses. Consistent with previous reports[4], we found anti-*Pf*CSP antibodies induced in response to both GA2-MB and GA1-MB. This suggests that the differential arrest in the liver did not impact anti-sporozoite antibody responses quantitatively. Lack of plasma antibodies against late-liver-stage antigens, *Pf*AMA1 and *Pf*MSP1, indicates that their exposure is insufficient to mount a measurable antibody response.

Unlike antibody responses, *Pf*-specific cellular immune responses differed between GA2-MB and GA1-MB-immunized participants. While we observed a strong pro-inflammatory

CD4+ T cell response in both groups, induction of *Pf*-specific polyfunctional effector memory CD4+ T cells was more robust in GA2-MB compared to GA1-MB. GA2-MB also more efficiently induced mono- and poly-functional Vδ2+ γδ T cells with a strong pro-inflammatory signature. These findings are in line with previous attenuated sporozoite studies reporting Vδ2+ γδ T cells as a marker of protective immunity[16,26].

The induction of γδ T cells is observed not only after immunization with parasites co-administered with chemoprophylaxis, but also after high dose immunization with radiation attenuated sporozoites where the duration of liver-stage antigen exposure is limited[26,31]. However, a recent study reported that Vδ2+ γδ T cell induction was dose-dependent in an early arresting chemo-attenuation strategy[9]. In contrast, late chemo-attenuation induced Vδ2+ γδ T cells irrespective of the dose. This suggests an important independent role of late-liver-stage antigens in inducing γδ T cell responses[9].

Studies with attenuated sporozoites in mice and non-human primates have indicated a central role for effector memory-like liver resident CD8+ T cells[32,33]. However, detection of *Pf*-specifc CD8+ T cells in peripheral blood of participants is generally limited[18,25,34-36], although CD8+ T cells can be present at up to 100 times higher frequencies in the liver than in the blood[26,35]. Hence, it remains unclear whether there is any limitation in induction or recirculation of tissue resident *Pf*-specific CD8+ T cells in humans immunized with attenuated sporozoites. In line with these studies, we did not detect *Pf*-specific CD8+ T cells in peripheral blood in any of the immunized participants using general flow cytometric tools.

Safety and lack of blood-stage breakthroughs is an important feature for the further development of genetically attenuated vaccines for malaria. Because *mei2* knockout resulted in blood-stage breakthrough infections in rodent models (*P. yoellii* and *P. berghei*), we did a dose-escalation approach in case of functional redundancy of the MEI2 protein[21,37]. However, preclinical studies showed that *mei2* knockout in *Pf* did not result in breakthrough blood infections as tested in the humanized mouse model[21]. Our results support this attenuation phenotype in *Pf*, with no evidence of blood-stage parasites after (multiple) 50 MB doses in 25 participants.

Nonetheless, the conclusions from this study are limited by the small sample size. More studies with greater numbers of participants are required to better understand the safety profile of GA2. In addition, the immunological assessments were exploratory and the relevance of the parameters associated with GA2-induced protection needs confirmation. To translate the promising efficacy results to the broader population affected by malaria (**Supplementary Table 7**), the immunogenicity of GA2 needs to be assessed for durability

and against heterologous *Pf* strains in malaria endemic regions[7]. Previous studies showed that co-administration of parasites with chemoprophylaxis can induce durable protection[38] and protection against heterologous challenge[9]. Replicating such findings in malaria-endemic areas is critical to determine the potential for late-arresting attenuated sporozoites. If high levels of protection can be sustained in such settings, satisfying the recent WHO preferred product characteristics[39], late-arresting whole sporozoites will be considered as a next generation vaccine, complementing the two currently available subunit vaccines[2,3].

In summary, our findings suggest that parasites arresting late during the liver-stage (GA2) offer protection compared to early-arresting sporozoites (GA1) and provide a step towards a next generation malaria vaccine with potentially higher protective efficacy possibly from cellular immune mechanisms.

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Supplementary appendix

Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

1. Subject is aged ≥ 18 and ≤ 35 years and in good health.
2. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Subject is able to communicate well with the investigator, is available to attend all study visits.
4. Furthermore, the subject will remain within the Netherlands from day -1 till day +35 after each parasite exposure. After exposure to parasites, subjects have to be reachable by phone (24/7) from day -1 until day 35.
5. Subject agrees that his/her general practitioner (GP) will be informed about participation in the study.
6. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to Sanquin guidelines (3 years minimum, depending on serology).
7. Non-pregnant, non-lactating, fertile (i.e., have a uterus and are neither surgically sterilized nor post-menopausal) female subjects agree to use adequate contraception and to not breastfeed for the duration of study.
8. Subject agrees to refrain from intensive physical exercise (disproportionate to the subjects' usual daily activity or exercise routine) for twenty-one days following each immunization and during the malaria challenge period.
9. Subject signs informed consent.

Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immune-deficient, psychiatric or other disorders, which could compromise the health of the subject during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:

a. Body weight <50 kg or Body Mass Index (BMI) <18.0 or >30.0 kg/m^2 at screening.
b. A heightened risk of cardiovascular disease, defined as:
    i. An estimated 10-year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE).
    ii. History, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or
    iii. A positive family history of cardiac events in first- or second-degree relatives (according to the system used in medical genetics) <50 years old.
c. Functional asplenia, sickle cell trait/disease, thalassemia trait/disease or G6PD deficiency.
d. History of epilepsy in the period of 5 years prior to study onset, even if no longer on medication.
e. Positive HIV, HBV or HCV screening tests.
f. Chronic use of i) immunosuppressive drugs, ii) antibiotics, iii) or other drugs that might have an influence on the immune system (excluding inhaled and topical corticosteroids and incidental use of oral anti-histamines), within 3 months prior to study onset or expected use of such during the study period.
g. History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years.
h. Any history of treatment for severe psychiatric disease by a psychiatrist in the past year.
i. History of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset, positive urine toxicology test for cocaine, amphetamines or cannabis at screening.

2. For female subjects: breastfeeding, or positive urine pregnancy test at screening or prior to immunization or prior to controlled human malaria infection (CHMI).
3. Any history of malaria, positive serology for *Pf,* or previous participation in any malaria (vaccine) study or CHMI.
4. Known hypersensitivity to or contra-indications (including co-medication) for use of atovaquone/proguanil or artemether/lumefantrine, or history of severe (allergic) reactions to mosquito bites (MB).

5. Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 8 weeks thereafter. Exceptions are made for influenza vaccination and for vaccination against the coronavirus SARS-CoV2 or any other vaccination which cannot be reasonably withheld or postponed.
6. Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.
7. Being an employee or student of the department of Parasitology, Medical Microbiology or Infectious Diseases of the LUMC or RUMC.
8. Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol or would compromise the integrity of the data.

Materials and methods

Trial procedures

Parasites and mosquito rearing procedures

The generation and characterization of GA2 and GA1 from the *Plasmodium falciparum (Pf)* NF54 strain has been previously described[1-3]. *Pf* parasites were cultured in standard conditions using a static culture system and subsequently fed to female *Anopheles stephensi* mosquitoes by standard membrane feeding[4]. *An. stephensi* mosquitoes were reared according to standard procedures at the Radboudumc instectiary[5,6].

Exposure of participants to infected mosquitoes

Exposure of participants to infected MB was carried out according to previously described procedures[7]. In short, mosquitoes were inspected after the blood meal to confirm feeding. If necessary, participants were exposed to additional mosquitoes until the predefined number of MB was reached. For practical reasons, a margin of error of 20% was accepted for the immunizations, hence 12-18 MB were accepted for the targeted 15 MB dose, whereas 40-60 MB were accepted for the targeted 50 MB dose. For CHMI, participants were exposed to exactly 5 *Pf*-infected MB. All procedures were supervised by an experienced technician and a study physician. After exposure, participants were observed for at least 15 minutes.

Schedule of immunization and follow-up

Stage A

In stage A of the trial, participants visited the trial centre daily, from days 6 to day 21 after exposure. On day 28 after exposure, all participants received rescue treatment with a standard regimen of atovaquone/proguanil. Complete blood counts were done at inclusion, on days 6, 14, 21, 28, 31 and 35 after exposure to GA2-MB, whereas biochemistry tests were done on days 6, 14, 28, 31 and 35 after exposure to GA2-MB. Platelet counts, lactate dehydrogenase and highly sensitive troponin T were assessed at each ambulatory visit, except for days 100 and 188. Blood samples for immunological analysis were taken at baseline, on days 6, 14, 21, 100 and 188.

Stage B

In stage B of the trial, visits were planned on day 14 after every immunization and on the day prior to each immunization. Phone calls were scheduled on days 7 and 21 after each immunization. After the first immunization, 3 additional ambulatory visits were planned on days 7, 9 and 11. Three weeks after the last immunization, participants underwent CHMI with 5 unattenuated *Pf*3D7-MB. After CHMI, participants visited the trial centre daily from days 6 to 21 post CHMI. Participants received rescue treatment with atovaquone/proguanil as soon as they developed parasitemia or, at the latest, at day 28 post CHMI.

During the immunization period, complete blood counts and biochemistry tests were done at inclusion, on day 14 after each immunization and on the day prior to each subsequent immunization and prior to CHMI. After CHMI, complete blood counts were performed on days 6, 14, 21, 28 and 31 if the participant had not yet received rescue treatment and always on day 35, whereas biochemistry tests were performed on days 6, 14, 28 and 31 if the participant had not yet received rescue treatment and always on day 35.

Platelet counts, lactate dehydrogenase and highly sensitive troponin T were assessed at every ambulatory visit, except for days 100 and 188 post CHMI. Blood samples for immunological analysis were taken at baseline, on day 14 after each immunization, on the day prior to each new immunization, prior to CHMI and after CHMI on days 6, 14, 21, 35, 100 and 188 (**Fig. 1**).

Blood stage parasitemia

Blood stage parasitemia was assessed by qPCR, which was carried out according to previously described methods[8]. Samples were considered positive if >100 *Pf*/ml.

In stage A qPCR analysis was performed at inclusion, daily on days 6 to 21, 28, 31 and on day 35. whereas in stage B qPCR was performed at inclusion, prior to each new immunization and prior to challenge and on days 7, 9, 11, 14 after the first immunization and on day 14 after the second and third immunization. After CHMI, qPCR was carried out daily from days 6 to 21 and on days 28, 31 and 35. If participants developed parasitaemia before rescue treatment, qPCR was also repeated on the afternoon of qPCR positivity and on the third day after treatment initiation (**Fig.1**).

Adverse events

Solicited and unsolicited adverse events (AEs) were recorded at every visit until day 35 after exposure to GA2-MB in stage A and until day 35 after CHMI in stage B of the trial. Solicited local AEs were: bruising, erythema, induration, pain, pruritus, swelling and tenderness. Solicited systemic AEs were: arthralgia, chills, oedema, fatigue, fever, headache, malaise, pruritus, rash and urticaria. Participants were instructed to fill in a diary with daily temperature and AEs. AEs were graded according to the following scale:

- Mild (grade 1): awareness of symptoms that are easily tolerated and do not interfere with usual daily activity;
- Moderate (grade 2): discomfort that interferes with or limits usual daily activity;
- Severe (grade 3): disabling, with subsequent inability to perform usual daily activity, resulting in absence or required bed rest.

For additional information regarding AE toxicity please refer to the study protocol. Causality of the AEs was assessed by the investigator and classified as definitively, probably, possibly, unlikely or not related. In a dichotomous analysis, the first 3 categories were classified as related, the latter 2 as unrelated. AEs were considered unrelated if there was a more plausible explanation or if timing made the relatedness unlikely.

ELISA to measure antibody responses

Plasma samples taken at the indicated time points were assessed for IgG antibodies targeting the circumposporozoite protein (*Pf*CSP), apical membrane antigen 1 (*Pf*AMA1) and merozoite surface protein 1 (*Pf*MSP1) by enzyme-linked immunosorbent assay (ELISA). Full length CSP was recombinantly expressed in *Escherichia coli* at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. Full length recombinant AMA1 and a fragment of MSP-1, both expressed in *Pichia pastoris* were kindly offered by Dr. B. Faber and Dr. C. Kocken (Biomedical Primate Research Center,Rijswijk, The Netherlands). 96-well Microplates (Corning® Half Area Clear Flat Bottom Polystyrene

High Bind) were coated overnight at 4 °C with 1 µg/ml of antigen at 25 µl/well in 0.1 M sodium carbonate buffer (pH 9.6). Plates were washed 3 times with washing buffer (0.05% Tween in phosphate buffered saline (PBS)) and blocked with 50 µl 5% skim milk in PBS for 2 h at 37 °C. Plasma samples were serially diluted in 0.5% skim milk with the initial dilution of 1:500 for 8 steps each by 1:2.5. After washing the plates, diluted plasma samples were added at 25 µl/well and incubated at room temperature for 2h. Plates were washed 5 times and incubated with 25 µl/well of goat-anti-human immunoglobulin G (IgG) conjugated with horseradish peroxidase at a concentration of 0.2 µg/ml (in 0,5% skim milk in PBS and 0.05% EDTA) for 1 hour at room temperature. After 6 washes, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added. Colour development was blocked with 10% sulfuric acid after 8 minutes. Plates were read with the Multiskan EX reader at 450 nm. Measurements were normalized to a standard curve, consisting of polyclonal IgGs with a known concentration, purified from human plasma (Sigma-Aldrich/ Merck). Measurements were repeated in at least 2 independent experiments with overall coefficient of variance below 30%.

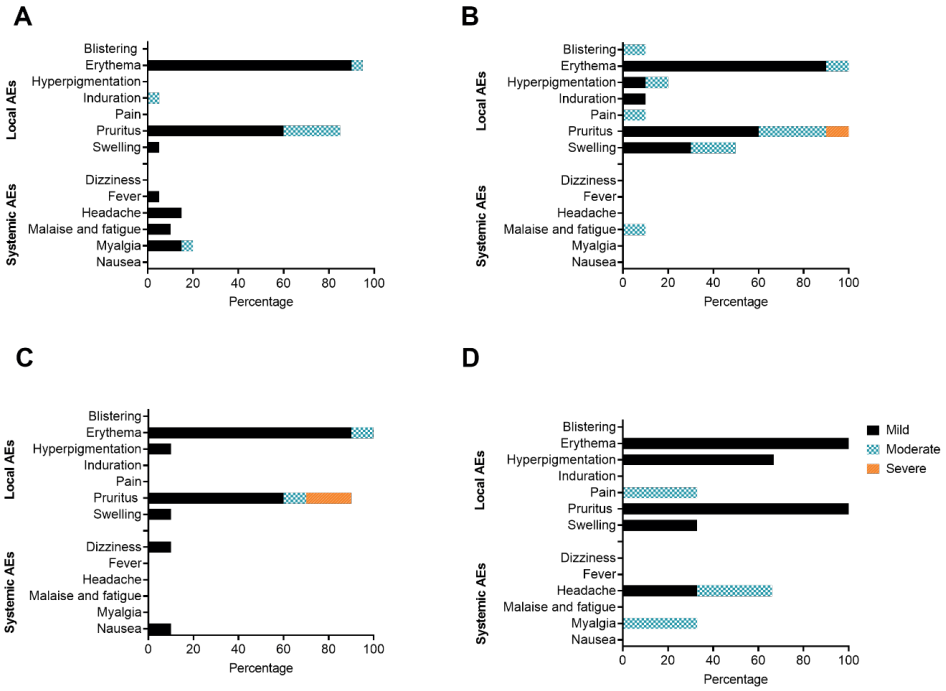
Peripheral blood mononuclear cells (PBMCs) restimulation and flow cytometry to measure T cell responses

Pf stimulation and flow cytometry analyses were performed as described before[2]. In brief, frozen PBMCs were thawed incubated overnight at 37°C and 5% CO_2 in complete Roswell Park Memorial Institute (RPM) medium (cRMPI consisting of RPMI 1640 (Invitrogen), 100 U/ml Penicillin (Euroco-Pharma), 100 µg/ml Streptomycin (Sigma), 1 mM Pyruvate (Sigma) and 2 mM L-Glutamate (Sigma), supplemented with 20% heat-inactivated fetal calf serum (FCS) (Bodinco). After incubation, cells were resuspended in cRPMI for 4 h, 10 µg/ml Brefeldin A (Sigma) was added to the culture. PBMCs from 1 malaria-naïve donor (Sanquin, the Netherlands) and a donor previously infected with malaria were used as negative and positive controls, respectively.

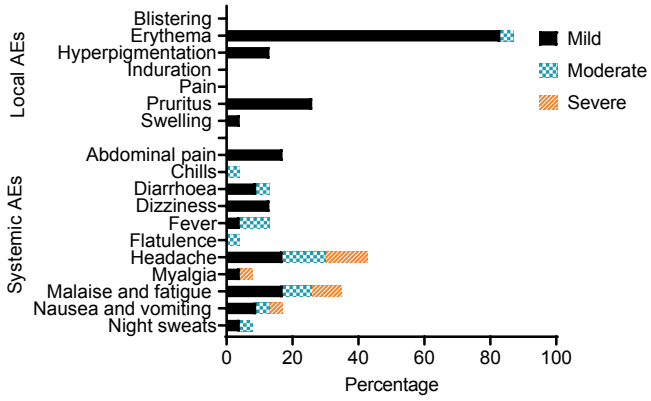
After stimulation for 24 h, the cells were transferred into a V bottom plate and all the flow cytometry staining steps were performed on ice using the panel described in **Supplementary Table 8**. Cells were treated with Fc block and surface-stained for the pan-γδ T cell receptor for 20 min in the dark and followed by a 20-min surface-staining for the markers CD56, γδ-Vδ2 TCR, CCR7, CD3, CD4, CD25 and CD11c. Aqua Live/ Dead dye (Invitrogen) was used as per the manufacturer's instructions to stain the dead cells. Upon washing, fixing, and permeabilizing using the Intracellular Fixation & Permeabilization Buffer Set (Invitrogen), cells were stained intracellularly for the markers CD45RA, IFN-γ, TNF-α, IL-10, IL-2, IL-4, IL-5, IL-13, FoxP3, and CD8 for 30

min. The cells were resuspended in fluorescence activated cell sorting (FACS) buffer (PBS (Invitrogen), 0.5% BSA, 2mM EDTA) and acquired on the 3-laser spectral analyzer Aurora (configuration 16V-14B-8R) and the data were analyzed using FlowJo 10.8.2 as described in **Supplementary Fig. 3**. Fluorescence Minus Many were used to define gates for cytokine positive cells. *Pf*-specific cytokine positive frequencies in *Pf*-infected red blood cells (PfRBC) stimulated samples are reported upon subtraction of the frequencies from the same gate on the same sample stimulated with uninfected red blood cells (unRBC).

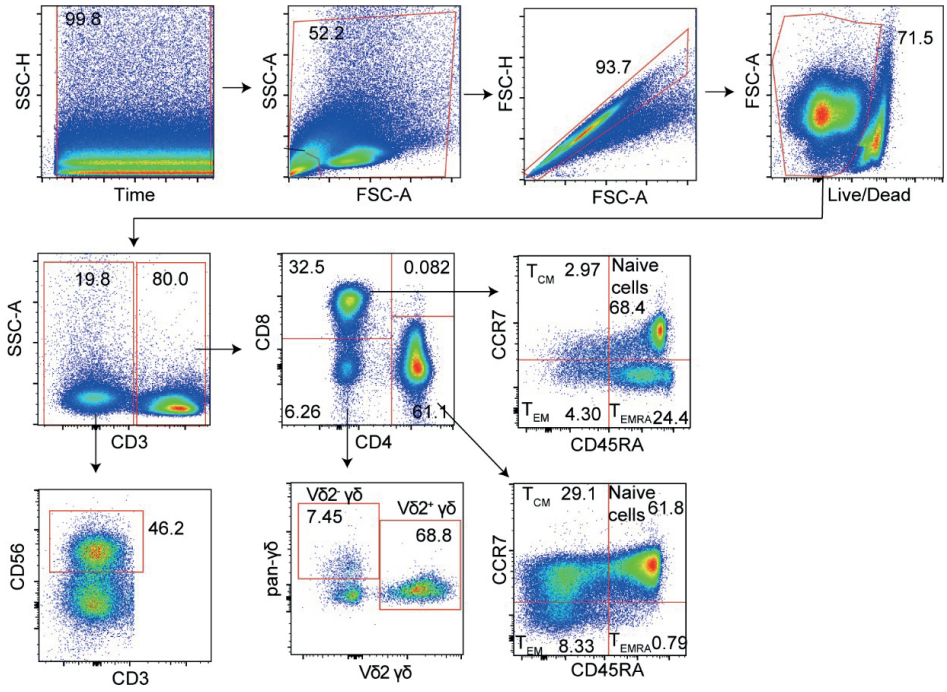
Supplementary figures



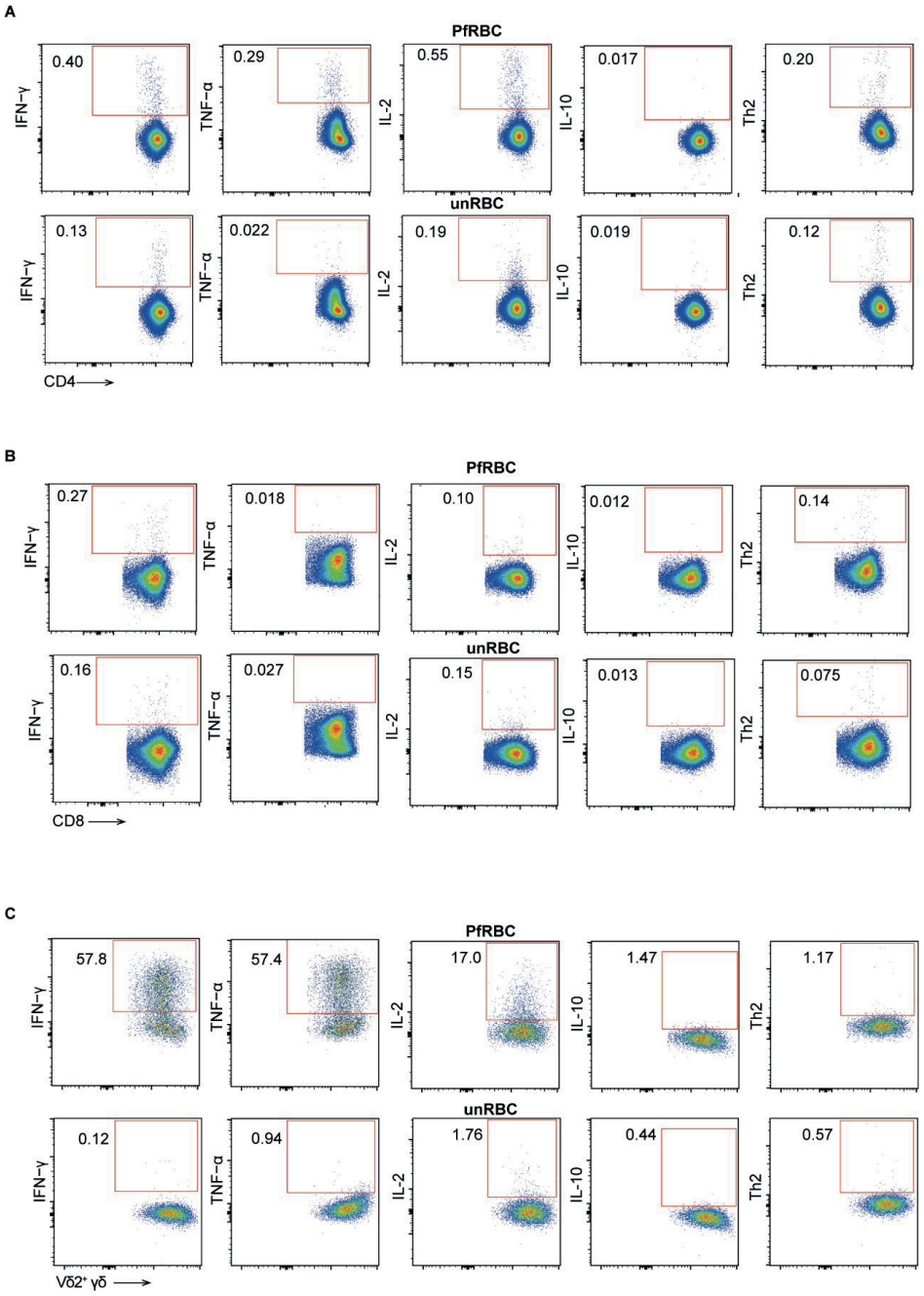
Supplementary Figure 1. Adverse events (AEs) reported during stage A and the immunization phase of stage B of the clinical trial
Stacked bars showing percentage of participants reporting any AEs related to GA2-MB in stage A (**A**) and GA2-MB, GA1-MB and uninfected-MB (placebo) groups in stage B (**B-D**), of mild (black), moderate (blue) and severe (orange) intensity.



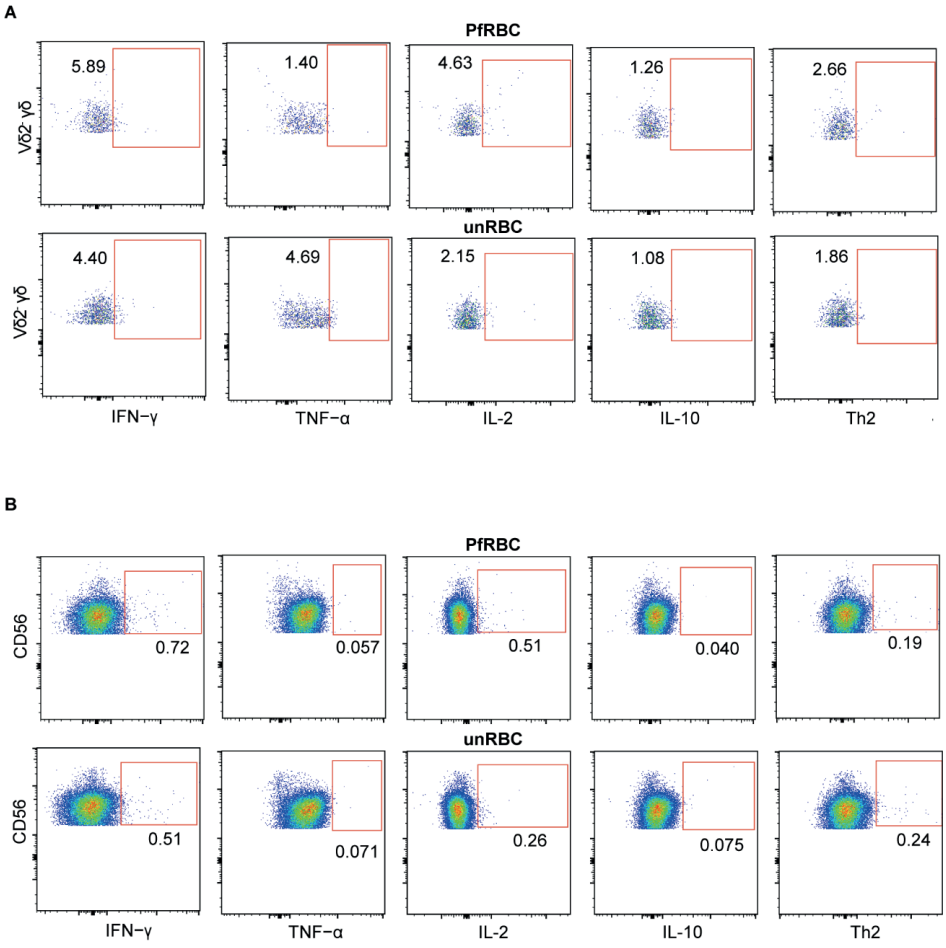
Supplementary Figure 2. Adverse events (AEs) reported after controlled human malaria infection (CHMI) in stage B of the clinical trial
Stacked bars showing percentage of participants reporting any AEs related to CHMI in stage B, of mild (black), moderate (blue) and severe (orange) intensity.



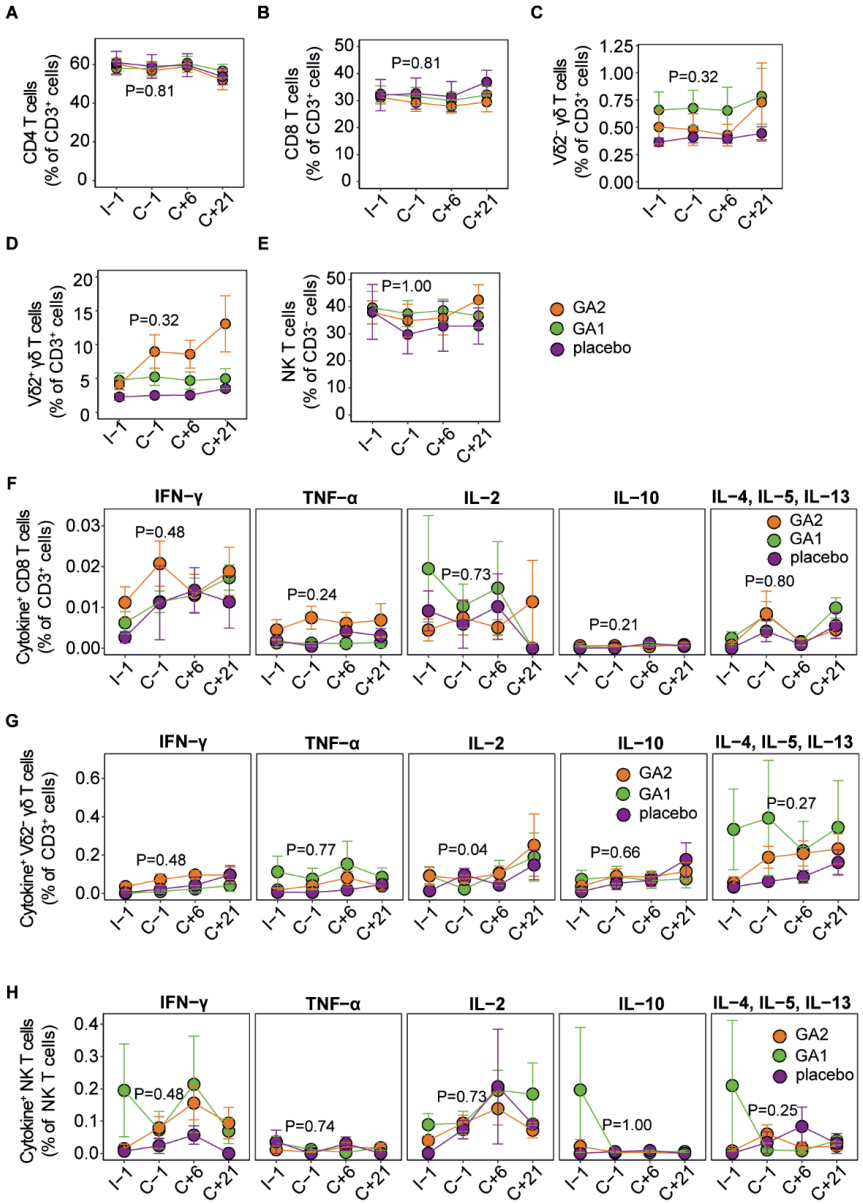
Supplementary Figure 3. Representative gating panels indicating identification of target T cell populations



Supplementary Figure 4. Cytokine expression in CD4⁺, CD8⁺ and Vδ2⁺ γδ T cell populations A-C. PBMCs stimulated either with PfRBC (top) or uninfected RBC (bottom) measured for the indicated cytokine expression in CD4⁺ (A), CD8⁺ (B) and Vδ2⁺ γδ (C) T cell populations.



Supplementary Figure 5. Cytokine expression in Vδ2$^-$ γδ and NK T cell populations
A,B. PBMCs stimulated either with PfRBC (top) or uninfected RBC (bottom) measured for the indicated cytokine expression in Vδ2$^-$ γδ (A) and NK (B) T cell populations.



Supplementary Figure 6. T cell analysis

A-E. Percentage of CD4+ (A), CD8+ (B), Vδ2– γδ (C), Vδ2+ γδ (D) and NK T cells at baseline (I-1), one day before challenge (C-1), 6 and 21 days post challenge (C+6 and C+21) upon stimulation with medium control.

F-H. Percentage of CD8+ (F) and Vδ2– γδ (G) and NK (H) T cells secreting indicated cytokines at baseline (I-1), one day before challenge (C-1), 6 and 21 days post challenge (C+6 and C+21).

Data corresponding to GA1-MB, GA2-MB and uninfected-MB (placebo) immunization groups are indicated in green, orange and purple colour, respectively.

A-H: Filled circle indicates arithmetic mean and error bars indicate standard error mean. $P < 0.05$ is considered significant, two tailed Mann-Whitney test.

Supplementary tables

Supplementary Table 1. Number of participants with related adverse events (AEs) reported during stage A of the clinical trial (percentage in brackets, N=20)

Adverse events	Mild	Moderate	Severe	Total
Local				
Erythema	18 (90%)	1 (5%)		19 (95%)
Induration		1 (5%)		1 (5%)
Pruritus	12 (60%)	5 (25%)		17 (85%)
Swelling	1 (5%)			1 (5%)
Systemic				
Fever	1 (5%)			1 (5%)
Headache	3 (15%)			3 (15%)
Malaise and fatigue	2 (10%)			2 (10%)
Myalgia	3 (15%)	1 (5%)		4 (20%)

Supplementary Table 2. Number of participants with unrelated adverse events (AEs) reported during stage A of the clinical trial (percentage in brackets, N=20)

Adverse events	Mild	Moderate	Severe	Total
Abdominal pain	6 (30%)	2 (10%)		8 (40%)
Acute kidney abnormality	1 (5%)			1 (5%)
Acute pharyngitis	7 (35%)			7 (35%)
Gastroenteritis	2 (10%)			2 (10%)
Change in bowel habit	1 (5%)			1 (5%)
Chills	1 (5%)		1 (5%)	2 (10%)
Cough	1 (5%)			1 (5%)
Diarrhea	3 (15%)	1 (5%)		4 (20%)
Dizziness	1 (5%)			1 (5%)
Epistaxis	1 (5%)			1 (5%)
Fever	1 (5%)			1 (5%)
Headache	3 (15%)	5 (25%)	1 (5%)	9 (45%)
Hematoma	3 (15%)			3 (15%)
Limb pain	1 (5%)			1 (5%)
Low back pain	1 (5%)			1 (5%)
Lymph node swelling	1 (5%)			1 (5%)
Malaise and fatigue	8 (40%)	2 (10%)		10 (50%)
Myalgia	2 (10%)	1 (5%)		3 (15%)
Nausea and vomiting	9 (45%)	2 (10%)	1 (5%)	12 (60%)
Night sweats	1 (5%)			1 (5%)
Otitis media	1 (5%)			1 (5%)
Restlessness	1 (5%)			1 (5%)

Supplementary Table 3. Number of participants with related adverse events (AEs) reported during the immunization phase of stage B of the clinical trial (percentage in brackets)

GA2 (immunization) (N=10)				
Adverse event	**Mild**	**Moderate**	**Severe**	**Total**
Local				
Blistering		1 (10%)		1 (10%)
Erythema	9 (90%)	1 (10%)		10 (100%)
Hyperpigmentation	1 (10%)	1 (10%)		2 (20%)
Induration	1 (10%)			1 (10%)
Pain		1 (10%)		1 (10%)
Pruritus	6 (60%)	3 (30%)	1 (10%)	10 (100%)
Swelling	3 (30%)	2 (20%)		5 (50%)
Systemic				
Malaise and fatigue		1 (10%)		1 (10%)
GA1 (immunization) (N=10)				
Adverse event	**Mild**	**Moderate**	**Severe**	**Total**
Local				
Erythema	9 (90%)	1 (10%)		10 (100%)
Hyperpigmentation	1 (10%)			1 (10%)
Pruritus	6 (60%)	1 (10%)	2 (20%)	9 (90%)
Swelling	1 (10%)			1 (10%)
Systemic				
Dizziness and giddiness	1 (10%)			1 (10%)
Nausea and vomiting	1 (10%)			1 (10%)
Placebo (immunization) (N=3)				
Adverse event	**Mild**	**Moderate**	**Severe**	**Total**
Local				
Erythema	3 (100%)			3 (100%)
Hyperpigmentation	2 (66%)			2 (66%)
Swelling	1 (33%)			1 (33%)
Pain		1 (33%)		1 (33%)
Pruritus	3 (100%)			3 (100%)
Systemic				
Headache	1 (33%)	1 (33%)		2 (66%)
Myalgia		1 (33%)		

Supplementary Table 4. Number of participants with unrelated adverse events (AEs) reported during the immunization phase of stage B of the clinical trial (percentage in brackets)

GA2 (N=10)				
Adverse events	**Mild**	**Moderate**	**Severe**	**Total**
Acute pharyngitis	5 (50%)			5 (50%)
COVID-19	3 (30%)	1 (10%)		4 (40%)
Diarrhea	1 (10%)		1 (10%)	2 (20%)
Dysmenorrhea	1 (10%)			1 (10%)
Elevated troponin	2 (20%)			2 (20%)
Epistaxis	1 (10%)			1 (10%)
Headache	1 (10%)	1 (10%)		2 (20%)
Influenza		1 (10%)		1 (10%)
Insomnia	1 (10%)			1 (10%)
Irritability and anger	1 (10%)			1 (10%)
Malaise and fatigue	1 (10%)	1 (10%)		2 (20%)
Myalgia	1 (10%)			1 (10%)
Nausea and vomiting	1 (10%)		1 (10%)	2 (20%)
Palpitations	1 (10%)			1 (10%)
Scabies		1 (10%)		1 (10%)
Unspecified feeling of malaise		1 (10%)		1 (10%)
Urinary tract infection	1 (10%)			1 (10%)
Urticaria			1 (10%)	1 (10%)
GA1 (immunization) (N=10)				
Adverse events	**Mild**	**Moderate**	**Severe**	**Total**
Abdominal pain		1 (10%)		1 (10%)
Acute pharyngitis	2 (20%)	2 (20%)		4 (40%)
Allergic rhinitis	1 (10%)	1 (10%)		2 (20%)
Chest pain	1 (10%)			1 (10%)
COVID19	severity not reported			1 (10%)
COVID19	2 (20%)			2 (20%)
Diarrhea			2 (20%)	2 (20%)
Enuresis	1 (10%)			1 (10%)
Epistaxis	1 (10%)			1 (10%)
Fecal abnormality	severity not reported			1 (10%)
Foot fracture		1 (10%)		1 (10%)
Headache	3 (30%)	2 (20%)	1 (10%)	6 (60%)
Malaise and fatigue	severity not reported			1 (10%)
Placebo (immunization) N=3				
Adverse events	**Mild**	**Moderate**	**Severe**	**Total**
Acute pharyngitis	2 (66%)			2 (66%)
Chills	1 (33%)			1 (33%)
COVID19	2 (66%)			2 (66%)
Dysmenorrhea	1 (33%)			1 (33%)
Headache	1 (33%)			1 (33%)
Headache	2 (66%)			2 (66%)
Influenza	1 (33%)			1 (33%)
Nausea and vomiting	1 (33%)			1 (33%)

Supplementary Table 5. Number of participants with related adverse events (AEs) reported after controlled human malaria infection (CHMI) in stage B of the clinical trial (percentage in brackets, N=23)

Adverse events	Mild	Moderate	Severe	Total
Local				
Erythema	19 (83%)	1 (4%)		20 (87%)
Hyperpigmentation	3 (13%)			3 (13%)
Pruritus	6 (26%)			6 (26%)
Swelling	1 (4%)			1 (4%)
Systemic				
Abdominal pain	4 (17%)			4 (17%)
Chills		1 (4%)		1 (4%)
Diarrhoea	2 (9%)	1 (4%)		3 (13%)
Dizziness	3 (13%)			3 (13%)
Fever	1 (4%)	2 (9%)		3 (13%)
Flatulence		1 (4%)		1 (4%)
Headache	4 (17%)	3 (13%)	3 (13%)	10 (43%)
Malaise and fatigue	4 (17%)	2 (9%)	2 (9%)	8 (35%)
Myalgia	1 (4%)		1 (4%)	2 (9%)
Nausea and vomiting	2 (9%)	1 (4%)	1 (4%)	4 (17%)
Night sweats	1 (4%)	1 (4%)		2 (9%)

Supplementary Table 6. Number of participants with unrelated adverse events (AEs) reported after controlled human malaria infection (CHMI) in stage B of the clinical trial (percentage in brackets, N=23)

Adverse events	Mild	Moderate	Severe	Total
Abdominal pain			1 (4%)	1 (4%)
Abnormal findings of blood chemistry	1 (4%)			1 (4%)
Acute pharyngitis	7 (30%)	3 (15%)		10 (43%)
Allergic rhinitis	4 (17%)	1 (4%)		5 (22%)
Anorexia	1 (4%)			1 (4%)
Diarrhea	1 (4%)		1 (4%)	2 (9%)
Dysmenorrhea	2 (9%)			2 (9%)
Dyspnea	1 (4%)			1 (4%)
Headache	3 (13%)	1 (4%)		4 (4%)
Malaise and fatigue		1 (4%)		1 (4%)
Migraine			1 (4%)	1 (4%)
Myalgia	2 (9%)			2 (9%)
Nausea and vomiting	2 (9%)	1 (4%)	1 (4%)	4 (17%)
Night sweats		1 (4%)		1 (4%)
Oral aphthae		1 (4%)		1 (4%)
Otalgia		1 (4%)		1 (4%)

Supplementary Table 7. Representativeness of study participants

Category	Details
Disease under investigation	*Plasmodium falciparum*, agent of malaria.
Special considerations related to:	
Sex and gender	Malaria affects both men and women. However, women may be more vulnerable to the effects of the disease, especially during pregnancy[9,10].
Age	Approximately 78% of malaria-related casualties globally are children under the age of 5[11].
Race or ethnic group	While malaria vulnerability does not depend on race or ethnicity, the disease is more prevalent in Africa and therefore Africans are disproportionally affected[11].
Geography	Africa has the highest incidence of *Plasmodium falciparum* malaria: in 2022 Africa accounted for 94% of morbidity and 95% of mortality worldwide. The 4 most affected African countries are Nigeria, the Democratic Republic of Congo, Uganda and Mozambique[11].
Other considerations	About 10.000 travelers acquire malaria each year, although this number may in reality be higher[12].
Overall representativeness of this trial	Participants of this clinical trial were malaria-naive healthy individuals residing in The Netherlands, aged 18 to 35. Gender and age were self-reported by the participants during the screening process. The male to female ratio was 49% to 51%. While this population differs from that most affected by malaria, this study was meant as a proof of concept to assess the safety and preliminary protective efficacy of a new late-arresting GAP, relative to that of an early-arresting GAP. Further studies will be required to confirm the efficacy of GA2 against naturally-acquired malaria in younger, pre-exposed populations.

Supplementary Table 8. Staining antibody panel used in multi-parameter spectro-flow cytometry.

Marker	Fluorophore	Dilution	Manufacturer	Cat. Number	Clone
TCR γδ	PerCP-Cy5.5	1:50	Biolegend	331224	B1
CD56	BV786	1:200	BD	744222	R19-760
TCR γδ Vδ2	BV605	1:200	BD	743751	B6
CCR7	AF700	1:100	Biolegend	353244	G043H7
CD3	APC-ef780	1:800	Invitrogen	47-0038-42	UCHT1
CD4	PE-Cy7	1:800	BD	557852	SK3
CD25	PE-fire 640	1:200	Biolegend	356148	M-A251
CD11c	AF532	1:100	Invitrogen	58011641	ITGAX
CD45RA	BV480	1:100	BD	746799	5H9
INF-γ	BV421	1:500	Biolegend	502532	4S.B3
TNF-α	BV650	1:500	Biolegend	502938	MAB11
IL-10	BV711	1:125	BD	564050	JES-9D7
IL-2	APC	1:100	Biolegend	500310	MQ1-17H12
IL-4	PE	1:20	BD	340451	3.010.211
IL-5	PE	1:250	Biolegend	504304	TRFK5
IL-13	PE	1:100	Biolegend	501903	JES10-5A2
FoxP3	PE-CF594	1:50	BD	562421	259D/C7
CD8	FITC	1:400	BD	555366	RPA-T8

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Chapter 3

The path from early- to late-liver stage arresting genetically attenuated parasites as a malaria vaccination strategy

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Published: NPJ Vaccines, 2025
DOI: 10.1038/s41541-025-01265-z

Abstract

Genetically attenuated parasites (GAPs) that arrest during liver stage development have shown significant potential as malaria vaccines. Compared to *Plasmodium falciparum* GAPs that arrest after 24-48 hours (early-arresters), parasites arresting after 6-7 days (late-arresters) have shown superior efficacy, highlighting the importance of liver stage immunity in promoting sterile protection. Here, we describe GAPs tested in humans and the pre-clinical research that led to their creation. We discuss safety and efficacy of existing GAPs with particular focus on their large-scale implementation as malaria vaccines.

Introduction

Decades of malaria research have led to WHO recommendations for two malaria vaccines, RTS,S and R21, both based on the circumsporozoite protein (CSP), the most abundant protein on the surface of *Plasmodium* sporozoites[1-3]. The mechanism of action of these subunit vaccines is thought to be solely mediated by antibodies preventing sporozoite invasion of hepatocytes[4]. Hence, efficacy, defined as the percentage reduction of malaria incidence in vaccinated trial participants compared to unvaccinated controls[5,6], of both RTS,S and R21, is variable (30-80%) and declines over time with waning antibody titres[1,7,8]. While approval of the two above-mentioned vaccines certainly represents an important step in the fight against malaria, it is insufficient to reach the WHO goal of reducing mortality by 90% in 2030[9].

Whole sporozoite (WSp) vaccine candidates consist of live *Plasmodium falciparum* (*Pf*) parasites that have been attenuated by either radiation, co-administration of chemoprophylaxis or genetic modification[10]. Protection, defined as the parasite's ability to prevent the onset of malaria after exposure to unattenuated parasites, can be tested by means of controlled human malaria infections (CHMIs). During CHMI, live unattenuated malaria parasites are administered in a controlled setting to healthy participants[11].

Though radiation-attenuated sporozoites (RAS) achieve high protection when administered in high doses to malaria-naïve individuals[12], in endemic areas their performance remains suboptimal[13-15]. Chloroquine chemoprophylaxis with *Pf* sporozoites (CPS) is the most potent WSp vaccination strategy to date, inducing 100% protection in malaria-naïve individuals after mosquito bite or intravenous immunization[16,17]. However, the requirement for drugs and the administration of unattenuated parasites to healthy individuals makes this method unattainable for large-scale implementation.

Genetically attenuated parasites (GAPs) are another type of WSp vaccine where attenuation is achieved by gene deletion. Genetic modification allows the generation of a homogeneous parasite population that arrests at a targeted life cycle stage to induce an immune response[18].

In the following, we will review the current global portfolio of GAP vaccine candidates which arrest during liver stage development with a particular focus on those that have or will soon be tested in clinical trials (**Table 1**, **Table 2** and **Table 3**). Depending on which genes have been removed from the *Plasmodium* genome, currently existing liver stage GAPs fall into two distinct phenotypic categories: arresting either early or late during liver stage development. GAPs will be described based on this categorization and in order of creation, starting from early- and ending with late-arresting GAPs.

Table 1. Overview of studies on murine liver-arresting GAPs.

Author	Parasite	Mother line
Murine liver-arresting GAPs that did not progress to clinical studies		
Mueller et al., 2005[19]	*PbΔuis3*	*Pb* NK65
Mueller et al., 2005[20]	*PbΔuis4*	*Pb* NK65
Tarun et al., 2007[21]	*PyΔuis3*	*Py* 17XNL
	PyΔuis4	*Py* 17XNL
Jobe et al., 2007[22]	*PbΔuis3Δuis4*	*Pb* NK65
Yu et al., 2008[23]	*PbΔFabl*	*Pb* ANKA
Butler et al., 2011[24]	*PyΔFabB/F*	*Py* 17XNL
Annoura et al., 2012[25]	*PbΔFabB/F*	*Pb* ANKA
Murine liver-arresting GAPs that progressed to clinical studies		
Van Dijk et al., 2005[26]	*PbΔp52*	*Pb* ANKA
Labaied et al., 2007[27]	*PyΔp52Δp36*	*Py* 17XNL
Aly et al., 2011[28]	*PyΔsap1*	*Py* 17XNL
Kublin et al., 2017[29]	*PyΔp52Δp36Δsap1*	*Py* 17XNL
Annoura et al., 2012[25]	*PbΔp52Δp36*	*Pb ANKA*
Annoura et al., 2014[30]	*PbΔb9*	*Pb* ANKA
Van Schaijk et al., 2014[31]	*PbΔb9Δslarp*	*Pb* ANKA
Dankwa et al., 2016[32]	*PyΔmei2*	*Py* 17XNL
Franke-Fayard et al., 2022[33]	*PbΔmei2*	*Pb* ANKA
Goswami et al., 2024[34]	*PyΔmei2Δlinup*	*Py* 17XNL

Table 2. Overview of studies in humans using liver-arresting GAPs.

Author	Parasite	Mother line	Number of participants	Location
Spring et al., 2013[35]	*PfΔp52Δp36* (2KO)	*Pf*NF54	6 participants	Silver Spring, USA
Kublin et al., 2017[29]	*PfΔp52Δp36Δsap1* (3KO)	*Pf*NF54	10 participants	Seattle, USA
Murphy et al., 2022[36]	*PfΔp52Δp36Δsap1* (3KO)	*Pf*NF54	Cohort 1: 10 participants Cohort 2: 6 participants Control: 12 participants	Seattle, USA
Roestenberg et al., 2020[37]	*PfΔb9Δslarp* (GA1)	*Pf*NF54	Phase 1: 19 participants Phase 2: 26 participants Control: 9 participants	Leiden and Nijmegen, The Netherlands
Lamers et al., 2024[38]	*PfΔb9Δslarp* (GA1) *PfΔmei2* (GA2)	*Pf*NF54	Phase 1: 20 participants Phase 2: 20 participants Control: 3 participants	Leiden and Nijmegen, The Netherlands
Roozen et al., 2025[39]	*PfΔmei2* (GA2)	*Pf*NF54	Cohort 10 participants Control: 5 participants	Leiden, The Netherlands

Table 3. Overview of test status of all GAPs intended for clinical testing.

Parasite	Approved for clinical testing	Test-status	Further development
PfΔp52Δp36 (2KO)	Yes	Tested	Not planned
PfΔp52Δp36Δsap1 (3KO)	Yes	Tested	Not planned
PfΔb9Δslarp (GA1)	Yes	Tested	Not planned
PfΔmei2 (GA2)	Yes	Tested	Planned
PfΔmei2Δlinup (LARC2)	Yes	In progress	Not applicable yet

GAPs that did not progress to clinical testing

Double knock-out based on Δuis3Δuis4

The search for essential genes for *Plasmodium* liver stage development identified upregulated in infective sporozoites (*uis*) genes as initial targets (**Table 1**)[19,20]. These genes are upregulated in salivary gland sporozoites, yet absent in oocysts[40]. Single deletion *P. berghei* (*Pb*) mutants *PbΔuis3* and *PbΔuis4* showed attenuation during liver stage development[19,20].

No breakthrough blood stage infections were observed for *PbΔuis3* parasites when inoculated into rats in doses of up to 1×10^5 sporozoites[19]. In C57BL/6 mice, immunization with 1×10^4 *PbΔuis3* plus two boosts of 2.5×10^4 provided complete protection against challenge with 1×10^4 wild-type (WT) parasites (**Table 4**). A reduced dose (twice 1×10^4) still protected 70% of mice[19]. The *P. yoelii* (*Py*) analog also conferred 100% protection after three immunizations with $1x10^4$ sporozoites (**Table 4**)[21].

For *PbΔuis4* parasites, breakthroughs were observed in 59% of C57BL/6 mice upon administration of $5x10^4$ sporozoites (**Table 5**)[20]. A three-dose regimen of 1×10^4 *PbΔuis4* at 14-day intervals conferred 100% protection[20]. In *Py*, a threefold immunization of 1×10^4 *PyΔuis4* also induced protection (**Table 4**)[21].

Due to concerns about *PbΔuis4* breakthrough infections, a double knock-out *PbΔuis3Δuis4* was created (**Table 1**)[22]. The parasites were completely attenuated during liver stage development. Immunizing C57BL/6 mice three times with $1x10^4$ *PbΔuis3Δuis4* parasites achieved sterile protection against challenge[22], which was maintained even after rechallenge six months later (**Table 4**)[22]. Attempts to translate these findings to *Pf* failed because *Pfuis3* proved refractory to deletion and the syntenic gene to *uis4*, *Pfetramp10.3*, was both functionally distinct, being expressed across multiple developmental stages, and refractory to deletion[41,42]. Hence, no studies have been conducted with *uis3* or *uis4* in the context of a *Pf* GAP and attempts shifted towards other candidates.

Single knockouts based on ΔFabB/F or ΔfabI

Further gene deletion candidates have been selected based on their essentiality during late liver stage development. Type II fatty acid synthesis (FASII) is crucial for *Plasmodium* development in hepatocytes due to the parasite's exponential replication[43,24,23]. Hence, FASII-pathway members were selected for deletion, such as *fabB/F* and *fabI* (**Table 1**). Deletion of *fabB/F* in *Py* showed full attenuation during late liver stage development. When *PyΔfabB/F* were injected intravenously in BALB/c mice in doses up to $5x10^4$ sporozoites, no breakthrough infections occurred. Immunization with *PyΔfabB/F* parasites (three times $1x10^4$) achieved sterile protection[43]. Protection was maintained even when parasites were administered intradermally or subcutaneously, albeit at higher doses (three times $5x10^4$ parasites) (**Table 4**)[24]. Immunization studies with *PbΔfabI* mutants were never performed as injection of $1x10^4$ *PbΔfabI* parasites resulted in breakthrough infections in 31-100% of C57BL/6 mice (**Table 5**)[23]. The results of the *PyΔFabB/F* failed to translate to *Pf* as both *PfΔfabI* and *PfΔfabB/F* showed severe defects in sporozoite production and salivary gland invasion, making development of a *Pf*GAP based on these genes impossible[44].

Table 4. Overview of the protection rate of liver-arresting GAPs in mice.

Author	Parasite	Mice	Dose	Challenge	Protection
Mueller et al., 2005[19]	*PbΔuis3*	C57BL/6	$5x10^4$ and then $2,5x10^4$ IV 14 and 21 days after first immunization	$1x10^4$ IV 7 days later	10/10 (100%)
	PbΔuis3	C57BL/6	$1x10^4$ and then $1x10^4$ IV 14 and 21 days after first immunization	$1x10^4$ IV 7 days later	10/10 (100%)
	PbΔuis3	C57BL/6	$5x10^4$ and then $2,5x10^4$ IV 34 and 45 days after first immunization	$1x10^4$ IV 30 days later	5/5 (100%)
	PbΔuis3	C57BL/6	$1x10^4$ and then $1x10^4$ IV 34 and 45 days after first immunization	$1x10^4$ IV 30 days later	5/5 (100%)
	PbΔuis3	C57BL/6	$5x10^4$ and then $5x10^4$ 14 days and $1x10^4$ IV 21 days after first immunization	10 mosquitos/ mouse/ 10min. 38 days later	5/5 (100%)
	PbΔuis3	C57BL/6	$1x10^4$ and then $1x10^4$ IV 14 days and $1x10^4$ 21 days after first immunization	10 mosquitos/ mouse/ 10 min. 38 days later	5/5 (100%)
	PbΔuis3	C57BL/6	$1x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 7 days later	7/10 (70%)
	PbΔuis3	C57BL/6	$5x10^4$ and then 2,5 x 10^4 IV 14 and 21 days after immunization	$1x10^4$ blood stage IV 30 days later	0/5 (0%)

Author	Parasite	Mice	Dose	Challenge	Protection
	PbΔuis3	C57BL/6	$1x10^4$ and then 1 x 10^4 IV 14 and 21 days after immunization	$1x10^4$ blood stage IV 30 days later	0/5 (0%)
Mueller et al., 2005[20]	*PbΔuis4REP*	C57BL/6	$5x10^4$ and then 2,5 x 10^4 IV twice at 14-day intervals	$5x10^4$ IV 10 days later	8/8 (100%)
	PbΔuis4REP	C57BL/6	$1x10^4$ IV three times at 14-day intervals	$5x10^4$ IV 10 days later	8/8 (100%)
	PbΔuis4REP	C57BL/6	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV blood stages 34 days later	0/4 (0%)
Jobe et al., 2007[22]	*PbΔuis3Δuis4*	C57BL/6	$7,5x10^4$ and then $2x10^4$ IV at 7-day intervals	$1x10^4$ IV 7 days later	27/27 (100%)
			NA	$1x10^4$ IV rechallenge 180 days later	6/6 (100%)
	PbΔuis3Δuis4	C57BL/6	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 7 days later	14/14 (100%)
	PbΔuis3Δuis4	C57BL/6	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 118 days later	14/14 (100%)
Tarun et al., 2007[21]	*PyΔuis3*	BALB/c	$5x10^4$ IV once	$1x10^4$ IV 7 days later	0/4 (0%)
	PyΔuis3	BALB/c	$5x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis3	BALB/c	$1x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis3	BALB/c	$1x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 30 days later	4/4 (100%)
	PyΔuis3	BALB/c	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis3	BALB/c	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 60 days later	4/4 (100%)
	PyΔuis3	BALB/c	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 180 days later	8/12 (67%)
	PyΔuis4	BALB/c	$5x10^4$ IV once	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis4	BALB/c	$5x10^4$ IV once	$1x10^4$ IV 30 days later	4/4 (100%)
	PyΔuis4	BALB/c	$5x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis4	BALB/c	$1x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis4	BALB/c	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 7 days later	4/4 (100%)
	PyΔuis4	BALB/c	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 60 days later	4/4 (100%)
	PyΔuis4	BALB/c	$1x10^4$ IV three times at 14-day intervals	$1x10^4$ IV 180 days later	8/8 (100%)

Author	Parasite	Mice	Dose	Challenge	Protection
Butler et al., 2011[24]	*PyΔsap1*	Swiss Webster	2x10^4 IV once	1x10^3 IV > 80 days later	8/20 (40%)
			Boost with 2x10^4 IV	1x10^3 IV > 60 days later	8/19 (42%)
	PyΔsap1	C57BL/6	2x10^4 IV once	1x10^3 IV > 60 days later	Not determined
			Boost with 2x10^4 IV 111 days later	1x10^3 IV > 60 days later	0/10 (0%)
	PyΔsap1	BALB/c	1x10^4 IV twice at 14-day intervals	1x10^4 IV 30 days later	2/10 (20%)
			1x10^4 IV three times at 14-day intervals	1x10^4 IV 30 days later	10/10 (100%)
	PyΔFabB/F	Swiss Webster	2x10^4 IV once	1x10^3 IV > 80 days later	8/20 (40%)
			Boost with 2x10^4 IV 111 days later	1x10^3 IV > 60 days later	18/20 (90%)
	PyΔFabB/F	C57BL/6	2x10^4 IV once	1x10^3 IV > 60 days later	1/10 (10%)
			Boost with 2x10^4 IV	1x10^3 IV > 60 days later	20/20 100%
	PyΔFabB/F	BALB/c	1x10^4 IV twice at 14-day intervals	1x10^4 IV 30 days later	10/10 (100%)
	PyΔFabB/F	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 30 days later	10/10 (100%)
	PyΔFabB/F	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 30 days later	8/8 (100%)
			NA	1x10^4 IV rechallenge 120 days later	8/8 (100%)
			NA	1x10^4 IV rechallenge 210 days later	8/8 (100%)
	PyΔFabB/F	BALB/c	1x10^4 IV three times at 14-day intervals	1 x 10^4 IV 14 days later	8/8 (100%)
			NA	1x10^4 IV rechallenge 210 days later	8/8 (100%)
	PyΔFabB/F	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 100 days later	5/5 (100%)
	PyΔFabB/F	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 300 days later	8/8 (100%)
	PyΔFabB/F	BALB/c	5x10^4 IV three times at 14-day intervals	1x10^4 IV 210 days later	8/8 (100%)
	PyΔFabB/F	BALB/c	5x10^4 ID three times at 14-day intervals	1x10^4 IV 30 days later	5/5 (100%)
			NA	1x10^4 IV 210 days later	5/5 (100%)
	PyΔFabB/F	BALB/c	5x10^4 SC three times at 14-day intervals	1x10^4 IV 30 days later	5/5 (100%)

Author	Parasite	Mice	Dose	Challenge	Protection
			NA	1x10^4 IV 210 days later	5/5 (100%)
	PyΔFabB/F	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 *Pb* IV 210 days later (heterologous)	5/5 (100%)
Van Dijk et al., 2005[26]	*PbΔp52*	BALB/c	1x10^5 IV once	5x10^4 IV 10 days later	18/20 (90%)
	PbΔp52	BALB/c	5x10^4 IV once	2,5x10^4 IV 10 days later	15/15 (100%)
	PbΔp52	BALB/c	5x10^4 IV once	1x10^4 IV 10 days later	5/5 (100%)
	PbΔp52	BALB/c	5x10^4 IV once	1x10^4 IV 30 days later	5/5 (100%)
	PbΔp52	BALB/c	5x10^4 IV once	1x10^4 IV 60 days later	5/5 (100%)
	PbΔp52	BALB/c	5x10^4 IV once	1x10^4 IV 120 days later	5/5 (100%)
	PbΔp52	BALB/c	5x10^4 IV once	1x10^6 infected RBCs IV 10 days later	0/4 (0%)
	PbΔp52	C57BL6	5x10^4 IV once	1x10^4 IV 10 days later	0/1 (0%)
	PbΔp52	C57BL6	5x10^4 and then 2x10^4 IV at 7-day intervals	1x10^4 IV 10 days later	1/4 (25%)
	PbΔp52	C57BL6	5x10^4 once and then 2x10^4 IV twice at 7-day intervals	1x10^4 IV 10 days later	4/4 (100%)
	PbΔp52	C57BL6	5x10^4 once and then 2x10^4 IV twice at 7-day intervals	1x10^4 IV 30 days later	5/5 (100%)
Labaied et al., 2007[27]	*PyΔp52Δp36*	BALB/c	1x10^4 IV three times at weekly intervals	1x10^4 IV 7 days later	8/8 (100%)
	PyΔp52Δp36	BALB/c	1x10^4 IV three times at weekly intervals	1x10^4 IV 30 days later	7/7 (100%)
	PyΔp52Δp36	BALB/c	1x10^4 IV three times at 7-day intervals	15 mosquitoes/ mouse/ 10 min 7 days later	5/5 (100%)
Aly et al., 2011[28]	*PyΔsap1*	Swiss Webster	2x10^4 IV four times at 14-day intervals	1x10^4 IV 60 days later	5/5 (100%)
	PyΔsap1	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 90 days later	8/8 (100%)
	PyΔsap1	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 180 days later	7/7 (100%)
	PyΔsap1	BALB/c	1x10^4 IV three times at 14-day intervals	1x10^4 IV 270 days later	6/6 (100%)
	PyΔsap1	BALB/c	5x10^4 IV three times at 14-day intervals	1x10^4 IV 60 days later	5/5 (100%)
	PyΔsap1	BALB/c	1x10^5 SC three times at 14-day intervals	1x10^4 IV 60 days later	5/5 (100%)

Author	Parasite	Mice	Dose	Challenge	Protection
	PyΔsap1	BALB/c	$1x10^5$ IV four times at 14-day intervals	20 mosquitoes/ mouse/ 10 min 360 days later	5/5 (100%)
Kublin et al., 2017[29]	*PyΔp52Δp36Δsap1*	BALB/cJ	$1x10^4$ IV twice at 14-day intervals	1 x 10^4 IV 7 days later	5/5 (100%)
	PyΔp52Δp36Δsap1	BALB/cJ	$1x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 30 days later	5/5 (100%)
	PyΔp52Δp36Δsap1	BALB/cJ	$1x10^4$ IV twice at 14-day intervals	$1x10^4$ IV 180 days later	5/5 (100%)
Van Schaijk et al., 2014[31]	*PbΔb9*	BALB/c	$1x10^3$ IV once	$1x10^4$ IV 10 days later	8/10 (80%)
	PbΔb9	BALB/c	$5x10^3$ IV once	$1x10^4$ IV 10 days later	18/20 (90%)
	PbΔb9	BALB/c	$1x10^4$ IV once	$1x10^4$ IV 10 days later	10/10 (100%)
	PbΔb9	C57BL/6	$5x10^4$ and then $2x10^4$ IV at 7-day intervals	$1x10^4$ IV 10 days later	4/4 (100%)
	PbΔb9	C57BL/6	$5x10^4$ and then $2x10^4$ IV at 7-day intervals	$1x10^4$ IV 90 days later	5/5 (100%)
	PbΔb9	C57BL/6	$5x10^4$ and then $2x10^4$ IV at 7-day intervals	$1x10^4$ IV 180 days later	9/9 (100%)
	PbΔb9	C57BL/6	$5x10^4$ and then 2x10 IV at 7-day intervals	$1x10^4$ IV 365 days later	5/11 (46%)
	PbΔb9Δslarp	BALB/c	$1x10^3$ IV once	$1x10^4$ IV 10 days later	20/20 (100%)
	PbΔb9Δslarp	BALB/c	$5x10^3$ IV once	$1x10^4$ IV 10 days later	10/10 (100%)
	PbΔb9Δslarp	BALB/c	$1x10^4$ IV once	$1x10^4$ IV 10 days later	20/20 (100%)
	PbΔb9Δslarp	C57BL/6	$5x10^4$ and then $2x10^4$ IV at 7-day intervals	$1x10^4$ IV 180 days later	6/6 (100%)
	PbΔb9Δslarp	C57BL/6	$1x10^3$ IV three times at 7-day intervals	$1x10^4$ IV 10 days later	6/10 (60%)
	PbΔb9Δslarp	C57BL/6	$1x10^4$ IV three times at 7-day intervals	$1x10^4$ IV 10 days later	10/10 (100%)
Dankwa et al., 2016 [32]	*PyΔmei2*	BALB/cJ	$1x10^4$ IV three times, 35 days and then 45 days after the first immunization	$1x10^4$ IV 46 days later	9/9 (100%)
			NA	$1x10^4$ IV rechallenge 56 days later	9/9 (100%)
	PyΔmei2	BALB/cJ	$1x10^4$ IV three times, 31 and 45 days after the first immunization	$1x10^4$ IV 39 days later	12/12 (100%)
Goswami et al., 2024 [34]	*PyΔmei2Δlinup*	BALB/cJ	$1x10^4$ IV, twice at 1-month intervals	$1x10^4$ IV 30 days later	10/10 (100%)
		BALB/cJ	$2x10^4$ IM, twice at 1-month intervals	$1x10^4$ IV 30 days later	7/10 (70%)
		BALB/cJ	$5x10^4$ IV, twice at 1-month intervals	$1x10^4$ IV 180 days later	9/10 (90%)

Author	Parasite	Mice	Dose	Challenge	Protection
		BALB/cJ	5x10^4 IV, twice at 1-month intervals	1x10^4 IV 360 days later	6/10 (60%)
		BALB/cJ	5 x 10^4 cryopreserved IV	1x10^4 IV 30 days later	10/10 (100%)
		BALB/cJ	1x10^4 IV, 3 times at 1-month intervals	1x10^4 IV blood stage parasites 30 days later	10/10 (100%)
		BALB/cJ	5x10^4 cryopreserved sporozoites IV, 2 times at 15-day intervals	1x10^4 cryopreserved sporozoites IV 25 days later	12/12 (100%)
		Swiss Webster (outbred)	5x10^4 IV, at 2-month intervals	1x10^4 IV 60 days later	7/10 (70%)

IV: intravenous, SC: subcutaneous, ID: intradermal, IM: intramuscular

Table 5. Overview of liver-arresting GAP breakthroughs in mice.

Author	Parasite	Mice	Dose	Breakthrough
Mueller et al., 2005[20]	*PbΔuis4*	C57BL/6	5x10^4 IV	33/56 (59%)
	PbΔuis4REP	C57BL/6	5x10^4 IV	27/75 (36%)
Yu et al., 2008[23]	*PbΔFabl*	C57BL/6	1x10^3 IV	5/16(31%)
	PbΔFabl	C57BL/6	1x10^4 IV	13/15 (87%)
	PbΔFabl^{REC}	C57BL/6	1x10^3 IV	15/15 (100%)
	PbΔFabl	C57BL/6	20 mosquitoes / mice	4/5 (80%)
	PbΔFabl^{REC}	C57BL/6	20 mosquitoes / mice	5/5 (100%)
Van Dijk et al., 2005[26]	*PbΔp52*	BALB/c	Not reported	1/26 (4%)
	PbΔp52	C57BL/6	Not reported	5/48 (10%)
Annoura et al., 2012[25]	*PbΔfabb/f-*	C57BL/6	5x10^4 IV	25/25 (100%)
	PbΔp36Δp52	C57BL/6	5x10^4 IV	3/20 (15%)
	PbΔp36Δp52-br 'breakthrough'	C57BL/6	5x10^4 IV	7/12 (58%)
	PbΔfabb/f-	BALB/c	5x10^4 IV	22/25 (88%)
Annoura et al., 2014[30]	*PbΔp36Δp52*	C57BL/6	2x10^5 IV	10/10 (100%)
	PbΔb9Δp36Δp52	C57BL/6	5x10^4 IV	1/10 (10%)
	PbΔb9	C57BL/6	5x10^4 IV	3/20 (15%)
	PyΔb9	BALB/c	2x10^5 IV	1/8 (13%)
Vaughan et al., 2018 [45]	*PyΔmei2*	BALB/cByJ	2x10^5 IV	3/30 (10%)
	PyΔmei2	BALB/cByJ	5x10^5 IV	4/30 (13%)
Franke-Fayard et al., 2022[33]	*PbΔmei2*	C57BL/6	2x10^5 IV	3/10 (33%)
	PbΔmei2-breakthrough-a	C57BL/6	2x10^5 IV	5/11 (45%)

IV: intravenous

Early-arresting GAPs

Double knock-out parasites based on Δp36Δp52 (2KO)

Other studies focused on deletion of *p52* (also referred to as *p36p*), which encodes a protein member of the 6-Cys domain family and plays an important role in the formation of the parasitophorous vacuole during liver stage development[27]. *p52* is expressed in pre-erythrocytic stages and deletion is associated with reduced parasite infectivity[27]. Immunization with *PbΔp52* induced up to 100% protection in mice (**Table 4**), yet in other experiments it led to breakthrough blood-stage infections in a subset of the animals (**Table 5**)[26,46].

For this reason, *p36* was selected as an additional candidate for deletion (**Table 1**). *p36* belongs to the same 6-Cys domain family as *p52*, has an analogous function and is similarly expressed in sporozoites[27]. Simultaneous deletion of *p36* and *p52* in *Py* led to a fully attenuated phenotype, preventing breakthrough blood stage infections[25,27]. Additionally, threefold immunization with $1x10^4$ *PyΔp52Δp36* consistently protected against challenge in murine models (**Table 4**)[27].

Given the safety and protection profile of *PyΔp36Δp52* in mice, a double knock-out *Pf*GAP, henceforth referred to as 2KO (*PfΔp36Δp52*), was generated[35,47,48]. *PfΔp36Δp52* failed to infect livers of mice engrafted with human hepatocytes (SCID Alb-UpA humanized mice), indicating full attenuation[47]. These results prompted approval for clinical testing.

The administration of 2KO parasites for safety testing was the first-in-human study using GAPs and occurred in six malaria-naïve participants, who were exposed to escalating numbers (five and then 200) of mosquito bites (**Table 2**)[35]. While the first exposure to five mosquito bites was well-tolerated, one participant developed malaria on day 12 after receiving 200 mosquito bites, putting a premature end to the study (**Table 6** and **Table 7**). Sequencing revealed that the breakthrough was caused by the incomplete attenuation of the GAP rather than by reversal to WT[35].

Table 6. Overview of liver-arresting GAP breakthroughs in humans.

Author	Parasite	Dose	Breakthroughs
Spring et al., 2013[35]	*PfΔp52Δp36 (2KO)*	200 mosquito bites	1/6 (17%)

Table 7. Overview of the protection rate of liver-arresting GAPs in humans.

Author	Parasite	Dose	Challenge	Protection
Spring et al., 2013[35]	*PfΔp52Δp36 (2KO)*	5 mosquito bites	NA	NA
	PfΔp52Δp36 (2KO)	200 mosquito bites	NA	NA
Kublin et al., 2017[29]	*PfΔp52Δp36Δsap1 (3KO)*	150-200 mosquito bites	NA	NA
Murphy et al., 2022[36]	*PfΔp52Δp36Δsap1 (3KO)*	200 mosquito bites 3 times (2 immunizations at monthly intervals and the third 8 weeks later)	5 *PfNF54* mosquito bites 4 weeks later	3/6 (50%)
	NA	NA	5 *PfNF54* mosquito bites rechallenge 180 days later	0/3 (0%)
	PfΔp52Δp36Δsap1 (3KO)	200 mosquito bites 5 times (4 immunizations at 4-week intervals and the fifth 8 weeks later)	5 *PfNF54* mosquito bites 4 weeks later	4/8 (50%)
	NA	NA	5 *PfNF54* mosquito bites rechallenge 180 days later	1/4 (25%)
Roestenberg et al., 2020[37]	*PfΔb9Δslarp* (PfSPZ-GA1)	$1.35x10^5$ cryopreserved sporozoites IV	NA	NA
	PfSPZ-GA1	$4.5x10^5$ cryopreserved sporozoites IV	NA	NA
	PfSPZ-GA1	$9x10^5$ cryopreserved sporozoites IV	NA	NA
	PfSPZ-GA1	$4.5x10^5$ cryopreserved sporozoites IV three time at 2-month intervals	5 *PfNF54* mosquito bites 3 weeks later	1/12 (8%)
	PfSPZ-GA1	$9x10^5$ cryopreserved sporozoites IV 3 times at 2-month intervals	5 *PfNF54* mosquito bites 3 weeks later	2/13 (15%)
Lamers et al., 2024[38]	*PfΔmei2* (GA2)	15 mosquito bites	NA	NA
	PfΔmei2 (GA2)	50 mosquito bites	NA	NA
	PfΔb9Δslarp (GA1)	50 mosquito bites 3 times at monthly intervals	5 *Pf3D7* mosquito bites 3 weeks later	1/8 (13%)
	PfΔmei2 (GA2)	50 mosquito bites 3 times at monthly intervals	5 *Pf3D7* mosquito bites 3 weeks later	8/9 (89%)
Roozen et al., 2025[39]	*PfΔmei2* (GA2)	50 mosquito bites	5 *Pf3D7* mosquito bites 6 weeks later	9/10 (90%)

IV: intravenous

Triple knock-out parasites based on Δp36Δp52Δsap1 (3KO)

To reduce the likelihood of a breakthrough, a 3KO parasite, lacking *sap1* in addition to *p36* and *p52,* was generated (**Table 1**)[49]. *sap1* (also referred to as *slarp*) encodes the sporozoite asparagine-rich protein 1 (SAP1) and is a post-transcriptional regulator involved in liver

infectivity[28]. *PyΔsap1* parasites are completely attenuated in highly susceptible BALB/cByJ mice in doses of up to 7.5x10^4 sporozoites[49]. Threefold immunization of BALB/c mice with 1x10^4 sporozoites resulted in 100% protection even when challenged 210 days after the last immunization and independently of whether unattenuated parasites where administered intravenously, by mosquito bite or as blood stages (**Table 4**)[50]. Sequential gene deletion of *sap1* in the previously established *PfΔp36Δp52* parasite was carried out to generate *PfΔp36Δp52Δsap1*[49]. Injection with 1x10^6 of this GAP into humanized FRG huHep mice that had been supplemented with human erythrocytes on day 7 post-infection, did not cause a breakthrough blood stage infection[49]. These observations helped obtain approval for clinical testing of the *PfΔp36Δp52Δsap1*.

The first clinical trial with the *Pf*3KO GAP saw the administration of 150-200 mosquito bites to ten malaria-naïve participants to test for safety (**Table 2**): none of the participants developed malaria in the 28-day follow-up period[29]. A further trial assessed the protection of *Pf*3KO-immunization against challenge: malaria-naïve participants were immunized by either 3x200 or 5x200 *Pf*3KO mosquito bites, followed by CHMI four weeks later[36]. The protection rate was 50% in both groups after CHMI, demonstrating that additional immunizations beyond three times does not result in higher protection rates (**Table 7**). To investigate duration of protection, a second CHMI of protected participants was carried out six months after the last immunization. Protection was 15% in both groups after the second CHMI, indicating that immunity waned over time (**Table 7**)[36].

Double knock-out based on Δb9Δslarp (GA1)

Another early-arresting GAP was based on genetic deletions of *b9* and *slarp* (also referred to as *sap1*) *b9* also encodes a member of the *Plasmodium* 6-Cys family (**Table 1**)[28,30]. Breakthrough blood stage infection was absent upon injection of Swiss or BALB/c mice with 5x10^4 single-deletion mutant *PbΔb9*. However, 20% of C57BL/6 mice became parasite-positive after inoculation with 5x10^4 *PbΔb9* sporozoites (**Table 5**)[30].

Based on these results, a double knock-out was generated, i.e. *PbΔb9Δslarp*. No breakthrough infection occurred after injection of 2x10^5 *PbΔb9Δslarp* sporozoites. Immunization of BALB/c mice with as few as 1x10^3 sporozoites induced protection against challenge with WT sporozoites. In C57BL/6 mice three immunizations with 1x10^4 sporozoites were required to induce protection, which was maintained for at least 180 days (**Table 4**)[31].

Subsequently, a *Pf*GAP, *PfΔb9Δslarp,* henceforth referred to as GA1, was generated. Survival within hepatocytes *in vitro* was completely abrogated by day 4 and parasites

were absent at day 5 when assessed in immunodeficient SCID Alb-uPA mice engrafted with human hepatocytes[31].

The promising results of the preclinical experiments warranted further assessment of GA1 in humans (**Table 2**). The biotechnology company Sanaria produced the GAP as an injectable cryopreserved product, referred to as PfSPZ-GA1. The PfSPZ-GA1 product was deemed safe, as no blood stage infections were observed after administering up to $9x10^5$ sporozoites to humans[37]. Immunizations of malaria-naïve participants were carried out three times at two-month intervals with either $4.5x10^5$ or $9x10^5$ sporozoites and followed by homologous challenge with five *Pf*NF54-infected mosquito bites three weeks later[51]. Three of the 25 PfSPZ-GA1 immunized participants were protected against CHMI, one in the low-dose ($4.5x10^5$ sporozoites) and two in the high-dose ($9x10^5$ sporozoites) group (**Table 7**)[37].

Late arresting GAPs

It had been previously demonstrated that prolonging GAP survival in the liver could improve protection rates through increased antigenic exposure[24]. The knowledge that CPS, where the concomitant administration of live unattenuated sporozoites with antimalarial agents causes the parasites to die just after reaching the blood stage, achieved the highest protection (100%)[16,17] of all WSp candidates to date corroborated this hypothesis by confirming the importance of prolonged antigenic exposure for protection in humans.

Single knock-out GAP based on Δmei2

The gene meiosis inhibited 2 (*mei2*) was identified as a promising target for a late-arresting GAP (**Table 1**)[32,33]. *mei2* encodes an RNA-binding protein expressed only during liver stage development and is involved in DNA segregation[32]. Removal of *mei2* resulted in a late-arresting phenotype with parasites incapable of producing infectious merozoites[32]. A breakthrough rate of 10% and 13% was observed in BALB/cByJ mice after intravenous administration of $2x10^5$ and $5x10^5$ sporozoites, respectively[45]. Similar results were reported for *Pb,* with C57BL/6 mice becoming blood stage patent after administration of $2x10^5$ sporozoites (**Table 5**)[33]*.* Immunizing BALB/c mice three times with $1x10^4$ *PyΔmei2* sporozoites resulted in 100% protection against challenge, showing promise as a late-arresting GAP (**Table 4**)[32].

The *PfΔmei2* was created by two different research groups and termed GA2 or late arresting replication competent 1 (LARC1) (**Table 2**)[33,52]. *PfΔmei2* parasites exhibited normal infection of human hepatocytes *in vitro* for up to nine days[33,52]. When FRG huHep

mice were infected with 1x10^6 GA2 or LARC1 sporozoites and supplemented with human erythrocytes, parasites were undetectable in blood from GAP-infected mice for up to 28 days post-infection[33,52]. The lack of parasitic genetic material in blood of GA2-infected mice and the absence of their growth *in vitro* suggests that either parasites are cleared in the liver stage therefore never entering the bloodstream or that aberrant, non-viable merozoites are released from the liver yet are incapable of infecting erythrocytes[33,52]. Based on these findings, GA2 was approved for clinical testing (**Table 2**).

In the first part of a phase 1/2a trial, which aimed to confirm the attenuation phenotype and safety profile of GA2, two sequential cohorts were exposed to 15 and then 50 GA2-infected mosquito bites (**Table 2**)[38]. No blood stage infections occurred, and adverse events were limited. The second part saw the three-fold immunization of ten participants with 50 GA2-infected mosquito bites at 28-day intervals with a homologous CHMI three weeks after the last immunization. Controls consisted of GA1- and placebo-immunized participants. Efficacy of GA2 was 89%, whereas only 13% of GA1- and none of the mock-immunized participants were protected (**Table 7**)[38], demonstrating for the first time in humans that late-arresting GAPs are superior to early-arresting GAPs.

Next, GA2-immunized participants (n=10) and placebo-exposed participants (n=5) were exposed once to 45-55 mosquito bites and challenged in a CHMI with five 3D7-infected mosquitoes six weeks later. 90% of the GA2-exposed individuals were protected and did not develop blood stage parasitemia (**Table 7**), whilst all placebo-exposed participants became parasite-positive. This indicates that a single dose of GA2-parasites with 50 mosquito bites is sufficient to elicit 90% protection in a small cohort of individuals[39].

Double knock-out based on Δmei2Δlinup (LARC2)

Since *PyΔmei2* parasites were incompletely attenuated when administered in high doses to susceptible BALB/cByJ mice (**Table 5**)[45], a double gene deletion mutant was generated in the form of *PyΔmei2Δlinup* (LARC2) (**Table 1**). LARC2 may have similar immunogenicity as GA2, but an enhanced safety profile. *linup* encodes the liver stage nuclear protein, which is a conserved protein localizing to the nucleus in liver stage *Plasmodium* parasites[53]. Single deletion mutant *PyΔlinup* showed that the gene is essential during late liver stage development[53]. Injection of susceptible BALB/cByJ with 2x10^5 or 5x10^5 double deletion mutants *Py*LARC2 parasites did not cause breakthrough infections[34]. *Py*LARC2 elicited durable protection with 90% of BALB/cJ mice being protected when immunized twice with 5x10^4 *Py*LARC2 sporozoites at one-month intervals and subsequently challenged six months later[34]. Administration of 5x10^4 cryopreserved sporozoites at two-week intervals

was equally protective. Finally, animals immunized with *Py*LARC2 completely cleared the infection within 12 days upon injection of blood stage parasites (**Table 4**)[34].

In the same study, *Pf*LARC2 was described (**Table 2**)[34]. To assess the risk of breakthrough infections *in vivo*, FRG NOD huHep mice were injected with one million cryopreserved *Pf*LARC2 or WT parasites and then supplemented with human erythrocytes six and seven days after infection. Blood collected from *Pf*LARC2-injected mice was parasite-negative up to experiment termination, in contrast to blood cultures from WT-injected mice that became parasite-positive on days 1-3 after transition[34]. These promising results prompted the approval for clinical testing of aseptic cryopreserved PfSPZ-LARC2. Current planning of clinical trials is underway to start testing *Pf*LARC2 in healthy participants as well as in individuals in endemic countries (**Table 3**).

Possible immunological mechanisms of protection

Both humoral and cellular immune responses play a role in protection against malaria, with $CD4^+$ and $CD8^+$ T-cells being important for establishing liver immunity[54]. Late-arresting parasites may be more efficient at activating and recruiting liver-resident $CD8^+$ T-cells through increased antigenic exposure. Rodent work has shown that immunization with late-arresting *PyΔFabB/F* induced a higher absolute count of $CD8^+$ T cells that also persisted for a longer period of time compared to early arresting RAS[24]. Analysis of immune responses from C57BL/6 mice that had been immunized twice with $5x10^4$ late-arresting *Py*LARC2 sporozoites showed that $CD8^+$ T-cells and specifically $CD69^{hi}CXCR6^{hi}$ $CD8^+$ T_{RM} cells were significantly increased in immunized mice compared to controls[34].

A correlation between $CD8^+$ T-cells and protection against malaria has been reported in human studies as well[17,55-57]. Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) producing $CD4^+$ T cells and, to a lesser extent, $CD8^+$ T were increased after immunization with early-arresting 2KO parasites. These responses exhibited a dose-dependent pattern, with significant increases after 200 mosquito bites compared to 5 mosquito bites[35]. Similar findings were reported after immunization with GA1: the frequency of IFN-γ producing $CD4^+$ and $CD8^+$ T cells was significantly increased after three immunizations, yet responses did not correlate with protection[37].

Immunization with GA2, whether it happened once or multiple times, resulted in an increased frequency of $CD4^+$ and Vδ2 γδ T-cells with a memory phenotype, preferentially expressing IFN-γ, TNF-α and IL-2[38,39,58]. However, circulating $CD8^+$ and NK cells were not increased upon immunization[44,45]. The difficulties in detecting $CD8^+$ T cells may not necessarily disprove their involvement in antimalarial immunity. As *Pf*-specific $CD8^+$

T-cells may exhibit liver-resident phenotypes, they are undetectable in peripheral blood and therefore difficult to identify[59]. Liver-residency of immune cells would also explain the superior protection of late-arresting compared to early arresting GAPs[24,38], as their prolonged permanence in the liver allows for the recruitment of tissue-resident $CD8^+$ T cells[24].

Humoral immune responses also contribute to protection against malaria[54]. Anti-CSP antibodies are frequently measured as they are directed against the most abundant protein on the sporozoite surface[60]. Indeed, all reviewed studies found an increase in CSP titres in humans after immunization[29,36-39,58], which however did not correlate with protection[36-39]. Interestingly, serum from 3KO-immunized participants was able to block *in vitro* hepatocyte traversal and invasion[35,47], suggesting inhibitory capacity of formed antibodies in immunized participants. This was confirmed *in vivo* by administering serum from participants to FRG huHep mice[35]. Subsequent challenge with WT parasites showed an 88% reduction of liver stage burden. The serum samples with the highest functional capacity *in vivo* had the lowest anti-CSP antibody titers, suggesting other sporozoite proteins may be important in hepatocyte invasion and subsequent development[29].

Timing of GAP arrest may also play a role in antibody production: when humoral responses were compared between GA1- and GA2-immunized participants, more and different antigens were detected in the GA2-immunized group, indicating increased antigenic exposure during late liver stage development[61]. The exact role of antibodies in liver immunity remains to be elucidated, although they interfere with parasite movement, traversal and hepatocyte invasion[54].

Discussion

This review presents the currently existing *Pf*GAPs, including research that was instrumental to their generation. These novel malaria vaccine candidates should be further explored, and specifically questions regarding biosafety, large scale production and implementation need to be addressed.

Biosafety

Safety is key for the implementation of any GAP. Despite extensive preclinical testing, instances of incomplete attenuation have led to hesitancy for the large-scale use of GAPs[35]. Breakthrough infections in rodent malaria species may not always predict a similar phenotype in humans, as blood stage infections were detected in highly susceptible BALB/cByJ mice after administration of $2.5\text{-}5 \times 10^5$ *PyΔmei2* sporozoites[45], but not for *PfΔmei2* in

humanized mice and preliminary clinical trials[33,52]. Further testing in larger groups may be necessary to define the risk of breakthroughs in clinical settings. However, if the chance of such an event occurring is infinitesimal, it may not be detected in phase 2 and 3 clinical trials and still can never be proven to be absent. Hence, humanized mice injected with human erythrocytes remain the best approximation for safety predictions before clinical testing in humans.

Biosafety concerns are pertinent to the use of genetically modified organisms (GMOs) in medicine, with varying regulations worldwide. One concern is escape from the controlled conditions of the laboratory, potentially disrupting ecosystems. All GAPs created so far arrest prior to reaching the blood stage, making propagation past the liver and transmission to other humans and mosquitoes effectively impossible[29,31,33,36,52]. Furthermore, no transposable genetic elements have been identified in *Plasmodium*, meaning that DNA cannot spontaneously be exchanged between two parasites unless it happens during sexual reproduction, a process only occurring in the mosquito midgut[62].

Another voiced concern is that of horizontal gene transfer from GMOs to host cells[63]. However, in the case of GAPs, vectors are not self-mobilizable and not present in the vaccine product. Removal of all exogenous genetic material used to guide the deletion of the GAP means that no new DNA is being introduced in the host[18,31,33]. GMO use in medicine is not novel, adenovirus-based vaccines for SARS-CoV-2 and Ebola are genetically engineered[64]. Given the proven success of such vaccines, GAPs share similar potential.

Protective capability

For any GAP to implemented, it must be safe but also confer high protection in malaria-endemic areas, where it may be hampered by vaccine hyporesponsiveness[65]. Most GAPs have achieved lower than desired efficacy and have therefore not proceeded to further clinical testing[35,37,66]. However, the advent of late-arresting GAPs (i.e. GA2 and LARC2), open the road to new scientific possibilities. The direct comparison of GA2 with GA1 unequivocally confirmed the importance of the late liver stage in achieving protection against malaria[38] (**Fig. 1**). An important question is whether late-arresters maintain protection rates against heterologous challenge. If protection were lower than desired, one possibility would be to generate GAPs with different genetic backgrounds to match the parasites in circulation. This approach is limited, as the number of existing *Pf* strains is considerable and new ones continuously evolve[67]. Improving protection over time could require boosts, although the frequency and timing remains to be determined. Duration of

protection also needs to be assessed. In this regard results from CPS studies conducted in malaria-naïve individuals show potential with protection lasting up to two years after immunization[68]. Despite the many open interrogatives, the superior protection of GA2 compared to previously developed early-arresting GAPs is promising and makes late liver stage arresting parasites potential candidates for further vaccine development and large-scale implementation.

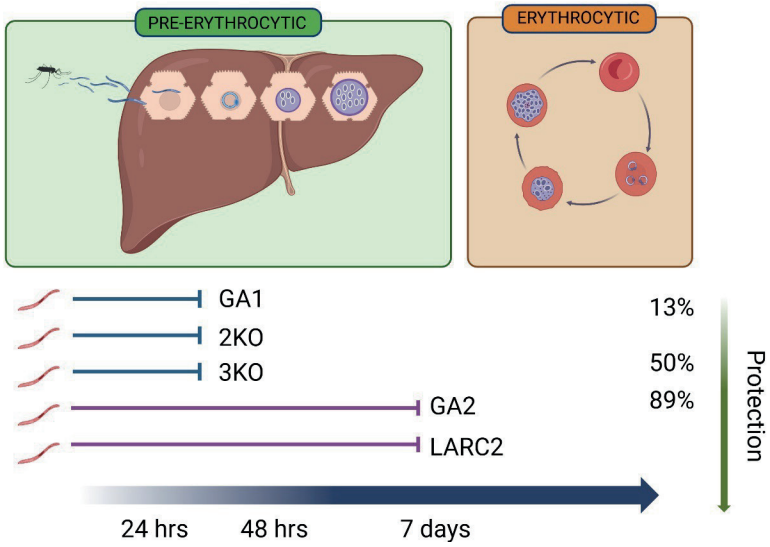

Figure 1. Schematic representation of liver stage arresting GAPs that have or will soon be tested in clinical trials. Length of development of each GAP in relation to protection in humans is shown. Candidates include the early arresters GA1 (*PfΔb9Δslarp*), 2KO (*PfΔp36Δp52*) and 3KO (*PfΔp36Δp52Δsap1*). GA2 (*PfΔmei2*) and LARC2 (*Pf Δmei2Δlinup*) are late arresters.

Implementation of GAPs

Another important question pertains to the feasibility of introducing GAPs as a large-scale vaccination strategy. The first potential obstacle is the cost of manufacturing of cryopreserved sporozoites, as it still requires rearing of aseptic mosquitoes, manual dissection of salivary glands and extraction of sporozoites. However, dissection-independent production of sporozoites[69] and *in vitro* culturing of sporozoites has been reported[70], allowing a complete bypass of the dissection step. Storage of sporozoites at −150°C could pose logistical challenges in endemic areas, but liquid nitrogen systems already exist in Africa and require minimal infrastructure. The additional advantage of a liquid nitrogen-based cold chain is that, except for the generation of liquid nitrogen, it is independent of electricity supply[71]. While logistical hurdles exist, recent technological advances offer viable solutions.

Future directions: Remaining problems and potential solutions

Despite the promise of efficacy and deliverability of GAPs, some questions remain open. The route of administration of GAPs is one such discussion point. Intravenous injection of sporozoites is more efficacious as large proportions of sporozoites reach the liver[72], yet intradermal and/or intramuscular administration would be more practical[73]. Intradermal or intramuscular injections could also provide the advantage of early immune system activation[74,75], as skin-draining lymph nodes play an important role in the initiation of the immune response against malaria[76].

Another question is whether approved malaria vaccines may act synergistically or antagonistically with GAP vaccine candidates. If part of the African population is immunized with RTS,S and R21, could efficacy of GAPs be decreased due to pre-existing immunity? The two subunit-based malaria vaccines induce mostly humoral immunity targeted to CSP. However, since antibodies do not distinguish between GAPs or WT parasites, GAPs may be intercepted before reaching the liver where important immune responses occur. Further clinical studies involving GAPs in combination with RTS,S or R21 are necessary to understand the interactions between the existing vaccines.

Conclusion

In conclusion, GAPs have shown to be safe, well-tolerated and highly efficacious. What emerges from this review is that any future research should focus on late-arresting GAPs, as they have achieved the highest protection rates in malaria-naïve individuals (**Table 3, Fig. 1**). While the results of phase 1/2 clinical trials are promising, some aspects remain to be investigated: efficacy of all late-arresting GAPs must be confirmed in larger trials, against heterologous challenge and in pre-exposed populations; duration of protection needs to be examined also. Despite these questions being unanswered to date, this field of research is just beginning to evolve. Many of the current hurdles may be easily overcome soon, making GAPs increasingly promising vaccine candidates against malaria.

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Chapter 4

What motivates SARS-CoV-2 vaccine trial participants? A pre- and post-participation survey study

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Published: Trials, 2024
DOI: 10.1186/s13063-024-08582-z

Abstract

Background: Scientific advancement, including the testing and licensing of new drugs, relies heavily on clinical trials with healthy individuals. The motivations of clinical trial participants have been discussed intensively, as some worry that financial compensation may distract from the intrinsic risk of clinical research. Herein, we investigated the motivations and decisional factors influencing SARS-CoV-2 clinical trial participants. Moreover, since most surveys are administered after clinical trial participation, we were interested in whether the results were tainted by recall bias.

Methods: This was a cross-sectional observational study. Participants were administered a survey on two occasions, once before and once after participation in a clinical trial. The primary outcomes were the motivations and decisional factors of SARS-CoV-2 vaccine trial participants and the difference between the surveys collected before and after clinical trial participation.

Results: The survey response rate was 149/200 (75%). SARS-CoV-2 vaccine trial participants were mostly motivated by the desire to contribute to science and help others. Answers collected before and after the trial were not statistically different, indicating the absence of recall bias.

Conclusion: The decision-making process of clinical trial participants is complex and multi-faceted. Previous studies have shown that clinical trial participants have mixed motivations, but never to the extent reported in the current survey**.** Here we present a theoretical framework that attempts to explain how different motivational factors may contribute to decision forming.

Introduction

Clinical trials, involving the testing of novel vaccines or drugs on humans, are a cornerstone of medical innovation. Any clinical research is tightly regulated, as laid down in the Nuremberg Code, the Declaration of Helsinki and the Declaration of Belmont: key principles include that participation should always be voluntary and the well-being and integrity of the participants should outweigh other considerations[1-3].

Clinical trials involving healthy individuals have received special scrutiny, because participants do not gain any benefits except for a financial compensation for the invested time and effort. The undue influence of payment has been questioned: is well-informed and voluntary participation possible if individuals are being remunerated[4-6]? Specifically, there are concerns that economically-motivated participants may not sufficiently weigh the downsides of participation because they receive payment[7,8]. However, it has been shown that financial reward is not the sole incentive for healthy volunteers, as they also hope to contribute to medical science or help others through their participation[9,10].

The SARS-CoV-2 pandemic represented an exceptional situation, which put the health care system and society in general under high pressure. Clinical trials with healthy participants were pivotal for rolling out an effective vaccine. SARS-CoV-2 vaccine trials are an interesting case study, because they combined social benefit with the personal advantage of being vaccinated early. However, the motivations and decision-making process of SARS-CoV-2 vaccine trial participants have not been characterized yet.

Most surveys investigating motivational factors of participants are compiled at the end of a clinical trial. Answers collected in such a post-hoc manner may be influenced by the experience of the trial itself or be tainted by recall bias. Questioning participants both before and after study completion can address these differences but has not been performed thus far.

This study employed a survey to investigate why individuals decided to participate in SARS-CoV-2-related clinical trials and how they experienced said trials, investigating their motivations both before and after study participation.

Materials and methods

Participants

This study was approved by the medical ethical committee Leiden Den Haag Delft.

Healthy participants of SARS-CoV-2 vaccine trials, conducted at the Leiden University Medical Center (LUMC) in Leiden, The Netherlands, between 14-SEP-2020 and 02-JUN-

2022 were invited to fill in the survey. Forty people participated in phase 2 of the Ad26.COV2.S SARS-COV-2 vaccine trial (NCT04535453), 93 in a post-licensure fractional dose study with the mRNA-1273 SARS-COV-2 vaccine comparing intramuscular and intradermal administration (EUCTR2021-000454-26-NL), and 16 in a post-licensure fractional dose study investigating the immunogenicity and safety of needle free delivery of the mRNA-1273 SARS-COV-2 vaccine (NCT05315362). All volunteers took part in a screening visit before participation in the original clinical trial, which consisted of signing of the informed consent forms after receiving study information including risks, possible side effects and appointments and the opportunity to ask questions, followed by an anamnestic screening, physical examination and laboratory tests.

There were no exclusion criteria for this survey study and participation was voluntary.

Survey

The survey investigated factors influencing the motivation and decision-making process of clinical trial participants and was designed as previously described[10]. Briefly, survey items were chosen based on earlier research[11] and by discussion with researchers involved in the screening and recruitment of clinical trial participants. This survey has been implemented and evaluated in one other study[10] and can be found in the **Supplementary Appendix**.

Ten main domains were investigated: risk propensity, motivation, decision making, influence of others, experience of the clinical trial, severity of symptoms and trust in the medical staff, informed consent, right to withdraw and financial compensation.

The survey was anonymous and sent before start of the clinical trial on the inclusion day and on the day of the last trial visit. The electronic data capture system Castor was used to distribute the survey and record the responses. Participants who failed to fill in the survey were sent a maximum of two reminders.

Statistical analysis

The primary endpoint consisted of answers to the survey, expressed as a ranking of motivational factors and factors influencing the decision to participate. Secondary outcomes were comparison between pre- and post-survey answers, and answers to multiple choice questions on expectations, experience and opinion on ethical principles. Surveys that were filled in more than one day after start of the clinical trial and/or did not answer the primary endpoint were excluded from the analysis. Ordinal variables were

ranked from highest to lowest. Frequencies were calculated for all items. Concerning the motivation, decision-making process, the importance of the screening interview and the right to withdraw, we report the answers provided before clinical trial participation. In contrast, questions regarding the experience and evaluation of the participation and of the financial compensation, were derived from the questionnaire administered after the clinical trial. To analyze the pre- and post-trial differences, ranked variables were dichotomized as follows: items marked as important or somewhat important were combined into one (overall important) and the same was done for items marked as a little important or not important (overall not important). The percentage of answers falling into the category "overall important" was then compared on the pre- and post-questionnaire by means of the McNemar test. The Bonferroni correction was used to adjust for multiple testing. All calculations were carried out in IBM© SPSS© Statistics, version 25 and all graphs were made in GraphPad Prism, version 9.3.1.

Results

The survey response rate was 149/200 (75%). Most respondents were male (53%), students (53.7%) aged between 18 and 24 years old (57%). Moreover, 80.5% had not previously participated in clinical research and 79.9% did not work in a health-care related field (**Table 1**).

Table 1. baseline characteristics of the participants.

Baseline table	N (%)
Number of volunteers	149
Sex	
Male	79 (53)
Female	70 (47)
Age	
18-24 years	85 (57)
25 to 30 years	35 (23.5)
31-64 years	12 (8.1)
65+	17 (11.4)
Employment	
Student	80 (53.7)
Working	48 (32.2)
Other	21 (14.1)
Previously participated in research	
Yes	29 (19.5)
No	120 (80.5)
Employed in healthcare or healthcare related study	
Yes	30 (20.1)
No	119 (79.9)

Motivation for participation and decision to participate

When asked before their participation about motivational factors, the majority of respondents answered that “contributing to science” was either very important (43%) or somewhat important (46.3%). This was closely followed by the desire to “help people in the Netherlands”, which was deemed very important by 35.8% and somewhat important by 46.6% of respondents. ‘’Financial compensation’’ and ‘’personal experience with the disease’’ were the two factors most often classified as only a little important (40.9% and 31.5%, respectively) and not important (14.1% and 58.4% respectively). 18.8% of participants scored financial compensation as ‘very important’. (**Fig. 1 A**).

In 26.2% of cases, “contributing to science” was the single most important motivational factor, closely followed by “financial compensation” with 20.1% of responses (**Fig. 1 B**).

Respondents were asked to indicate which factors influenced their decision to participate in the clinical trial: “trust in the study team” was considered somewhat important by 40.5% and very important by 23% of respondents. In second place came the topic of the clinical trial, in this case “infectious diseases”, which was considered somewhat important by 35.1% and very important by 10.1% of respondents. “Chance of developing symptoms” and “opinion of others” were the two options that respondents weighted the least, being scored as somewhat important by 27.5% and 12.2% and very important by 8.7% and 2.7%, respectively (**Fig. 1 C**).

Similarly, the two single most important factors influencing the decision to participate were “research about infectious diseases” (22.3%) and “trust in the study team” (18.9%) (**Fig. 1 D**).

Before and after

The median time between administration pre- and post-trial questionnaires was nine weeks. Generally, answers did not differ before and after the clinical trial (**Supplementary Table 1** and **Supplementary Fig. 1**). Most importantly, motivational and decisional factors were largely unchanged in the pre- and post-surveys (**Fig. 1 E-F**). Items that did show significant differences were the views on the screening interviews and the financial compensation. As far as the screening is concerned, fewer respondents remembered the appointments discussed during the initial interview after trial completion compared to before (65.3% pre-trials vs. 42.1% post-trial). Awareness of the clinical trial schedules, as discussed during the screening interview, is important for participant compliance during the trial. However, as a decision-making factor, the awareness of the schedule before

the trial is most important. The perception of what participants considered the financial compensation to be intended for also changed to some degree, as fewer respondents saw it as a motivation for participation (56.2% pre-trial vs. 33.6% post-trial) and as a compensation for general expenses (52.1% pre-trial vs. 27.9% post-trial) after trial completion compared to before.

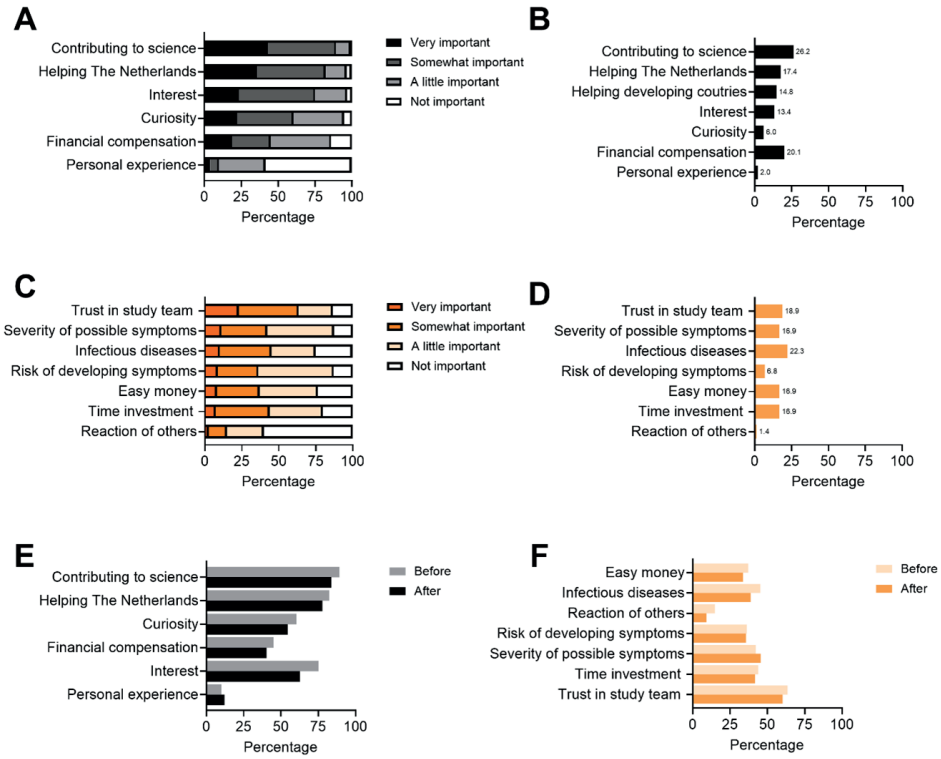

Figure 1. Motivational factors and factors influencing the decision to participate
Ranking of motivational factors (**A**) and single most important motivational factor for participating in clinical trials (**B**). Ranking of decisional factors (**C**) and single most important decisional factor (**D**) for participating in clinical trials. Bar graphs depicting motivational (**E**) and decisional factors (**F**) to participate before and after the clinical trial.

Reaction of others, screening, right to withdraw

Additional information regarding the participants' experience was collected prior to the clinical trial.

Generally, respondents received positive reactions about their participation (92.9%) (**Supplementary Fig. 2A**) and only few negative reactions (27.0%) (**Supplementary Fig. 2 B**). Others' opinion did not influence their decision to participate (85.8%) (**Supplementary Fig. 2 C**).

The screening was deemed as somewhat important and very important by only 19% and 2% of respondents, respectively (**Supplementary Fig. 3 A).** In line with this, 83.6% of respondents stated that the screening interview did not influence their opinion about the study, as they had already gathered enough information from the informed consent form they received beforehand **(Supplementary Fig. 3 B**). The most important thing respondents remembered from the screening were study related appointments (65.3%) (**Supplementary Fig. 2 C**). Most respondents agreed that the right to withdraw from the study at any time was either somewhat important (40.3%) or very important (32.6%) (**Supplementary Fig. 3 D**).

Evaluation of the experience and additional benefits

The evaluation of the experience was measured after completion of the clinical trial.

Receiving the vaccination was seen as positive by 49.0%, neutral by 34.3% and scary by 16.7% of the respondents (**Fig. 2 A**). The process of participating in the clinical trial was considered somewhat scary by only 1% of respondents; 52.1% and 18.8% found it somewhat or very interesting and 5.2% and 1.0% rated it as somewhat or very exciting, respectively (**Fig. 2 B**). In general, respondents were proud that they had participated in the clinical trial (87.5%) and said they would participate again (89.6%) and even advise others to participate (92.7%). 78.1% of respondents admitted that they gained a personal benefit, other than financial reward, from their participation (**Fig. 2 C-F**). When asked about the nature of such benefit (open questions), answers varied, but often focused on the health-related advantage of being vaccinated early. One participant wrote: “I was vaccinated, which was very nice. I also found it interesting to see how a clinical trial works and to learn about vaccines.” This answers exemplifies the dual motivations of clinical trial participants, who experienced the personal benefit of an early vaccination but also satisfied their scientific curiosity.

Financial compensation

Information about the financial compensation was derived from the post-trial questionnaire. As many as 44.7% of respondents would have taken part in the trial even without a financial compensation (**Fig. 3 A**). 87.5% rated the financial compensation as good, 9.4% found that it was too low and 3.1% too high (**Fig. 3 B**). The majority of participants used the financial compensation for “daily expenses” and “other” purposes and saw it as a “motivation for participation” (33.6%) or a “compensation for risk” (27.9%) (**Fig. 3 C, D**).

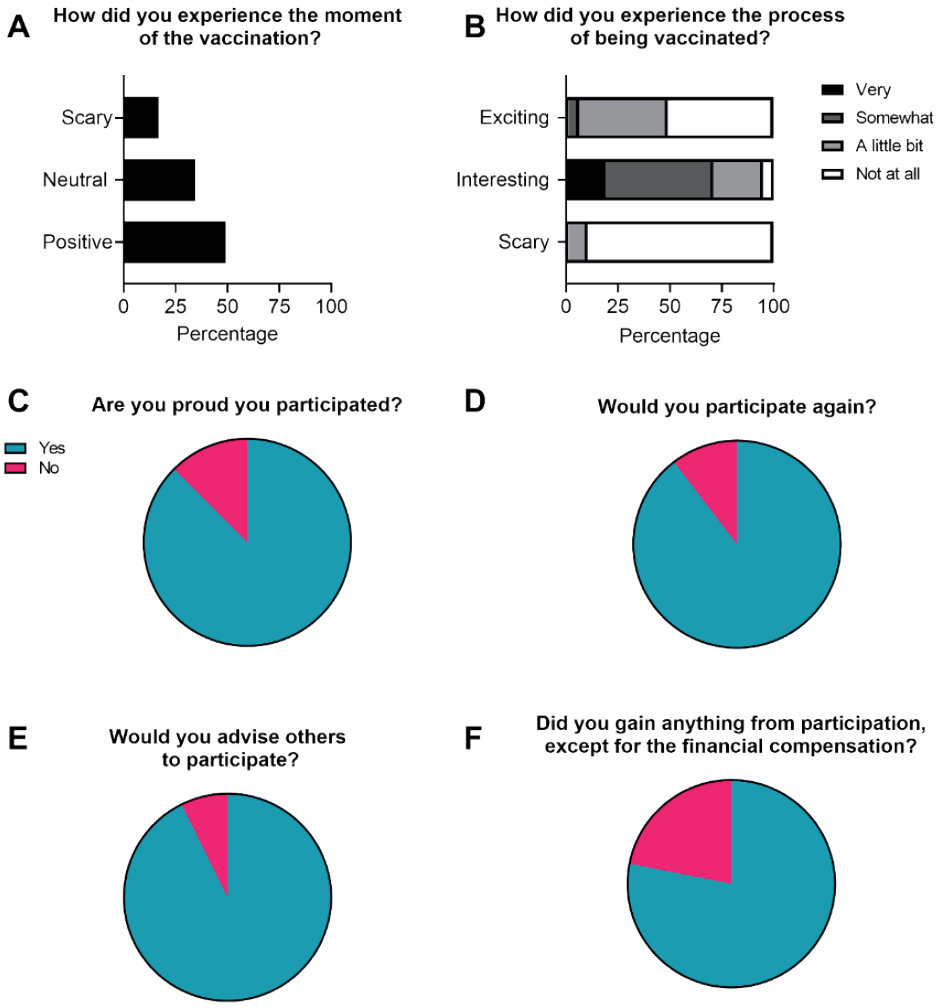

Figure 2. Evaluation of the participation
Bar graphs indicating how participants experienced the moment (**A**) and the process of the vaccination (**B**). Pie charts indicating whether survey respondents were proud they participated (**C**), would participate again (**D**), would advise others to participate (**E**) and whether they gained anything from participation except for the financial compensation (**F**).

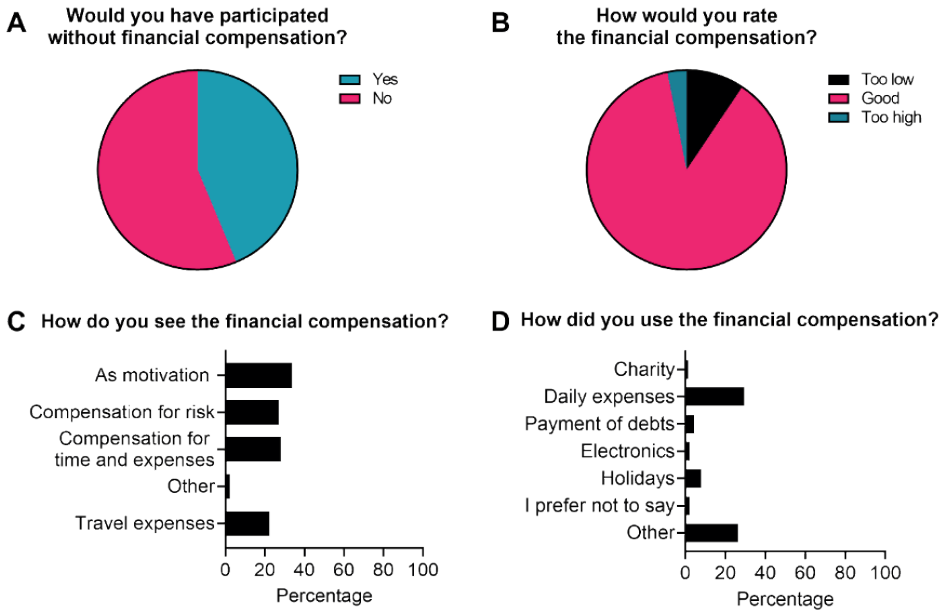

Figure 3. Financial compensation
Pie charts indicating how many respondents would have participated without financial compensation (**A**) and evaluation of the financial compensation (**B**). Bar graphs indicating what the respondents thought the financial compensation was intended for by the researchers (**C**) and its actual use by the respondents (**D**).

Discussion

In this study we described the characteristics and the motivational and decisional factors of SARS-CoV-2 clinical trial participants in the Netherlands. We also investigated whether answers were reported differently before and after participation.

We found that the majority of respondents were motivated by the desire to contribute to science or to helping the Netherlands. Financial compensation was classified as very or somewhat important by less than half of the respondents in a ranking of motivational factors, and was the single most important motivational factor for only 20% of participants. [10]. When deciding to take part in a trial, participants made a balanced decision involving trust in the study team, possible burdens and the importance of the research question. Reported motivational and decisional factors did not change after participation.

These results highlight the extraordinary conditions of the SARS-COV-2 pandemic. Mixed motivations have been reported in previous studies as well, but less pronounced than in the current instance[10,12]. While competence and friendliness of the study team were identified as important decisional factors in the past[11], financial reward is often indicated

as the main motivation for participating[11,12]. In this regard, it is interesting that as many as 44.7% of our survey respondents would have participated in the clinical trial even without a financial compensation.

The question then arises of whether SARS-CoV-2 study participants fall into a special category of medical volunteers, with distinct and mostly selfless motivations and, if so, what accounts for these characteristics.

We postulate that the underlaying motivations of medical volunteers participating in any clinical trial are fundamentally the same. However, external circumstances may affect how motivational factors are weighted, amounting to a different decisional outcome depending on the situation. According to our hypothesis, the decision to participate in a clinical trial is compounded by three elements: the reward of participating, the cost of inaction and the risk of suffering negative consequences from participation. If the sum of the rewards and the costs of inaction are higher than the risks, the decision will be in favor of participation, against it otherwise (**Fig. 4**).

The rewards of participating in a SARS-CoV-2 vaccination trial were high: besides the financial compensation, medical volunteers potentially derived a personal advantage from their participation, as it allowed them to be vaccinated before the rest of the population. Of course it should be noted here that while benefit from participation in clinical trials may be an added bonus, it is never guaranteed and one should be careful not to introduce therapeutic misconceptions about the scope of any type of clinical research.

Secondly, the cost of inaction was high, as the pandemic was a very tangible threat that directly affected the lives of clinical trial participants. In line with this, the proximity of the threat also accounts for its perceived danger and urgency. This concept has been most famously described by Peter Singer's drowning child thought experiment: humans feel more compelled to help when suffering is close by than when it is far removed[13,14].

It is not surprising then that the volunteer-led initiative 1DaySooner, whose members advocate for participation in high-risk, high-gain challenge studies [15], was founded during the pandemic. 1DaySooner continues to exist even now, yet it was the immediacy of the threat that brought it to life.

Any altruistic motivations, that is, the desire to help others independently of one's own gains[16], may also be considered an extension of this second postulate. In other words, the risk of inaction is determined by how heavily one weighs the lack of a cure for the disease under study. However, altruism remains a controversial philosophical and sociological concept and some have argued it to be a form of disguised egoism[17-19].

Finally, another factor that influences an individual's decision to partake in a clinical trial is the perceived risk. Most SARS-CoV-2 vaccine trial participants described in the current study were not scared of being vaccinated and judged the risk of the study to be absent or moderate. It is doubtful whether someone convinced that vaccinations would harm them would have participated in any SARS-CoV-2 trial, no matter what the benefits.

It should be noted that the evaluation of each of the above-mentioned factors, namely the rewards, the cost of inaction and the risks of participation, is subjective and highly dependent on personal beliefs and circumstances, which may be referred to as value system.

Comparing SARS-CoV-2 vaccination trials to the conceptual framework of other, "regular" clinical trials, shows how situational circumstances may affect the outcome of the decision-making equation.

Healthy individuals usually have little to no personal incentive for participating in clinical trials. The chances that they will directly benefit from the tested drugs is minimal and remote, making financial compensation the main reward. The risk of inaction is usually low for healthy individuals, as their lives are not directly affected by the outcome of the clinical trial. Conversely, the risk of participation is relatively high for healthy medical volunteers: if anything, they might be better off not taking part and avoiding the risk of side-effects.

In this model, financial compensation may be the only factor weighing against the risks and burdens of participation. We postulate that economic reward should not be considered an undue influence on participants, but rather a component of a weighted decision to do or do not take part in clinical research. While the absence of truly altruistic motivations may appear callous to some, it would be inappropriate to utter judgement. The framework presented herein is not meant as a moral guide, but rather as a tool to understand human behavior.

The second research question concerned the recall bias of surveys administered after clinical trial participation: little change in answers was observed comparing the pre- and post-participation surveys. The participants' judgement is unaffected by time and/or by the experience of the trial itself, further highlighting the consistency of our results over time.

The evaluation of the most important aspects of a clinical trial, are, among others, dependent on the education and information provided by the research team during the informed consent briefing. Providing extensive and adequate informed consent is of great importance to aid the potential participant in their decision to partake in the trial. Our study

shows that participants do weigh the provided information carefully and that the outlined expectations corresponded to reality, emphasizing the importance of the informed consent procedure.

Respondents were participants of a number of different types of trials, ranging from phase 2 vaccine studies to post-licensure fractional dose immunization studies. This might induce response bias, if answers differ depending on trial. Another concern is that self-reporting respondents may not be completely honest[20]. However, the surveys were anonymous, thereby alleviating the pressure to answer in a socially desirable way.

This study shows that the SARS-COV-2 pandemic induced clinical trial participants to behave differently than previously observed[10,21,22]. We propose a theoretical framework to explain the decision-making process that leads healthy individuals to participate in clinical trials. This framework shows that the underlaying motivations of medical volunteers participating in any clinical trial are fundamentally the same, but that context determines actual behavior. The framework can be a starting point for further research whether medical volunteers can identify with it.

Moreover, we show that recall bias of questionnaires administered after a clinical trial is negligible and that the answers to such questionnaires may be considered reliable. Overall, the opinions and impressions of medical volunteers are not easily swayed by time and the experience of the trial itself.

Our results highlight that the agency of medical volunteers who freely decide to participate in a clinical trial should be recognized. Especially in high-risk, high-gain situations, medical volunteers may consider a variety of factors when deciding to participate in a clinical trial. Just as it is important that individuals are not coerced into partaking in research against their will, their informed decision to do so, should also be honored.

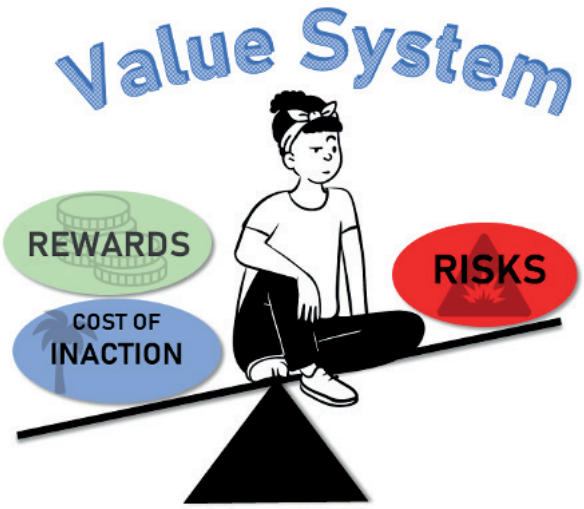

Figure 4. Theoretical model of the decision-making process of medical volunteers
The sum of the rewards and the cost of inaction must exceed the risks for a healthy individuals to decide to take part in a clinical trial.

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Supplementary appendix

Pre-participation survey

Risk Propensity Score

1. Please indicate the best score for each of the following statements (answers: 1 completely disagree to 9 completely agree). Do not think too long, often the first answer that comes to mind is the best.
    - Safety before everything else;
    - I do not take risks with my health;
    - I try to avoid risks as much as possible;
    - I regularly take risks;
    - I do not like not knowing what is going to happen;
    - I see risks as a challenge;
    - I see myself as: (1-risk-avoider 9-risk-taker).

General

2. Which study did you participate in?
3. Are you male or female?
4. How old are you? 18-24 / 25-30 / >30.
5. At the time of your participation in the trial were you: Student / Working / Unemployed.
6. Have you participated as a subject in medical research before? Yes / No.
7. Do you work in healthcare or do you follow a health-care related study? Yes / No.

Motivation

We want to investigate which factors influence the motivation to participate in our studies.

8. On a scale of 1 to 4 indicate how important the following factors were for your decision to participate (1=not important at all, 4=very important):
    - Curiosity 1 2 3 4
    - Contributing to science 1 2 3 4
    - Helping people in developing countries 1 2 3 4
    - The financial compensation 1 2 3 4
    - Interest in the subject 1 2 3 4
    - Personal experience with the disease 1 2 3 4
    - Other, namely…

    a. Which of these factors was most important to you?

Factors that influenced your decision to participate

9. On a scale of 1 to 4 indicate how much you weighted the following factors before deciding to participate:
    - Severity of possible symptoms 1 2 3 4
    - Chances of developing symptoms 1 2 3 4
    - How much time the study will cost 1 2 3 4
    - Easy money 1 2 3 4
    - Trust in the study team 1 2 3 4
    - The fact that the study is about parasites 1 2 3 4

    a. Which of these factors was most important to you?

Reaction of others

10. Did you discuss your participation with people around you? Yes / No.
    a. If no: why not (open question)?
    b. If yes: with whom?
    c. Did you receive positive reactions about your participation? Yes / No.
    d. Did you receive negative reactions about your participation? Yes / No.
    e. Did you receive other reactions about your participation?
    f. Did the reactions of others influence your decision to participate?
    g. Did you feel pressure to participate? Yes / No.
    h. If yes, why? I needed money / I did not want to say no after registering / I felt pressure from the study team / other.

How did you experience the vaccination?

11. How do you judge the risk of participating in this study? No risk / a little bit of risk / considerable risk / large risk.
12. Are you scared of complaints at the moment? Yes / No.

Complaints and trust in the study team

13. Do you think it is acceptable that a physician makes you sick for research purposes?
    - Yes, I am confident that I will be well looked after and that the trial will be safe.
    - Yes, if it contributes to science and to finding a treatment for serious diseases, then the advantages outweighs the disadvantages.
    - Yes, for both reasons above.
    - Yes, because of other reasons.

- No, that goes against the principle of doing no harm.
- No, that risk can never outweigh the benefits of the research.
- No, for both reasons above.
- No, because of other reasons.

Informed consent

14. How important was the screening for your decision to participate? Not important / a little important / somewhat important / very important.
15. What is the most important thing you remember from the screening? Possible complaints / risks of participation / appointments / lifestyle rules / other.
16. Did your opinion about the study change after the screening?
    - Yes, I thought the complaints would be more serious.
    - Yes, I thought the complaints would be less serious.
    - No, I had enough information from the informed consent form.
    - Other.
17. Can you briefly explain the scope of the study?

Right to withdraw

18. An important part of any study protocol is that volunteers can always withdraw from a study.
    a. How important is it to be able to withdraw from a study at any time? Not important / a little important / somewhat important / very important.
    b. In a controlled infection study, immediate withdrawal is often not possible, because treatment and follow-up must always take place for the safety of the volunteer. What do you think of this?
        - Logical, this is done for the participant's own safety and they know before the study starts.
        - This feels like a limitation to my right to withdraw at any time.
        - Other.

Financial compensation

19. Would you have participated without financial compensation? Yes / No.
20. How do you see the financial compensation? Compensation for time and expenses / travel expenses / compensation for risk / as motivation for participation / other.

21. What did you think of the height of the financial compensation? Good / too high / too low.
    a. If too high or too low: what would you consider a good amount?
22. If the risk of serious complications were twice as high in this study, what compensation would you consider appropriate?

Post-participation survey

Risk Propensity Score

1. Please indicate the best score for each of the following statements (answers: 1 completely disagree to 9 completely agree). Do not think too long, often the first answer that comes to mind is the best.
    - Safety before everything else;
    - I do not take risks with my health;
    - I try to avoid risks as much as possible;
    - I regularly take risks;
    - I do not like not knowing what is going to happen;
    - I see risks as a challenge;
    - I see myself as: (1-risk-avoider 9-risk-taker).

General

2. Which study did you participate in?
3. Are you male or female?
4. How old are you? 18-24/25-30/>30.
5. At the time of your participation in the trial you were: Student / Working / Unemployed.
6. Had you participated as a subject in medical research before? Yes / No.
7. Do you work in healthcare or do you follow a health-care related study? Yes /No.

Motivation

We want to investigate which factors influence the motivation to participate in our studies.

8. On a scale of 1 to 4 indicate how important the following factors were for your decision to participate (1=not important at all, 4=very important)
    - Curiosity 1 2 3 4
    - Contributing to science 1 2 3 4
    - Helping people in developing countries 1 2 3 4

- The financial compensation 1 2 3 4
- Interest in the subject 1 2 3 4
- Personal experience with the disease 1 2 3 4
- Other, namely…

a. Which of these factors was most important to you?

Factors that influenced your decision to participate

9. On a scale of 1 to 4 indicate how much you weighed the following factors before deciding to participate:
   - Severity of possible symptoms 1 2 3 4
   - Chances of developing symptoms 1 2 3 4
   - How much time the study will cost 1 2 3 4
   - Easy money 1 2 3 4
   - Trust in the study team 1 2 3 4
   - The fact that the study is about parasites 1 2 3 4

   a. Which of these factors was most important to you?

Reaction of others

10. Did you discuss your participation with people around you? Yes / No.
    a. If no: why not (open question)?
    b. If yes: with whom?
    c. Did you receive positive reactions about your participation? Yes / No.
    d. Did you receive negative reactions about your participation? Yes / No.
    e. Did you receive other reactions about your participation?
    f. Did the reactions of others influence your decision to participate?
    g. Did you feel pressure to participate? Yes / No.
    h. If yes, why? I needed money / I did not want to say no after registering / I felt pressure from the study team / other.

How did you experience the vaccination?

11. How do you judge the risk of participating in this study? No risk /a little bit of risk / considerable risk / large risk.
12. Were you scared of complaints at the moment? Yes / No.
    a. Did this change during the study? Yes / No
    b. If yes: did this increase or decrease?

13. How did you experience the moment of the vaccination? Positive/neutral/exciting/scary/other.
14. Indicate how you experienced being vaccinated
    - Exciting;
    - Interesting;
    - Frightening;
    - Otherwise.

Complaints and trust in the study team

15. How would you rate your complaints during the study? 0 no complaints, 10 was so severe that you had to quit the study.
16. Were the complaints as you had expected beforehand? Yes/No.
17. Do you think the complaints and risks of this research outweigh the expected benefit? Yes/No.
18. Do you think it is acceptable that a physician makes you sick for research purposes?
    - Yes, I am confident that I will be well looked after and that the trial will be safe.
    - Yes, if it contributes to science and to finding a treatment.
    - Yes for both reasons above.
    - No, that goes against the principle of do no harm.
    - No, that risk can never outweigh the benefits of the research.
    - No, for both reasons above.
    - No, other.

Informed consent

19. How important was the screening for your decision to participate? Not important / a little important / somewhat important / very important.
20. What is the most important thing you remember from the screening? Possible complaints / risks of participation / appointments / lifestyle rules / other.
21. Did your opinion about the study change after the screening?
    - Yes, I thought the complaints would be more serious.
    - Yes, I thought the complaints would be less serious.
    - No, I had enough information from the informed consent form.
    - Other.
22. Can you briefly describe the goal of the study?

Right to withdraw

23. An important part of any study protocol is that volunteers can always withdraw from a study.
    a. How important do is it to be able to withdraw from a study at any time? Not important / a little important / somewhat important / very important.
    b. In a controlled infection study, immediate withdrawal is often not possible, because treatment and follow-up must always take place for the safety of the volunteer. What do you think of this?
        - o Logical, this is done for the participant's own safety and they know before the study starts.
        - o This feels like a limitation to my right to withdraw at any time.
        - o Other.

Financial compensation

24. Would you have participated without financial compensation? Yes / No.
25. How do you see the financial compensation? Compensation for time and expenses / travel expenses / compensation for risk / as motivation / other.
26. What did you do with the financial compensation? Holidays / electronics / payment of debts / daily life / charity / I prefer not to say / other.
27. What did you think of the height of the financial compensation? Good/ too high / too low.
    a. If too low: what would you consider an appropriate amount?
28. If the risk of serious complications was twice as high as in the study you participated in, what compensation would you consider appropriate?
29. Did you gain anything from participation, except for the financial compensation? Yes / No.

Conclusion

30. Are you proud you participated? Yes / No.
31. Would you advise others to participate in similar research? Yes / No.
32. Would you participate again? Yes / No.
33. If not, why not? Too many complaints / time investment was too large / financial compensation was too low / other.

Supplementary tables

Supplementary Table 1

Question	Before	After
Risk propensity score		
Please indicate the best score for each of the following statements (answers: 1 completely disagree to 9 completely agree)		
Safety first	2.5 +/- 1.6	2.5 +/- 1.6
I do not take risks with my health	3.3 +/- 1.8	3.1 +/- 1.5
I try to avoid risks as much as possible	3.2 +/- 1.7	3.7 +/- 1.9
I regularly take risks	4.22 +/- 1.9	4.34 +/- 2.0
I hate not knowing what is going to happen	4.5 +/- 2.6	4.1 +/- 2.2
I see risks as a challenge	4.7 +/- 2.2	4.8 +/- 1.9
I see myself as a risk avoider	4.8 +/- 1.9	4.6 +/- 1.9
Motivation	**Pre**	**Post**
We want to identify which factors influence the motivation to participate in our studies. Please indicate how important the factors below were for your participation.		
Personal experience		
Not important	58.4 %	54.5 %
A little important	31.5 %	33.3 %
Somewhat important	6.0 %	11.1 %
Very important	4.0 %	1.0 %
Financial compensation		
Not important	14.1 %	13.1 %
A little important	40.9 %	46.5 %
Somewhat important	26.2 %	26.3 %
Very important	18.8 %	14.1 %
Curiosity		
Not important	5.4 %	5.1 %
A little important	34.2 %	40.4 %
Somewhat important	38.3 %	38.4 %
Very important	22.1 %	16.2 %
Interest		
Not important	3.4 %	3.0 %
A little important	21.5 %	34.3 %
Somewhat important	51.7 %	43.3 %
Very important	23.5 %	19.2 %
Aid in the Netherlands		
Not important	3.4 %	5.1 %
A little important	14.2 %	17.2 %
Somewhat important	46.4 %	51.5 %
Very important	35.8 %	26.3 %
Contributing to science		
Not important	0.7 %	1.0 %
A little important	10.1 %	15.2 %
Somewhat important	46.3 %	47.5 %
Very important	43.0 %	36.4 %
What was the single most important motivational factor?		

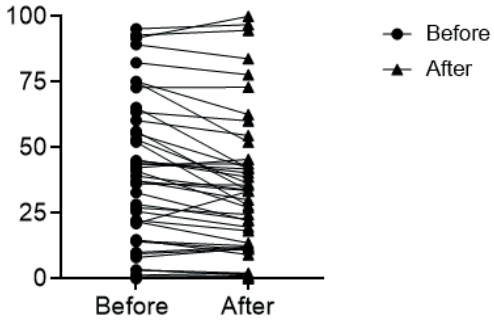
Contributing to science	26.2 %	37.4 %
Aid in The Netherlands	17.4 %	9.1 %
Aid in developing countries	14.8 %	15.2 %
Interest	13.4 %	6.1 %
Curiosity	6.0 %	6.1 %
Financial compensation	20.1 %	25.3 %
Personal experience	2.0 %	1.0 %
Participation		
Reaction of others		
Not important	60.1 %	62.2 %
A little important	25.0 %	28.6 %
Somewhat important	12.2 %	8.2 %
Very important	2.7 %	1.0 %
Time investment		
Not important	20.3 %	20.4 %
A little important	35.8 %	37.8 %
Somewhat important	36.5 %	33.7 %
Very important	7.4 %	8.2 %
Easy money		
Not important	23.6 %	31.6 %
A little important	39.2 %	34.7 %
Somewhat important	29.1 %	22.4 %
Very important	8.1 %	11.2 %
Risk of developing symptoms		
Not important	12.8 %	14.3 %
A little important	51.0 %	50.0 %
Somewhat important	27.5 %	31.6 %
Very important	8.7 %	4.1 %
Infectious diseases		
Not important	25.0 %	30.6 %
A little important	29.7 %	30.6 %
Somewhat important	35.1 %	28.6 %
Very important	10.1 %	10.2 %
Severity of possible symptoms		
Not important	12.8 %	9.1 %
A little important	45.0 %	45.5 %
Somewhat important	30.9 %	37.4 %
Very important	11.4 %	8.1 %
Trust in the study team		
Not important	13.5 %	14.3 %
A little important	23.0 %	25.5 %
Somewhat important	40.5 %	46.9 %
Very important	23.0 %	13.3 %
What was the single most important factor for your decision to participate?		
Severity of possible symptoms	16.9 %	17.3 %
Risk of developing symptoms	6.8 %	10.2 %
Time investment	16.9 %	16.3 %
Easy money	16.9 %	19.4 %
Trust in the study team	18.9 %	16.3 %
Infectious diseases	22.3 %	18.4 %

Reaction of others	1.4 %	2.0 %
Have you spoken to others about your participation?		
Yes	95.3 %	96.9 %
No	4.7 %	3.1 %
Who did you speak to about your participation?		
Colleague	28.4 %	22.5 %
Flat mate	22.3 %	18.3 %
Other	9.5 %	11.3 %
Parents	75.0 %	52.1 %
Partner	39.2 %	33.8 %
Study friend	25.7 %	19.7 %
Did you receive positive reactions about your participation?		
Yes	92.9 %	94.7 %
No	7.1 %	5.3 %
Did you receive negative reactions about your participation?		
Yes	27.0 %	24.5 %
No	73.0 %	75.5 %
Did the reactions you received influence your decision?		
Yes	14.2 %	11.2 %
No	85.8 %	88.8 %
Did you feel pressure to participate?		
Yes	0.7 %	1.0 %
No	99.3 %	99.0 %
If yes, why?		
Need money	0.0 %	0.0 %
Not wanting to say no	0.0 %	0.0 %
Pressure form the study team	0.0 %	0.0 %
Other	0.7 %	0.7 %
How do you judge the risk of participating in this study?		
Absent	26.4 %	31.1 %
Modest	72.3 %	66.7 %
Moderate	1.4 %	2.1 %
High	0.0 %	0.0 %
Are/were you scared of complaints at the moment?		
Yes	8.1 %	11.5 %
No	91.9 %	88.5 %
Did this change during the study?	NA	
Yes	NA	12.5 %
No	NA	87.5 %
Indicate how you experienced being vaccinated:	NA	
Exciting	NA	
Not at all	NA	51.0 %
A little	NA	42.7 %
Somewhat	NA	5.2 %
Very	NA	1.0 %
Interesting	NA	
Not at all	NA	5.2 %
A little	NA	24.0 %
Somewhat	NA	52.1 %
Very	NA	18.8 %
Frightening	NA	

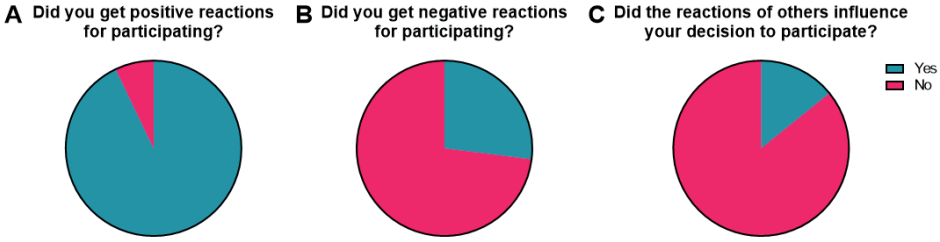
Not at all	NA	89.6 %
A little	NA	9.4 %
Somewhat	NA	1.0 %
Very	NA	0 %
Other	NA	NA
How did you experience the moment of the vaccination?	NA	
Positive	NA	49.0 %
Neutral	NA	34.4 %
Exciting	NA	16.7 %
Did you experience anything else?	NA	
Yes	NA	7.3 %
No	NA	92.7 %
How would you rate your complaints during the study?	NA	NA
Were the complaints as you had expected beforehand?	NA	
Yes	NA	79.2 %
No	NA	20.8 %
Do you think the complaints and risks of this research outweigh the expected benefit?	NA	
Yes	NA	88.5 %
No	NA	11.5 %
Do you think it is acceptable that a physician makes you sick for research purposes?		
Yes, I am confident that I will be well looked after and that the examination will be safe	34.9 %	NA
Yes, if it contributes to science and to finding a treatment serious diseases, then the disadvantage outweighs the advantages	15.6 %	NA
Yes for both reasons above	38.5 %	NA
Yes, because of other reasons	2.8 %	NA
No, that goes against the principle of do no harm	1.8 %	NA
No that risk can never outweigh the benefits of the research	2.8 %	NA
No, for both reasons above	0%	NA
No, because of other reasons	3.7 %	NA
How important was the screening for your decision to participate?		
Not important	32.7 %	28.1 %
A little important	46.3 %	38.5 %
Somewhat important	19.0 %	29.2 %
Very important	2.0 %	4.2 %
What is the most important thing you remember from the screening?		
Possible complaints	53.1 %	36.4 %
Risks	37.4 %	30.0 %
Appointments	65.3 %	42.1 %
Lifestyle rules	21.8 %	13.6 %
Other	3.4 %	1.4 %
Did your opinion about the study change after the screening?		
Yes, I thought the complaints would be more serious	11.0 %	10.4 %
Yes, I thought the complaints would be less serious	3.4 %	2.1 %
No, I had enough information from the informed consent form	83.6 %	86.5 %
Other	2.1 %	1.0 %
How important is it to be able to withdraw from a study at any time?		

Not important	4.2 %	4.2 %
A little important	22.9 %	22.9 %
Somewhat important	40.3 %	41.7 %
Very important	32.6 %	31.3 %
In a controlled infection study, immediate withdrawal is often not possible, because treatment and follow-up must always take place for the safety of the patient. What do you think of this?		
Logical, this is done for the participant's own safety and they know before the study starts	NA	NA
This feels like a limitation to my right to withdraw at any time	NA	NA
Other	NA	NA
Would you have participated without financial compensation?		
Yes	43.7 %	43.7 %
No	56.3 %	56.3 %
How do you see the financial compensation?		
Compensation for time and expenses	52.1 %	27.9 %
Travel expenses	32.9 %	22.1 %
Compensation for risk	41.1 %	27.1 %
As motivation	56.2 %	33.6 %
Other	3.4 %	2.1 %
What did you do with the financial compensation?	NA	
Holidays	NA	7.9 %
Electronics	NA	2.1 %
Payment of debts	NA	4.3 %
Daily expenses	NA	29.3 %
Charity	NA	1.4 %
I prefer not to say	NA	2.1 %
Other	NA	26.4 %
What did you think of the height of the financial compensation?		
Good	88.2 %	87.5 %
Too high	3.5 %	3.1%
Too low	8.3 %	9.4 %
Did you gain anything from your participation except for the financial compensation?	NA	
Yes	NA	78.1 %
No	NA	21.9 %
Are you proud you participated?	NA	
Yes	NA	87.5 %
No	NA	12.5 %
Would you advise others to participate in similar research?	NA	
Yes	NA	92.7 %
No	NA	7.3 %
Would you participate again?	NA	
Yes	NA	89.6 %
No	NA	10.4 %
If no, why not?	NA	
Too many complaints	NA	0.0%
Time investment too large	NA	4.3 %
Financial compensation too low	NA	0.0 %
Other	NA	3.6 %

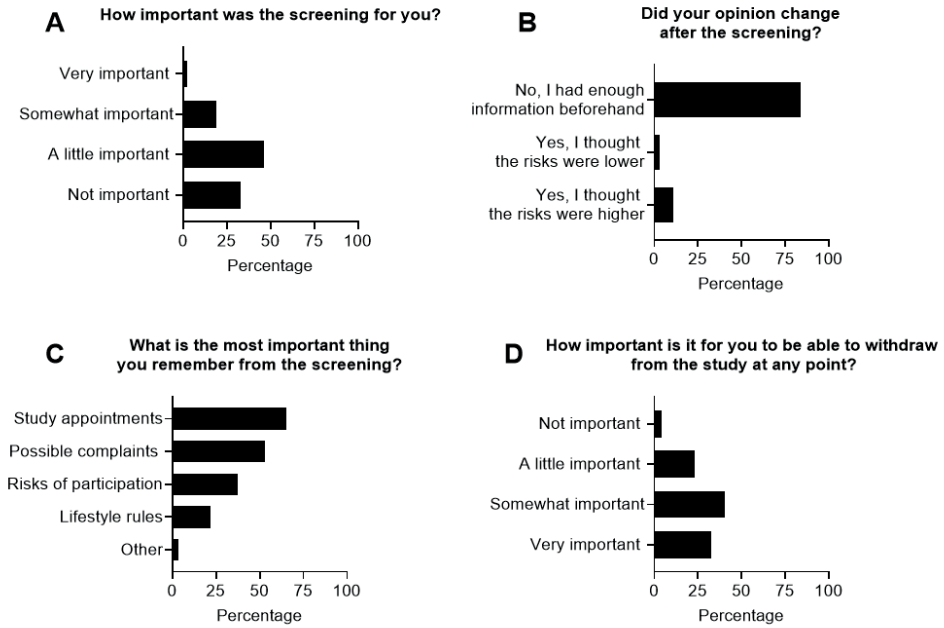
Supplementary figures



Supplementary Figure 1. Answers before and after participation in the clinical trial.



Supplementary Figure 2. **Reaction of others**
Pie charts indicating how many participants received a positive (**A**) or negative (**B**) reaction about their decision to participate and whether the influence of others' influenced their decision to participate (**C**).



Supplementary Figure 3. **Screening**
Bar graphs indicating how important the screening was (**A**), the most important information participants remembered from the screening (**B**), whether the screening changed their opinion about the trial they planned to participate in (**C**) and the importance of the right to withdraw **(D**).

Chapter 5

Risk in healthy volunteer studies: Does it matter if the agent is a drug or a bug?

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Submitted: Trials

Abstract

Clinical research, involving healthy human participants, has, because of its very nature, been the subject of extensive discussions, as adverse events can be minimized yet never completely avoided. Controlled human infection (CHI) studies where participants are exposed to infectious agents in a controlled setting have received additional scrutiny being perceived as particularly risky.

In the following we analyzed whether, and if so how CHI studies differ from phase 1 clinical trials in matters of risk to participants themselves and risks to others through disease transmission. We found CHI and phase 1 studies to be generally comparable, with risk to others may be the potentially most consequential difference between CHI and phase 1 studies. Finally, we propose a set of guidelines to facilitate the evaluation of CHI studies by medical ethical committees in the future.

Introduction

Phase 1 clinical trials are characterized by the administration of novel pharmacological compounds to humans to determine safety and tolerability profiles and pharmacodynamics and -kinetics. Without phase 1 clinical trials much of the current medical and pharmacological knowledge would not have been possible, as testing in humans is a prerequisite for the approval of any new drug[1,2].

CHI studies, involving the exposure of healthy participants to pathogens in a controlled setting, have been implemented for a long time [3] and are a generally well-established and accepted scientific tool[4]. Recent discussions have focused on CHI studies for novel pathogens (i.e. the Zika and the Sars-CoV-2 virus)[4,5]: it could be argued that CHI studies are most useful in these settings, accelerating both our understanding of new diseases as well as vaccine and therapy development. However, novel diseases may be unpredictable with no rescues treatment, thereby making potential risks to participants difficult to mitigate or counteract [6-8]. Additionally, CHI studies with infectious agents may not only involve risk for the participants themselves, but also affect bystanders through disease spread. While phase 1 clinical trials are never completely devoid of risk, they do not generally affect anyone other than the participants themselves[7]. Risk may therefore be seen as a factor a distinguishing CHI studies from phase 1 clinical trials.

In the following we will discuss the (perceived) risks of CHI studies compared to more traditional phase 1 clinical trials, focusing on the risks to the participants themselves and risk to bystanders.

Risks and burdens to participants

Despite extensive safety measures, clinical research always entails a risk of adverse events. However, healthy individuals should not suffer irreversible long-term sequelae from participating in research and this holds true for both phase 1 clinical trials and CHI studies[9-11]. In some publications risk is defined to include the burden to participants[12], whereas in others risk and burden are considered separate concepts[13]. For a more accurate comparison between studies, risk and burden will be discussed as separate entities in this paper.

Risk, defined as the danger to incur serious harm, is based on objective, measurable criteria. This has also been described as the “risk of harm”, a serious, but rare occurrence, which could be considered the “worst-case scenario” and needs to be mitigated as much as possible[13].

Burden or “risk of discomfort” refers to any negative experience of transient nature that is part of trial participation. Some degree of burden is inherent to trial participation and any study procedure can, in this definition, be considered as such. However, the subjective experience of burden will vary from person to person[13,14].

Phase 1 clinical trials assessing novel pharmacological compounds in humans generally have a low risk. Not all phase 1 clinical trials are necessarily first in human trials as well. In trials assessing different doses of a new compound, only the very first administration is considered as first in human. Similarly, testing the effectiveness of an old drug for a new disease may also be considered as a phase 1, yet not a first in human, clinical trial[15]. While symptoms may vary, and some participants may not experience any symptoms at all, systematic meta-analyses have shown that the most common adverse events in phase 1 clinical trials with healthy participants are mild to moderate and include headache, drowsiness and diarrhea[16,17]. Dosing of a novel compound is calculated based on the minimum anticipated biological effect level (MABEL) and no observed adverse events levels (NOAEL) and derived from animal experiments[18,19]. However, for some compounds there still can be a small factor of uncertainty in translating preclinical findings to clinical research as animal models may not always be representative, causing risks to humans to be overlooked[20]. Phase 1 clinical trials, where previously healthy participants have suffered irreversible consequences, have been reported in the past[21-23]. These incidents mostly stem from inaccurate translation from animals models to testing in humans or from the occurrence of serious unexpected adverse events[21]. Additional safety measures, such as careful dose-escalation and sentinel exposure of one participant prior to dosing others, are put in place to further reduce the risks of adverse events[21,24].

Prior to execution of a CHI study, information about the mechanisms of action and possible risks to participants are gathered: the course of disease and related adverse events are usually well-known and predictable[25]. In some CHI studies symptoms may even be used as outcome measures, such as in challenge models for gastro-intestinal pathogens[26-28]. It could nevertheless be argued that there are instances in which risks remain high, such as CHI studies involving highly pathogenic micro-organisms, for example Ebola or Anthrax. However, just like pharmacological compounds tested in phase 1 clinical trials undergo extensive screening prior to administration to humans, we argue that the same is true for pathogens. In theory, both pathogenic micro-organisms and highly toxic substances could be tested in humans in phase 1 clinical trials without prior risk assessment through preclinical research. Yet holding onto the principle of not causing irreversible damage to participants, neither drugs nor pathogens that are known to be harmful or even deadly to humans should go into clinical testing, no matter the scientific or social benefits[9-11]. An

exception are chemotherapeutic agents which may have potentially serious side effects and are therefore frequently tested in patient populations rather than healthy participants [29]. The argument that one would be more easily tempted to conduct high risk CHI studies rather than high risk phase 1 clinical trials is also null because both are equally possible and there is no intellectual nor practical incentive to prefer the one over the other.

The burdens of phase 1 clinical trials and CHI studies are variable: they can include lifestyle and dietary restrictions to optimally monitor pharmacokinetics and pharmacodynamics as well as more invasive procedures, such as such as lumbar punctures or pain stimuli[30]. For many trials, participants are expected to remain at the study facility for a period of time, ranging from a couple of hours to one or two weeks, to ensure safety and accuracy of measurements.

When comparing different trial types, it is helpful to consider the objective risk of harm and burden as separate concepts, which can be visualized as a spectrum of two intersecting axes, ranging from high to low in both directions (**Fig. 1**). Clinical research can then be localized on this matrix, according to the perceived consequences of any given study on its participants.

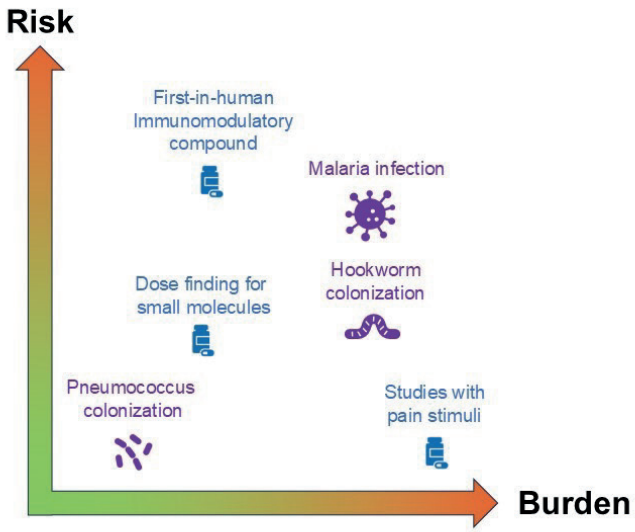

Figure 1. Risk and burden in phase 1 clinical trials and CHI studies
A first-in-human phase 1 clinical trial with a first in class immuno- or neuro-active compound will always, despite extensive pre-clinical studies, involve some degree of uncertainty simply because of its novelty. If such a study also includes invasive procedures, such as a lumbar puncture, it would classify as high risk and relatively high burden (upper right quadrant). In contrast, a dose-finding study for small molecules belonging to a class of compounds already tested in humans, has a low chance of mild adverse events and limited number of study visits, therefore classifying as low risk and low burden (lower left quadrant).

Pneumococcus colonization, which has been introduced to investigate vaccine efficacy[31] and to study factors that influence colonization[32], is a CHI model associated with a small chance of mild adverse events[33]. After being nasally instilled with *Streptococcus pneumoniae*, participants are monitored for symptoms and evidence of colonization [31]. Pneumococcal colonization itself is a natural occurrence and with a very small chance of treatable complications in healthy young individuals[34], therefore considered low risk. Burden is also low as except for recording potential symptoms, there are no lifestyle restrictions for participants [35]. In contrast, controlled human malaria infections (CHMI), consisting of the infection of healthy participants in a controlled setting[36], involve a higher risk to participants because, if left untreated, it is a potentially fatal disease[37]. Burden is also higher as participants are required to undergo blood draws on several occasions[38].

In conclusion and in line with the current WHO's stance on the matter[4], we argue that the risk to which CHI study participants are exposed is not inherently different from that of phase 1 clinical trials, as both risks and burden depend on individual study characteristics rather than their classification as phase 1 clinical trials or CHI studies. While this reasoning does not exclude the possibilities of specific subcategories of high risk and high burden, there is no reason to assume that these are more likely to belong to the larger CHI or phase 1 grouping. Both highly pathogenic organisms as well as highly poisonous chemical compounds exist and both could theoretically be tested in humans. To prevent harm to study participants a set of rules and regulations have been set in place, which apply in equal measure to clinical trials and CHI studies[9-11].

Risk to others: the right to withdraw, bystander risk and quarantining

Linked to the risks to the participants themselves are the risks to others. Bystander risk refers to third parties experiencing harm without participating in clinical research themselves and is often overlook by ethical regulations[39,40].

There are examples of phase 1 clinical trials with risks to others, such as studies involving potentially teratogenic substances. These clinical trials entail a risk for an unborn child, but may also be seen as burdensome as female partners of male participants are required to use double contraception[40]. However, the risks to bystanders are more prominent in CHI models[41,42] and it has been previously argued that some degree of bystander risk may be acceptable if justified by the social value of the research[7].

Closely linked to bystander risk is the necessity for quarantine. The right to withdraw from a clinical trial at any time is one of the fundamental stipulations of voluntary participation[9-11]. However, in CHI studies immediate withdrawal is not always possible, as treatment and follow-up visits may be necessary for the safety of the participants themselves but also to protect others[4].

Both phase 1 clinical trials and CHI studies occasionally require participants to stay in an in-patient facility. In phase 1 clinical trials, permanence in a facility serves to carefully monitor participants for both scientific and safety purposes. Withdrawal and refusal to remain in the research center could impact the quality of the collected data, but, from a safety perspective, it would affect the participants only[43]. Restrictions imposed on phase 1 clinical trial participants are therefore more practical in nature and good management, exhaustive informed consent and adequate monitoring during the study can limit early withdrawal. Most importantly, the right of the participants to withdraw at any time takes precedence over other matters, leaving them in charge of deciding whether and for how long they want to be part of research. In contrast, in certain CHI studies, quarantine may be necessary to prevent an infection from spreading beyond the boundaries of a controlled setting[44], resulting in risk to bystanders.

When evaluating bystander risk, it is important to determine risk of transmission, severity of transmission and background risk of contracting the disease[7,40]. Risk and severity of transmission vary per studied pathogen. *Vibrio cholerae* CHI studies are an example where the pathogen can be transmitted in the absence of proper hygiene and sanitation. Additionally, some patients may become severely ill and require intravenous rehydration[45]. Hence, if an infected participant were to leave the research center prior to study conclusion, this could, potentially, initiate a cascade of transmission and have negative consequences for bystanders.

However, it is important to realize that there are also many CHI models that either do not involve transmissible pathogens or that, when they do, have a low risk of transmission which only becomes problematic in specific instances[46,47]. For instance, hookworm colonization studies have a very low risk of transmission: eggs derived from feces can only hatch if they are inoculated in soil at warm temperatures, making transmission highly unlikely in areas with moderate climates and universal access to adequate sanitation[48] (**Fig. 2**).

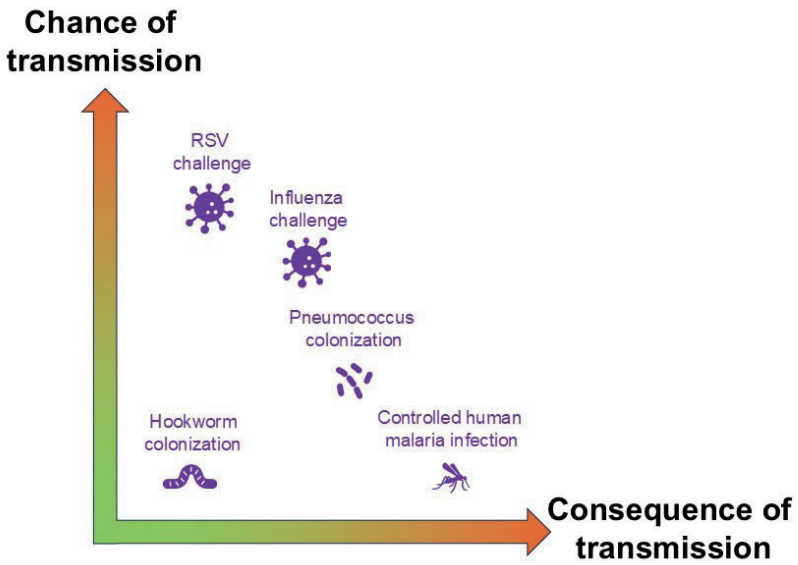

Figure 2. Chance and consequence of transmission without mitigating factors in CHI studies
When analyzing risk to bystanders through transmission in CHI studies, it is important to determine the chance of transmission of any disease and the consequence of such transmission occurring. For instance, hookworm colonization studies have a very low risk of transmission as long as adequate sanitation is present. The consequences of transmission are also limited as hookworm infection is not life-threatening. CHMIs are another example with a low chance of transmission (as long as the vector is not present), but with potentially fatal consequences if the transmission were to happen. Controlled infections with respiratory pathogens (*Pneumococcus,* influenza or respiratory syncytial virus) all have a higher chance of transmission, yet consequence are either treatable or generally mild. It should be noted that in all instances appropriate precautions are taken to prevent transmission to others[31,46,49].

Another factor that needs to be considered is the background risk of contracting a disease, that is, the chance of becoming ill independently of participation in clinical research. An example where background risk is applicable are CHI studies with respiratory viruses: especially in the winter months incidence increases among the general population meaning that the background risk of transmission is also higher[50]. However, the endemicity of an illness does not exonerate from identifying susceptible populations and devising strategies to protect them. In some studies involving respiratory viruses, such as rhinovirus or respiratory syncytial virus outpatient challenges, risks to third parties can be mitigated by implementing precautionary measures and specific hygiene rules (e.g. distancing and wearing a face mask)[51].

Despite the above exceptions, it has been argued that maintaining the quarantine until participants are no longer infectious in some CHI studies is pivotal for the safety of society and becomes a priority which may conflict with the right to withdraw of the single

participant. The fact that CHI study participants may be required to give up part of their autonomy, even if willingly so, distinguishes this type of research from phase 1 clinical trials[9-11].

However, it should be noted that the right to withdraw is never formally compromised in such cases. In other words, individuals are still allowed to interrupt participation at any desired timepoint, yet it is the dismissal from isolation that may be postponed, for their own and others' safety. Hence, one must distinguish between violations of the participants' autonomy as such and a delay in the physical freedom of movement. Another way to mitigate the infringement of personal freedom of movement is to allow participants to quarantine in their own home[52]. Extensive informed consent to explain the risks and consequences of CHI participation and the consequent requirement for quarantining can further alleviate the pressure on participants. Previous studies, investigating CHI participants' opinion on the matter, found that the majority understood and agreed with the need to treat a disease before allowing for definitive withdrawal[53].

Summarizing, there is a small subset of CHI studies that differs from other clinical research because there is a possibility of transmission to third-parties. Quarantining may be put in place to protect bystanders, affecting the ability of participants to withdraw at any desired timepoint. Medical ethical committees should carefully consider CHI studies with these characteristics, making sure that the risks do not exceed the benefits and that the measures imposed on participants remain reasonable. However, even in cases where quarantine may be necessary and participants may experience temporal limitations to their physical freedom, the integrity of the right to withdraw and personal autonomy is guaranteed.

Based on our risk analysis and the extensive comparison between phase 1 clinical trials and CHI studies, we have identified a set of key questions that researchers may consider prior to initiating a CHI study and which we have summarized in **Table 1**. This tool is meant to facilitate risk assessment of CHI studies with new and old pathogens. While this does not replace assessment by a qualified medical ethical committee, it may help researchers to enhance both the safety and overall risk analysis of CHI protocols.

Table 1. Guidance for potential questions to be added to the risk analysis of CHI studies

Risk to the participants
Have other CHI studies with the same type of pathogens been conducted in the past and, if so, what were the outcomes?
What are the expected symptoms and/or adverse events and how will they be mitigated?
What are the long-term effects of the pathogen under study?
Is there an effective treatment for the pathogen under study? Is the disease under study self-limiting?
Has a certain dose of the pathogen been tested before and with what outcomes?
Have participants been selected to minimize risks to their health?
What are the side effects and duration of treatment for the pathogen under study?
Risk to bystanders/risk of transmission
How infectious is the pathogen under study?
Is there a risk of transmission for bystanders?
What are the chances of transmission, should the infectious agent be released in the environment?
Are there susceptible members of the population that need to be protected from the disease under study?
What steps can be taken to protect these susceptible populations from infection?
What is the background risk of infection for this particular disease in the general population?
What measures need to be put in place in general to avoid transmission?
Are quarantine measures for participants necessary and if yes, how long and how much will quarantine measures affect participants?
Is the informed consent and participant information clear about which measures (e.g. quarantine) are put in place to protect bystanders and how this will affect participants' ability to retire from the study at any time?

Conclusion

Clinical trials have been and will remain invaluable for scientific innovation. CHI studies are likely to become even more important in the future given the rise of new pandemic and the necessity to quickly respond to them. In this paper we have extensively analyzed the differences and similarities between phase 1 clinical trials and CHI studies as far as risks to participants and risks to bystanders are concerned. While some of these points have been made earlier[7,8,54], we provide a comprehensive review risk assessment of CHI studies compared to phase 1 clinical trials.

Although we state that CHI studies are comparable to other research with healthy participants, we acknowledge that bystander risk and risk of disease transmission require additional scrutiny. Based on our analysis in this paper, we provide guidance to analyze the ethical acceptability of CHI studies. **Table 1** lists a number of questions that could be answered prior to conducting a CHI study. While this is not meant as a replacement of the local regulatory authorities, it is supposed to complement them and encourage reflection on behalf of the researcher. The guidelines provided in **Table 1** could facilitate and improve safety evaluation and risk analysis of CHI studies.

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Chapter 6

Creation and preclinical evaluation of genetically attenuated malaria parasites arresting growth late in the liver

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Published: NPJ Vaccines, 2022
DOI: 10.1038/s41541-022-00558-x

Abstract

Whole-sporozoite (WSp) malaria vaccines induce protective immune responses in animal malaria models and in humans. A recent clinical trial with a Wsp vaccine comprising genetically-attenuated parasites (GAP) which arrest growth early in the liver (PfSPZ-GA1), showed that GAPs can be safely administered to humans and immunogenicity is comparable to radiation-attenuated PfSPZ-Vaccine. GAPs that arrest late in the liver stage (LA-GAP) have potential for increased potency as shown in rodent malaria models. Here we describe the generation of four putative *Pf* LA-GAPs, generated by CRISPR/Cas9-mediated gene-deletion. One out of four gene-deletion mutants produced sporozoites in sufficient numbers for further preclinical evaluation. This mutant, *Pf*Δ*mei2,* lacking the *mei2-like RNA* gene, showed late liver growth-arrest in human liver-chimeric mice with human erythrocytes, absence of unwanted genetic alterations and sensitivity to antimalarial drugs. These features of *Pf*Δ*mei2* make it a promising vaccine candidate, supporting further clinical evaluation. *Pf*Δ*mei2* (GA2) has passed regulatory approval for safety and efficacy testing in humans based on the findings reported in this study.

Introduction

Whole-sporozoite (WSp) malaria vaccines can induce strong protective immune responses in animal models of malaria and in humans[1-4]. WSp vaccines consist of whole parasites, i.e. metabolically active *Plasmodium. Falciparum (Pf)* sporozoites (PfSPZ) that have the ability to migrate to and infect the human liver, but cannot transform into the symptomatic blood stage, and as such do not cause disease. The most advanced WSp vaccine candidate, the PfSPZ Vaccine, employs sporozoites that have been attenuated by radiation[5]. These sporozoites enter hepatocytes but are unable to replicate and thus abort development early in the liver. The PfSPZ Vaccine, consisting of cryopreserved, vialed radiation-attenuated sporozoites and produced by the biotech company Sanaria, Inc. (US), has entered phase 3 clinical development[6,7]. Sporozoite attenuation by genetic modification rather than by radiation, offers the advantage of a more homogeneous product, increased biosafety for sporozoite production and potentially increased potency[8,9]. In a recent clinical study, human volunteers were immunized by intravenous injection with multiple doses of cryopreserved, vialed sporozoites of either the PfSPZ Vaccine or a WSp vaccine, consisting of genetically-attenuated parasites (GAP) that arrest growth soon after invasion of hepatocytes (early-arresting GAP; EA-GAP)[10,11]. This study showed that the EA-GAP vaccine, termed PfSPZ-GA1, was safe and induced immune responses that were comparable to those induced by the radiation attenuated PfSPZ Vaccine[10]. Multiple clinical studies with the PfSPZ Vaccine in healthy volunteers from non-endemic areas (e.g. without prior exposure to malaria), have shown that this vaccine can induce levels of protection that are higher than those achieved with various subunit malaria vaccines (protein, peptide, or DNA-based)[3,12,13]. However, high sporozoite numbers are needed to achieve sufficient levels of efficacy. Moreover, the efficacy of the PfSPZ Vaccine appears to be lower in people living in areas of malaria endemicity compared to people with no previous history of malaria infection[14,15], creating a need for highly potent WSp vaccines to achieve the 75% protection against clinical disease for >1 year as targeted in the malaria vaccine technology roadmap (https://www.malariavaccine.org/malaria-and-vaccines/malaria-vaccine-roadmap). Chemo-attenuated PfSPZ immunization studies suggest that the WSp vaccine efficacy may be improved by delaying the growth arrest in the liver and thereby broadening the antigen repertoire[16]. A study using a mouse malaria GAP has shown that attenuated sporozoites that arrest growth late in the liver (late-arresting GAP; LA-GAP) through deletion of a gene encoding a protein involved in fatty acid synthesis (Fabb/f), induced stronger protective immune responses than immunization with EA-GAP sporozoites[17]. The increased potency can most likely be explained by the exposure to an increased (liver-and blood-stage) antigen repertoire and/or biomass of LA-GAP

liver-stages compared to those of EA-GAP. Unfortunately, removal of several fatty acid synthesis genes from the *P. falciparum* genome, results in the arrest of parasite growth in the mosquito preventing sporozoite formation[18,19]. These genes are therefore unsuitable targets for creating a *Pf* LA-GAP for human use. To generate a *Pf* LA-GAP, we selected in this study four additional genes (*palm, hcs1, cbr, mei2*) with a putative specific role in *Pf* late liver-stage development, based on published phenotypes of rodent malaria mutants that lack the orthologous genes. We report the creation and analysis of four *Pf* gene-deletion mutants that lack the *palm, hcs1, cbr* or *mei2* gene. We show that three gene-deletion mutants did not show the expected phenotype of sporozoite production, essential for further preclinical evaluation. Only the *Pf* mutant lacking the *mei2* gene (*Pf*Δ*mei2*) produced infective sporozoites that arrest growth late during development in the liver. We report results of preclinical evaluation of *Pf*Δ*mei2,* involving evaluation of late liver growth-arrest in cultured primary human hepatocytes and in chimeric mice with humanized liver and human red blood cells, genotype analysis by whole-genome sequencing and *in-vitro* drug-sensitivity testing of blood stages. Based on these preclinical studies, *Pf*Δ*mei2* has passed regulatory approval for clinical evaluation of safety and efficacy as a LA-GAP vaccine (GA2).

Results

Selection of genes with a role during late liver-stage *Plasmodium* development

Gene-deletion studies have identified multiple proteins that play a role during late liver-stage development of rodent malaria parasites. **Table 1** shows liver-stage growth phenotypes of published gene-deletion mutants that display a normal, wild type-like blood-stage and sporozoite development but present defects in late liver-stage development. Most mutants do not display full growth arrest in the liver as revealed by 'break-through' blood infections, although with a (strong) increase in prepatency. Deletion of genes encoding apicoplast-located proteins involved in fatty acid synthesis results in the strongest attenuation phenotype, specifically in *Py*, with complete growth arrest. However, FASII-pathway genes, involved in the transformation of Acetyl-CoA into Acyl-ACP, are unsuitable targets for generation of *Pf* LA-GAP because this pathway is essential for formation of viable sporozoites[18]. It can be assumed that genes encoding the apicoplast-located proteins of the pyruvate dehydrogenase (PDH) complex, which provides Acetyl-CoA, are also essential for *Pf* sporozoite formation. Therefore, we excluded FASII-pathway genes for generation of *Pf* LA-GAP. Four genes with a previously reported strong attenuation phenotype, *palm, hcs1, cbr,* and *mei2,* were selected for creating *Pf* LA-GAPs. This

selection was based on the absence of blood infections and/or compelling extension of the prepatent period after infection of mice with gene-deletion mutant sporozoites, while these sporozoites developed into late liver-stages in cultured hepatocytes (**Table 1**). The *Plasmodium berghei (Pb)* PALM protein, whose exact function is unknown but plays a role during the final steps of *Pb* liver-stage merozoite formation[20], has been shown to localize to the apicoplast[20]. HCS1, an ATP-dependent ligase that catalyzes biotin binding to biotin carboxylase, plays a role in adequate formation of *Pb* liver-stage merozoites[21] and is expressed in the cytoplasm of liver-stages[21]. For CBR, a putative cytochrome-b5 oxidoreductase, a location in the food vacuole of blood-stages has been reported[22]. *Pb* parasites lacking CBR, showed a slight reduction in the size of mature liver-stages and in detachment of cultured hepatocytes containing maturing liver-stages[23]. The MEI2 protein is a member of a family of RNA-binding proteins containing an RNA recognition motif[24]. *Plasmodium yoelii (Py)* parasites lacking MEI2 develop into late liver schizonts but have a complete attenuation phenotype in highly susceptible BALB/cByJ mice infected with 50.000 sporozoites[24] and only occasional breakthrough blood-infections were observed when >200.000 sporozoites were inoculated[25]. We confirmed this late liver-stage growth arrest and the strong attenuation phenotype in *Pb* by creating and characterizing *Pb* gene-deletion mutants lacking the *mei2* gene (*Pb*Δ*mei2;* **Supplementary Fig. 1**). We also observed occasional breakthrough blood-infections when mice were inoculated with high numbers ($2x10^5$) of *Pb*Δ*mei2* sporozoites. In one experiment we infected mice with $2x10^5$ sporozoites of a *Pb*Δ*mei2* line (*Pb*Δ*mei2-breakthrough-a)* that was collected from mice with a breakthrough blood-infection after the first infection with *Pb*Δ*mei2* sporozoites. In this experiment we found that the percentage of mice with a 'second' breakthrough blood infection was comparable to that of mice with a 'first' breakthrough infection and the mice that became positive had again a strongly prolonged prepatent period, i.e. five of the 11 mice did develop a blood stage infection with a prepatent period of 9-10 days, whereas mice infected with wild type parasites became blood-stage positive at day 4 or 5. These observations strongly indicate that the *Pb*Δ*mei2-breakthrough-a* parasites are not derived from parasites that had permanently switched to an efficient and Mei2-independent mechanism of liver stage development (**Supplementary Fig. 1**).

Table 1 - Published rodent malaria gene-deletion mutants with a 'late-liver stage' growth phenotype

berghei/yoelii gene ID	falciparum gene ID	product	blood-infection	Extended prepatency	Ref.
PBANKA_1125100	PF3D7_0626300	3-oxoacyl-acyl-carrier protein synthase I/II (FabB/FabF)	yes	yes	[70]
PY17X_1126500	PF3D7_0626300	3-oxoacyl-acyl-carrier protein synthase I/II (FabB/FabF)	no	-	[71]
PBANKA_1229800	PF3D7_0615100	enoyl-acyl carrier reductase (ENR,FabI)	yes	yes	[72]
PY17X_1342900	PF3D7_1323000	beta-hydroxyacyl-ACP dehydratase (FabZ)	no	-	[71]
PBANKA_1410500	PF3D7_1312000	malonyl CoA-acyl carrier protein transacylase (FabD)	no	-	[23]
PBANKA_0308200	PF3D7_0211400	beta-ketoacyl-ACP synthase III (FabH)	yes	yes	[23]
PBANKA_0823800	PF3D7_0922900	3-oxoacyl-[acyl-carrier-protein] reductase (FabG)	no	-	[23]
PY17X_0715100	PF3D7_0815900	dihydrolipoyl dehydrogenase, apicoplast (PDH E3)	no	-	
PBANKA_0923800	PF3D7_1124500	pyruvate dehydrogenase E1 component subunit alpha (PDH E1α)	no	-	[73]
PY17X_0934900	PF3D7_1114800	glycerol-3-phosphate dehydrogenase, putative (apiG3PDH)	no	-	[74]
PBANKA_0923800	PF3D7_1114800	glycerol-3-phosphate dehydrogenase, putative (apiG3PDH)	yes	yes	[73]
PY17X_1418400	PF3D7_1318200	glycerol-3-phosphate 1-O-acyltransferase (apiG3PAT)	no	-	[74]
PBANKA_0820900	PF3D7_0920000	long chain fatty acid elongation enzyme, putative (ELO3, ELO-A)	no	-	[23]
PBANKA_1357500	PF3D7_1344600	lipoyl synthase (LipA)	yes	yes	[23]
PBANKA_0707000	PF3D7_0823600	lipoate-protein ligase B (LipB)	yes	yes	[21]
PBANKA_1143400	PF3D7_1367500	NADH-cytochrome b5 reductase, putative (CBR)	yes	yes	[23]
PBANKA_0101100	PF3D7_0602300	liver merozoite formation protein (PALM)	Yes	Yes	[20]
PBANKA_0511000	PF3D7_1026900	biotin--protein ligase 1 (HCS1)	yes	yes	[21,23]
PBANKA_1122300	PF3D7_0623400	MEI2-like RNA-binding protein (MEI2)	Yes	Yes	[24]
PBANKA_0214400	PF3D7_0730300	a transcription factor with AP2 domain(s) (AP2-L)	Yes	Yes	[75]
PBANKA_1024600	PF3D7_1418100	putative liver stage protein 1 (LISP1)	Yes	Yes	[76]
PBANKA_1003000	PF3D7_0405300	liver-specific protein 2 (LISP2; sequestrin)	Yes	Yes	[77,78]
PBANKA_0506500	PF3D7_1022300	ZIP domain-containing protein (ZIPCO)	Yes	Yes	[79]
PBANKA_0505000	PF3D7_1020800	dihydrolipoamide acyltransferase component E2	yes	yes	[23]
PBANKA_1436200	PF3D7_1221000	histone-lysine N-methyltransferase, H3 lysine-4 specific	yes	yes	[23]
PBANKA_0304800	PF3D7_0207400	serine repeat antigen 7	yes	yes	[80]
PBANKA_0404100	PF3D7_0305600	DNA-(apurinic or apyrimidinic site) endonuclease	yes	yes	[81]
PBANKA_1021500	PF3D7_1421900	copper transporter, putative (CTR2)	yes	yes	[82]

Genes 1-7: Apicoplast-located proteins, involved in fatty acid synthesis (FAS II) - transformation of Acetyl-CoA into Acyl-ACP; Genes 8-12: Apicoplast-located proteins of the pyruvate dehydrogenase (PDH) complex, providing acetyl-CoA for fatty acid synthesis; Genes 13-17: (Putative) apicoplast-located proteins: not involved in the transformation of Acetyl-CoA into Acyl-ACP; Genes 16-19: genes selected for gene-deletion in *P. falciparum*

Pf mutants lacking the genes *palm*, *hcs1* or *cbr* do not produce viable sporozoites

To create *Pf* LA-GAP, independent mutants lacking the *palm, hcs1, cbr* or *mei2* genes (*Pf*Δ*palm, Pf*Δ*hcs1*, *Pf*Δ*cbr, Pf*Δ*mei2*) were generated using established methods of CRISPR/Cas9 gene-editing[26,27]. Correct deletion of the genes was confirmed by diagnostic PCR and/or Southern blot analysis of digested genomic DNA (**Supplementary Fig. 2-4; Fig. 1**). These four mutants showed blood-stage growth rates comparable to wild-type *Pf*NF54 (WT *Pf*NF54) parasites. *In vitro* gametocyte production and oocyst numbers in *An. stephensi* mosquitoes fed with cultured gametocytes were in the range of those of WT *Pf*NF54 (**Supplementary Fig. 2-4; Fig. 1; Table 2**). However, in mosquitoes infected with the *Pf*Δ*palm, Pf*Δ*hcs1* and *Pf*Δ*cbr* mutants, salivary gland sporozoites were either absent or their numbers were highly reduced. Midgut/hemocoel sporozoites were also absent or strongly reduced and most oocysts did not show clear signs of sporozoite formation, presenting an 'empty' or vacuolated appearance (**Supplementary Fig. 5**). These observations indicate a role of these three proteins in adequate sporozoite formation inside oocysts. Only *Pf*Δ*mei2* mutants produced numbers of salivary gland sporozoites comparable to those observed for WT *Pf*NF54 (**Table 2**).

Table 2: Gametocyte, oocyst and sporozoite production of four *P. falciparum* gene-deletion mutants and wild type WT *Pf*NF54

Lines	Stage V Gam. (%)[1]	Exflag./ 10^5 RBC[2]	Infection rate (%)[3]	Oocyst no. [4]	Spor. no ($\times 10^3$)[6]
	Average (s.d)	Average (s.d.)	Average (s.d.)	Average (s.d.)	Average (s.d.)
WT *Pf*NF54	2.04 (0.88)	804 (387)	66 (39)	92 (86)	53 (51)
	0.8 - 3.7 (11 exp.)	201 – 1459 (27 exp.)	68-100 (34 exp.)	21-247 (34 exp.)	4-155 (34 exp.)
PfΔpalm	2.85 (1.34)	1544 (116)	75 (18)	38 (27)	0
	1.9-3.8 (2 exp.)	1462-1626 (2 exp.)	56-93 (4 exp.)	19-64 (4 exp.)	(4 exp.)
PfΔcbr	1.6 (0.6)	440 (219)	53 (45)	65 (52)	0
	0.8-2.1 (4 exp.)	147-660 (9 exp.)	54-100 (13 exp.)	22-156 (13 exp.)	(11 exp.)
PfΔhcs1	2.6 (0.8)	459 (133)	92(14)	89 (42)	0
	1.8-3.3 (3 exp.)	365-554 (2 exp.)	76-100 (3 exp.)	63-138 (3 exp.)	(3 exp.)
PfΔmei2	2.1 (0.6)	288 (182)	73 (32)	63 (68)	31 (26)
	1.8-2-8 (3 exp.)	94-600 (9 exp.)	23-100 (14 exp.)	14-167 (13 exp.)	10-84 (9 exp.)

[1] Mean percentage of blood stage parasites developing into gametocytes in vivo
[2] Mean percentage of exflagellating males in vitro, 12-15 minutes after activation
[3] Mean percentage of infected mosquitoes at days 7-12 after feeding
[4] Mean number of oocysts per mosquito (days 7–12)
[5] Mean number of sporozoites per mosquito (days 21)
s.d.: standard deviation

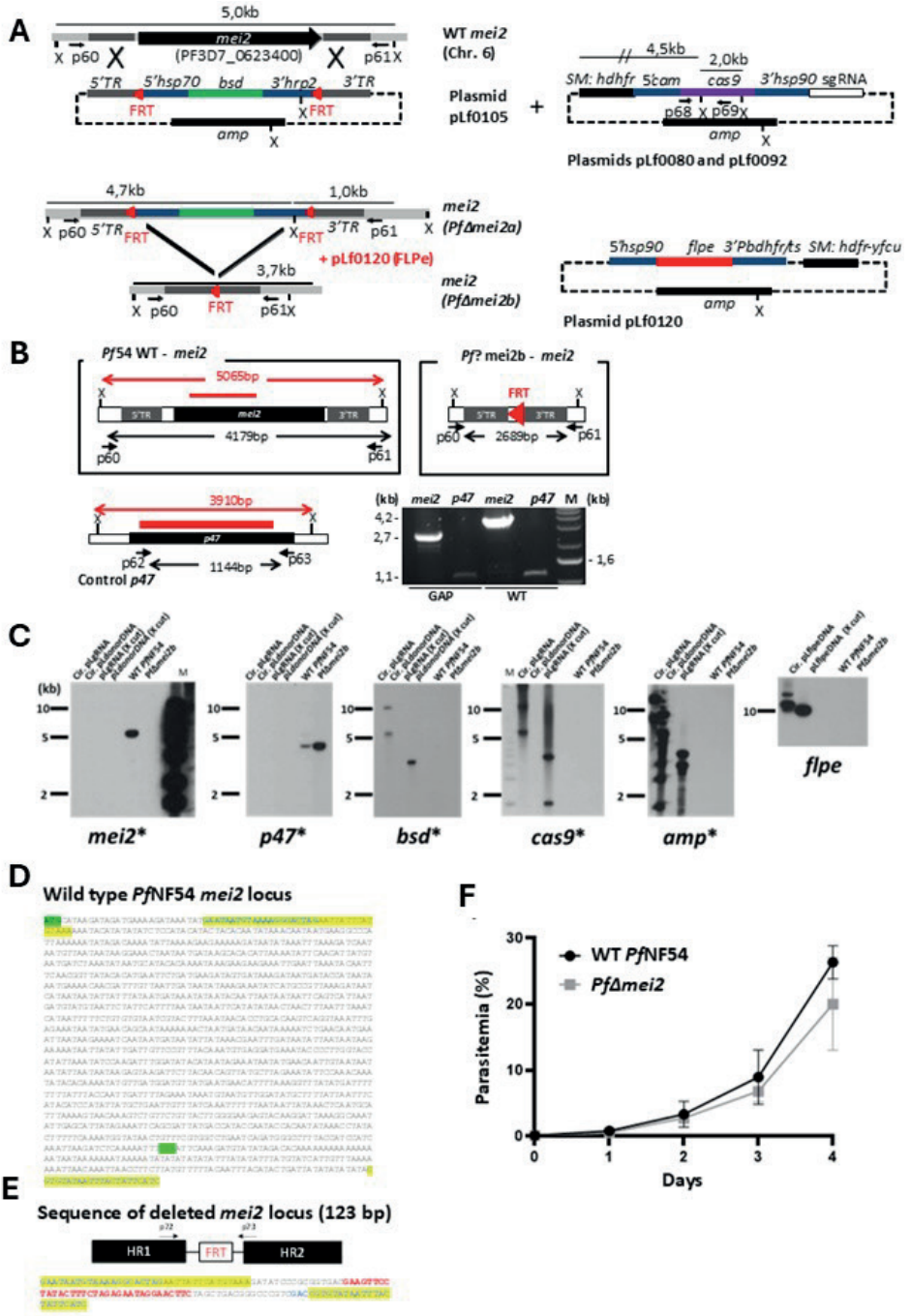

A
5,0kb
mei2
(PF3D7_0623400)
p60
p61
5'TR
5'hsp70
bsd
3'hrp2
3'TR
FRT
amp
WT mei2
(Chr. 6)
Plasmid
pLf0105
4,5kb
2,0kb
SM: hdhfr
5'cam
cas9
3'hsp90
sgRNA
p68
p69
Plasmids pLf0080 and pLf0092
4,7kb
1,0kb
3,7kb
mei2
(PfΔmei2a)
+ pLf0120 (FLPe)
mei2
(PfΔmei2b)
5'hsp90
flpe
3'Pbdhfr/ts
SM: hdfr-yfcu
Plasmid pLf0120
B
Pf54 WT - mei2
5065bp
4179bp
Pf? mei2b - mei2
2689bp
3910bp
p47
p62
p63
1144bp
Control p47
(kb)
mei2
p47
M
4,2-
2,7-
1,1-
1,6
GAP
WT
C
mei2*
p47*
bsd*
cas9*
amp*
flpe
D
Wild type PfNF54 mei2 locus
E
Sequence of deleted mei2 locus (123 bp)
HR1
FRT
HR2
F
WT PfNF54
PfΔmei2
Parasitemia (%)
Days

Figure 1. Generation, genotyping and blood-stage growth of *Pf*Δ*mei2* (LA-GAP, GA2)
A. Left: The *mei2* (PF3D7_ 0623400) genomic locus on chromosome 6 (Chr. 6) of wild type *Pf* NF54 (WT *Pf* NF54) and *Pf*Δ*mei2* parasites before (*Pf*Δ*mei2a*) and after (*Pf*Δ*mei2b*) FLPe-mediated removal of the *blasticidin-S-deaminase* (*bsd*) selectable marker (SM). The donor plasmid pLf0105 to delete *mei2* contains the *bsd* SM, flanked by two *frt* sites (red triangles) and *mei2* targeting sequences (5' TR and 3' TR) for double cross-over integration. Primer pairs **p60/p61** and PCR fragment size for diagnostic PCR are indicated (**panel B**); X (*Xmn*I): restriction site used for Southern blot analyses (**panel C**). *hsp70*, *heat shock protein 90; hrp2, histidine-rich protein II; amp, ampicillin.* Right top: sgRNA plasmids (pLf0080, pLf0092) containing the human *dihydrofolate reductase-thymidylate synthase* (h*dhfr*) SM and the *cas9* expression cassette. *Cam, calmodulin*. PCR primers (**p68/p69**) to amplify part of *cas9*, sizes of the sgRNA constructs after *Xmn*I (X) digestion and *mei2* and *cas9* probes are indicated (**panel C**). Right bottom: construct pLf0120 with the h*dhfr*-y*fcu* SM and the *flpe* expression cassette. pb*dhfr/ts,: Pb bifunctional dihydrofolate reductase-thymidylate synthase, putative*. See **Supplementary Table 1** for primers details.
B. WT *Pf* NF54 and *Pf*Δ*mei2b* genomic loci and the control gene *p47* (coding sequence shown as black boxes). Shown are the 5' and 3' *mei2* targeting regions (5'TR and 3'TR), used construct pLf0105 (**panel A**) and the *frt* site. PCR primers (in black) for amplifying *mei2* (**p60/p61**) and *p47* (**p62/63**), expected sizes of the full length *mei2* and *p47* genes and size of *mei2* locus after *mei2* deletion and removal of *bsd* SM cassette are shown. X (*Xmn*I): restriction site used for Southern analysis (**panel C**). DNA probes used in Southern analyses (**panel C**) and sizes of digested DNA fragments recognized by the probes (*mei2* and *p47*) are shown (in red). Red triangle: the 34 bp *frt* site in the *Pf*Δ*mei2*b genome after removal of the *bsd* SM cassette. PCR (right lower panel) analysis of WT *Pf* NF54 and *Pf*Δ*mei2*b genomic DNA confirms *mei2* deletion (control: amplification of *p47*). Primer pairs: **p60/61** for *mei2* and **p62/p63** for *p47*. See **Supplementary Table 1** for primer details). M, molecular weight marker; 1kb DNA ladder (Invitrogen).
C. Southern analysis of restricted genomic DNA from WT *Pf* NF54, *Pf*Δ*mei2*b and plasmids used to delete *mei2* (DNA digested with *Xmn*I (X)). DNA-samples/lanes: i) circular sgRNA plasmids (Cir. pLgRNA); ii) circular donor DNA plasmid pLf0105 (pLΔ*mei2*); iii) *Xmn*I-digested sgRNA plasmid (pLgRNA-X cut); iv) *Xmn*I-digested donor DNA plasmid; v) genomic WT *Pf* NF54 DNA; vi) genomic *Pf*Δ*mei2*b DNA. Probes: part of *mei2*, *p47* (control), *cas9*, *bsd*, *amp* and *flpe* (see **panel A** and **B** for probe location and expected fragment sizes). Hybridizations show correct *mei2* deletion and absence of *cas9, bsd, amp and flpe* in *Pf*Δ*mei2*b. M, molecular weight marker; 1kb DNA ladder (Invitrogen) labeled on the sides of the gels.
D. Sequence of the WT *Pf*NF54 *mei2* locus. Yellow: sequences present in the disrupted *mei2* locus of *Pf*Δ*mei2* (**E**); Green: start and stop codon of *mei2*.
E. The *mei2* locus in the *Pf*Δ*mei2* genome after *mei2* deletion by integration of pLf0105 and FLPe-mediated removal of the *bsd* SM marker The *mei2* targeting regions (HR1 and HR2) for double cross-over integration and the *frt* site are shown. In addition, the sequence of the PCR fragment of the *mei2* locus is shown, amplified using primers **p72/p73** (see **Supplementary Table 1** for primer details). Yellow: sequences present in the *mei2* locus of WT *Pf*NF54 and *Pf*Δ*mei2*. Red: the 34 bp FRT sequence, flanked by 16bp and 14bp cloning restriction sites.
F. *In vitro* growth rate of *Pf*Δmei2 and WT *Pf* NF154 asexual blood-stages. Parasitemia (%) during a 4-day culture period (mean and s.d. of three cultures). Error bars represent standard deviation.

*Pf*Δ*mei2* parasites develop into replicating liver-stages but fail to produce red blood cell-infectious liver-stage merozoites

The growth arrest of *Pf*Δ*mei2* liver-stages was investigated using primary human hepatocytes. First, *Pf*Δ*mei2* sporozoite infectivity and development was analyzed *in vitro* in cultures of cryopreserved primary human hepatocytes (from the company BioIVT) infected with isolated *Pf*Δ*mei2* and WT *Pf*NF54 sporozoites. A mean of 2,1% (s.d. 0.42) and 1.4% (s.d. 0.42) infected hepatocytes per well was observed for WT *Pf*NF54 WT and *Pf*Δ*mei2*, respectively, at day 3 post infection (p.i.) (**Fig. 2 A**). During *in vitro* liver-stage development (day 3, 5, 7 and 9 p.i.) the size and light-microscopy morphology of *Pf*Δ*mei2* parasites was comparable to those of WT *Pf*NF54 parasites and a strong increase in parasite size (**Fig. 2 B**) and nuclear content (**Fig. 2 C**) was observed in both WT *Pf*NF54 and *Pf*Δ*mei2* parasites. Liver-stages of WT *Pf*NF54 and *Pf*Δ*mei2* liver stages displayed similar staining patterns of the cytoplasmic marker HSP70 and of the parasitophorous vacuole marker EXP1. At days 7 and 9 p.i., liver-stages of WT *Pf*NF54 and *Pf*Δ*mei2* exhibited comparable staining of the merozoite surface protein 1 (MSP1) (**Fig. 2 C**). We then confirmed WT-like hepatocyte invasion and development into late liver-stages of *Pf*Δ*mei2* in cryopreserved primary human hepatocytes from the company Lonza (**Supplementary Fig. 6**) These analyses also showed that sporozoite infectivity and the size of *Pf*Δ*mei2* liver stages (at day 3, 5, 7 and 9) were comparable to those of WT *Pf*NF54 parasites. In addition, *Pf*Δ*mei2* and WT *Pf*NF54 liver stages displayed similar staining patterns with antibodies against the cytoplasmic marker HSP70 and the parasitophorous vacuole marker EXP1. All together, these results_show that *Pf*Δ*mei2* sporozoites can effectively infect cultures of human hepatocytes and are able to develop into large, replicating liver-stages that express merozoite-specific antigens.

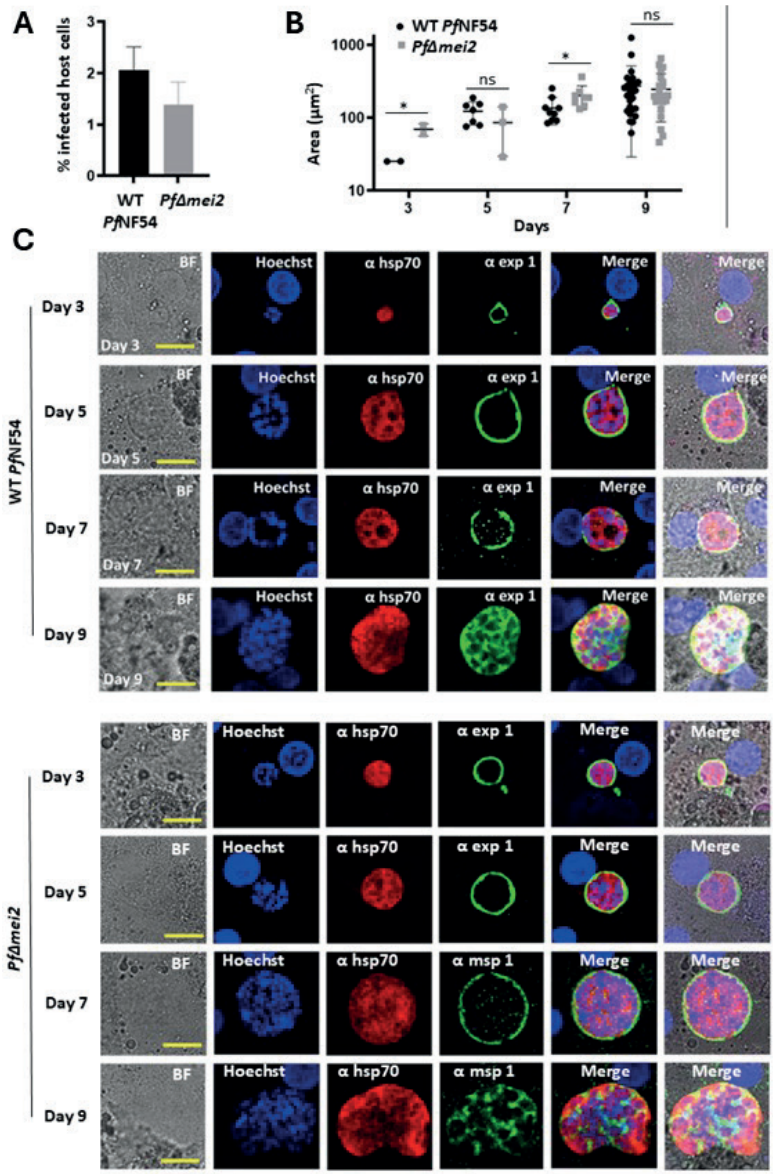
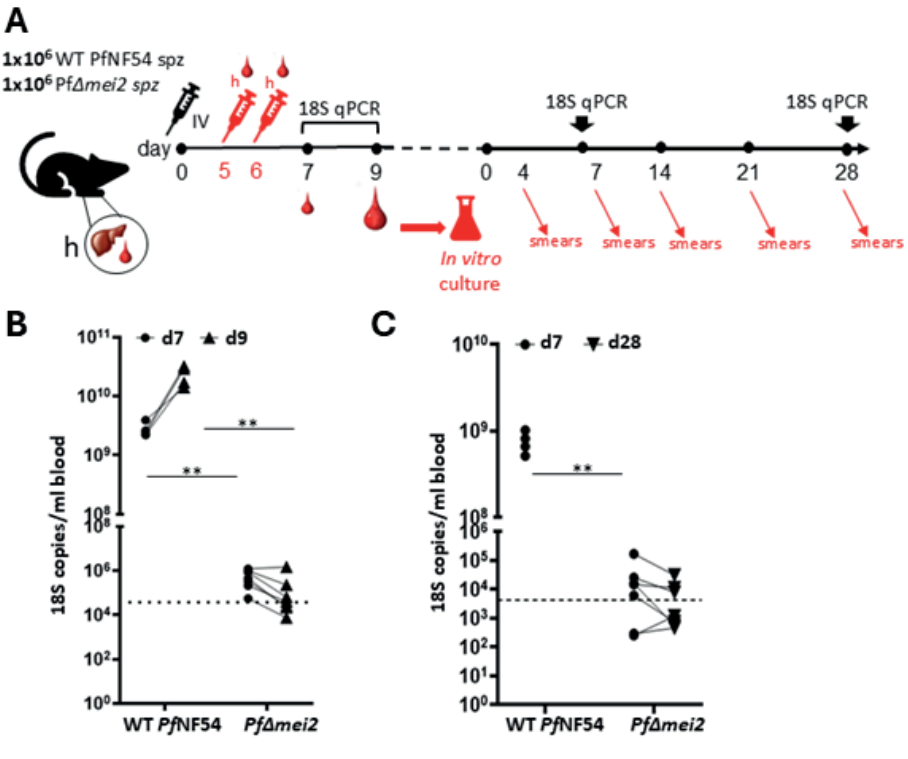


Figure 2. *Pf*Δ*mei2* liver-stage development in cultured human primary hepatocytes (BioIVT)
A. Percentage of hepatocytes infected with WT *Pf*NF54 and *Pf*Δmei2 at day 3 post-infection (p.i.) (p=0,002; unpaired Mann Whitney test).
B. Liver-stage size on day 3, 5, 7 and 9 p.i. (3-20 parasites measured in 2 wells). The average of the parasite's cytoplasm at its greatest circumference using HSP70-positive area (µm²), s.d. and significances values are shown (unpaired Mann Whitney test: *p < 0.05; ns: not significant).
C. Representative confocal microscopy images of liver-stages on days 3, 5, 7 and 9 p.i. Upper panel WT *Pf*NF54; lower panel *Pf*Δmei2. Fixed hepatocytes were stained with the following antibodies: rabbit anti-*Pf*HSP70 (α hsp70), mouse anti-*Pf*EXP1 (α exp1) and anti-*Pf*MSP1 (α msp1). Nuclei stained with Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor® 488 (green) 0.7 s; anti-IgG Alexa Fluor® 594 (red) 0.6 s; Hoechst (blue) 0.136 s; bright field (BF) 0.62 s (1x gain). Scale bar, 10µm. Error bars represent standard deviation.

Next, the liver-stage development of *Pf*Δ*mei2* and WT *Pf*NF54 parasites was analyzed in liver-chimeric humanized mice (FRG huHep mice) engrafted with high proportions of human red blood cells (RBC), injected intravenously (see **Fig. 3 A** for a schematic representation of these experiments). The use of FRG huHep mice to mimic *in vivo* hepatocyte infection with *Pf* sporozoites through to the blood-stage of infection has been previously described[28,29]. Briefly, mice were infected with $1x10^6$ sporozoites of WT *Pf*NF54 or *Pf*Δ*mei2* followed by injection of human red blood cells at days 5 and 6 p.i. Blood was collected for qRT-PCR analysis at days 7 and 9 p.i., and mice were euthanized at day 9 p.i. for blood collection and cryopreservation. In WT *Pf*NF54-infected mice an average of $1.4x10^{10}$ 18S copies per ml was detected at day 7 (range: $1.1\ x10^{10}$ to $2.0\ x10^{10}$) which increased on day 9 to an average of $1.2x10^{11}$ copies (range $7.0x10^{10}$ to $1.6x10^{11}$). In the *Pf*Δ*mei2*-infected mice the average number of 18S copies was much lower at day 7 ($2.4x10^6$; range $1x10^6$ to $4.9x10^6$) and dropped ten-fold at day 9 in 6 mice ($3.2x10^5$; range $3.7x10^4$ to $1.1x10^6$) while in one mouse the number of 18S copies remained similar ($5.8x10^6$ at day 7 and $7.3x10^6$ at day 9. (**Fig. 3 B**). Combined these observations show the presence of replicating parasites in WT *Pf*NF54-infected mice whereas in the *Pf*Δ*mei2*-infected mice replicating blood stages are absent. To determine if the WT *Pf*NF54 or *Pf*Δ*mei2* infected mice contained viable blood stage parasites, the cryopreserved blood of the FRG huHep mice collected at day 9 was cultured using standard *in vitro* culture conditions for blood-stage parasites. Samples from all WT *Pf*NF54-infected mice showed a >0.1% parasitemia after five days of culture. In contrast, none of the cultures of blood samples of the *Pf*Δ*mei2*-infected mice became blood-stage positive up to 28 days, as determined by microscopy of Giemsa stained smears and 18S qRT-PCR analyses (**Fig. 3 C,D**). The absence of parasites in the cultures of *Pf*Δ*mei2*-infected mouse blood suggests that the low number of 18S copies detected at days 7 and 9 in these mice most likely results from the presence of dead parasite material released from *Pf*Δ*mei2*-infected hepatocytes in the blood. Overall, our results show that *Pf*Δ*mei2* sporozoites can effectively infect human hepatocytes *in vitro*, develop into large, replicating liver-stages that express merozoite-specific antigens but these maturing liver-stages fail to produce human RBC-infective merozoites in the livers of liver/blood-humanized mice.



D

	WT *Pf*NF54				*PfΔmei2*						
	m1	m2	m3	m4	m1	m2	m3	m4	m5	m6	m7
Parasitemia[1]											
d4	0,7	1,3	0,2	0,4	0	0	0	0	0	0	0
d7	12	17	9,3	14,3	0	0	0	0	0	0	0
d28	NA	NA	NA	NA	0	0	0	0	0	0	0

NA: not applicable

[1] Parasitemia was determined in Giemsa stained thin blood films at day 4, 7 and 28 after start of the cultures

Figure 3. Development of *Pf*Δ*mei2* in FRG huHep mice containing human red blood cells
A. Timeline of experiments in liver-chimeric mice where mice were injected intravenously (IV) with 1x10^6 sporozoites on day 0 and then with human red blood cells (h) on days 5 and 6 prior to emergence of blood-stage parasites. Blood samples were taken at days 7 and 9 for qRT-PCR with day 9 samples used for 28-day *in vitro* culture of blood stages with subsequent parasitaemia readout by microscopy and qRT-PCR.
B. 18S qPCR analysis of blood samples from FRG huHep mice on day 7 and 9 after infection with 1x10^6 sporozoites of *Pf*Δ*mei2* (n=7 mice) and WT *Pf*NF54 (n=4 mice). Significance values (unpaired Mann Whitney test): **p < 0.001. Dotted line: the cutoff as used in controlled human malaria infection (CHMI) at OHSU of 5 parasites/ml, assuming 7400 18S copies/per parasite.
C. Analysis of blood samples from FRG huHep mice for presence of blood-stage parasites by *in vitro* cultivation of blood stages. Cultures, maintained in a semi-automated shaker system, were monitored for blood-stages for 28 days by microscopy analysis of Giemsa stained thin and thick blood smears (see **D**) and by 18S qPCR. Significance values (unpaired Mann Whitney test): **p < 0.001. Dotted line: the 10 parasites/ml cutoff used in CHMI at LUMC, assuming 425,2 18S copies per parasite.
D. Blood-stage parasites in cultured blood samples collected from FRG huHep mice (m) after infection with *Pf*Δmei2 and WT PfNF54sporozoites. Samples from *in vitro* cultures were analyzed at different days (d) for blood-stage parasitemia by microscopy (% of infected RBC).

*Pf*Δ*mei2* genotyping by whole-genome sequencing confirms correct deletion of *mei2* and indicates absence of major rearrangements or integration of heterologous DNA sequences

The promising results of late growth arrest of *Pf*Δ*mei2* liver-stages warranted further characterization of *Pf*Δ*mei2* parasites to obtain regulatory approval for clinical testing of this LA-GAP. Clinical evaluation involves safety testing, i.e. analysis of adverse events in humans resulting from injection of *Pf*Δ*mei2* sporozoites or resulting from possible break-through blood infections, similar to procedures used in safety evaluation of the GA1 *Pf* GAP[10]. Thorough identity testing and characterization of the genetic makeup of the parasites, in particular a confirmation of absence of unwanted genetic alterations which may impact the virulence of *Pf*Δ*mei2* parasites, is crucial for regulatory approval of their clinical use. We therefore analyzed the presence of undesirable integration of plasmid DNA sequences that were used to generate *Pf*Δ*mei2* and the presence of other genome rearrangements that might result from the genetic modification or mutations that may affect gene expression or drug-sensitivity. Four different plasmids with heterologous DNA sequences were used to create *Pf*Δ*mei2*, specifically, (i) a donor DNA plasmid containing two 34 bp *frt* (GAAGTTCCTATACTTTCTAGAGAATAGGAACTTC) sequences; (ii) two sgRNA plasmids containing the heterologous selectable marker genes *bsd*, h*dhfr* and y*fcu* flanked by 3'and 5'utr sequences of *Pf*-specific genes, employed to delete *mei2* by double cross-over integration; (iii) a fourth plasmid, containing a yeast FLPe recombinase expression cassette, which was used to remove nearly all heterologous DNA from the donor DNA plasmid that is introduced into the genome for deletion of *mei2*, specifically the *bsd* selectable marker cassette. These heterologous DNA sequences, located between the two *frt* sequences are excised in the presence of FLPe recombinase, leaving one *frt* sequence in the genome. This method of removal of heterologous DNA by FLPe recombinase is similar to that described for the generation of the GA1[10] that also contains a single *frt* site. First, diagnostic PCR analysis and Southern blot analyses of digested genomic DNA were used to confirm the correct deletion of *mei2* and subsequently the removal of the *bsd* selectable marker cassette from the *mei2* locus in *Pf*Δ*mei2* by (**Fig. 1 B-C**). In addition, PCR products that encompass the wild type *mei2* and deleted DNA regions were cloned and sequenced, revealing that i) the expected gene-targeting had occurred at the *mei2* locus resulting in deletion of *mei2*, ii) the *bsd* selection marker was absent in the *mei2* locus, and iii) only a single *frt* sequence was present in the *mei2* locus (**Fig. 1 D-E**). Next, the complete genome of *Pf*Δ*mei2* was sequenced and single nucleotide polymorphisms (SNPs), insertions and deletions were analyzed by comparison with the reference 3D7 *Pf* genome, which is a cloned line of *Pf*NF54. This analysis

showed that: i) No reads were mapped on the *mei2* coding sequence, while they mapped to the *mei2* 3' and 5' *utr* sequences, confirming correct deletion of *mei2* and showing preservation of the flanking targeting sequences in the *Pf*Δ*mei2* genome (**Fig. 4 A-B**); ii) None of the following plasmid sequences are present in the *Pf*Δ*mei2* genome: *cas9*, *ampicillin*, the drug selection marker genes *bsd*, h*dhfr*, y*fcu* and the *flpe recombinase* gene (**Fig. 4 C**); iii) The endogenous 3'- and 5'*utr* sequences that were used in plasmids to drive gene expression were unaltered in the *Pf*Δ*mei2* genome, showing the absence of unwanted recombination events in these genomic regions (**Fig. 4 D**). These sequences comprised 5'*utr* sequences of *calmodulin* (PF3D7_1434200), *Hsp70* (PF3D7_0818900), *Hsp90* (PF3D7_0708400) and 3'*utr* sequences of *Hpr2* (PF3D7_0831800); *Hsp86* (PF3D7_0708400) and *dhfr/ts* (PF3D7_0417200); iv) No detectable rearrangements in the *Pf*Δ*mei2* genome were observed by InDel analysis, using programs for identification of insertion and deletions; v) A total of 105 high-quality SNPs were identified in genes and only two of these 105 genes had a non-synonymous mutation: *eukaryotic translation initiation factor 2* (PF3D7_0107600, A>C position 3421, amino acid N114 to H) and a *Pfemp1* (PF3D7_0421100, A>T, position 914, amino acid Y350 to F). In conclusion, our analyses did not indicate the presence of unwanted integration of (heterologous) plasmid DNA sequences into the *Pf*Δ*mei2* genome or the presence of genome rearrangements, except for the deletion of mei2, that may impact other phenotypic/virulence characteristics of *Pf*Δ*mei2.*

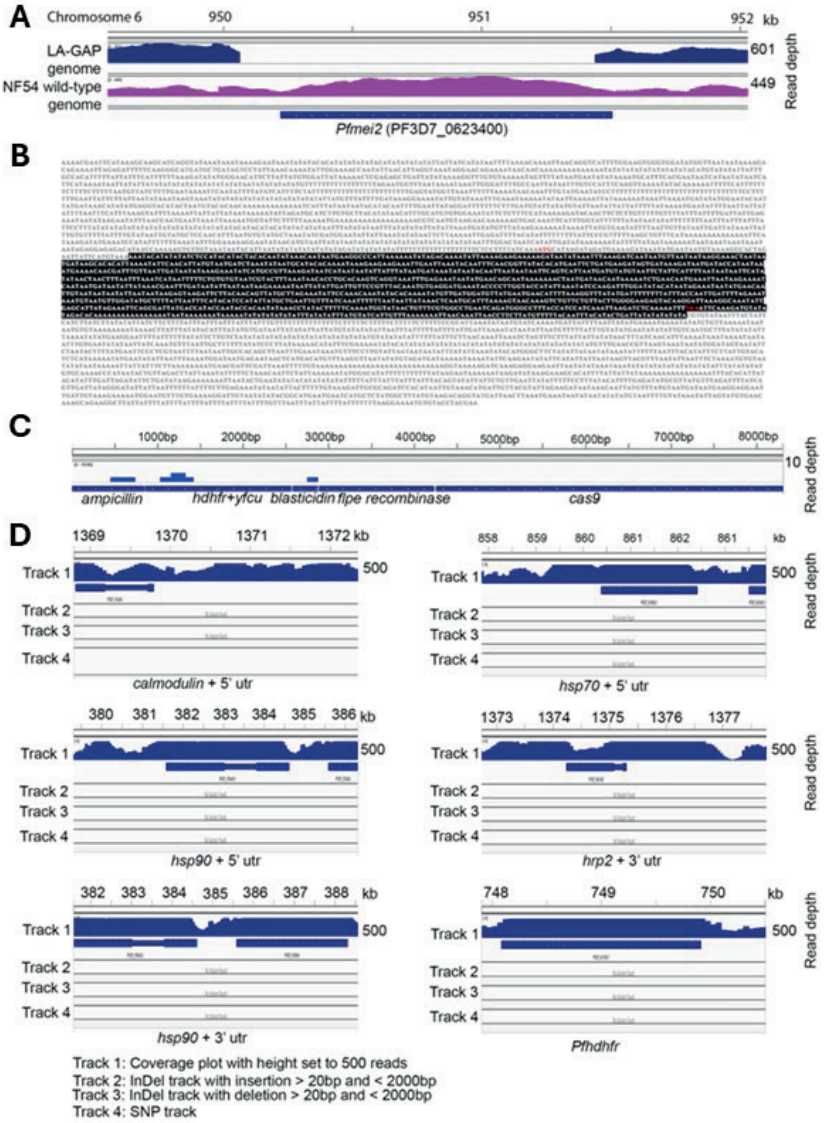
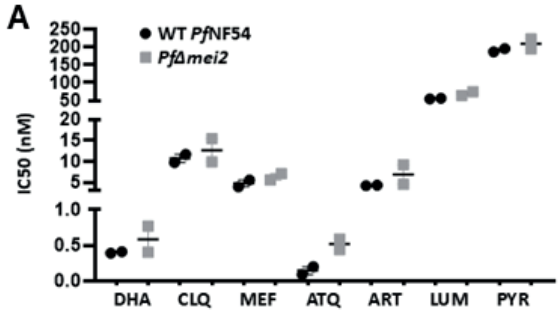


Figure 4. *PfΔmei2* genome sequence analysis
A. The *mei2* locus of *PfΔmei2*. No sequence reads are mapped on the *mei2* coding sequence while reads map in the *mei2* up- and downstream regions.
B. Sequence of the *mei2* locus of *PfΔmei2.* The *mei2* flanking regions are unaltered and the expected *mei2* deletion event is shown by preservation of the *mei2* targeting sequences (yellow). The *mei2* coding sequence (in red) is absent (start and stop codon in green).
C. Uniquely mapped sequence read (Illimina reads) coverage of heterologous sequences used in the DNA constructs/plasmids for generation of *PfΔmei2*. None of the sequences (*ampicilin,* h*dhfr-yfcu, blasticydin, flpe and cas9*) were mapped with sequence reads from the *PfΔmei2* genome.
D. Read coverage, InDel and SNP information of the following endogenous 5'- and 3'-*utr* sequences, used in DNA constructs/plasmids to drive gene expression. These sequences were unaltered in the *PfΔmei2* genome. The regions were: 5'UTR: *calmodulin*, PF3D7_1434200; *Hsp*70, PF3D7_0818900; *Hsp90*, PF3D7_0708400; 3'UTR: *Hpr2*, PF3D7_0831800; *Hsp90*, PF3D7_0708400; and the *dhfr/ts* locus, PF3D7_0417200.

Drug sensitivity of *PfΔmei2* blood-stage parasites is comparable to that of WT *Pf*NF54 blood stages

An inherent safety feature of attenuated parasites is the ability to quickly cure any breakthrough blood infection with a variety of drugs. As noted above, the comparison of the genome sequences of *Pf△mei2* and WT 3D7 *Pf* revealed the presence of a relatively low number of SNP's. Non-silent mutations in coding and non-coding sequences may affect genes that encode proteins involved in sensitivity/resistance of blood-stage parasites to commonly used antimalarials. We therefore determined drug sensitivity of *Pf△mei2* and WT *Pf*NF54 blood-stages to seven commonly used drugs for treating (experimental) malaria infections (dihydroartemisinin, chloroquine, mefloquine, atovaquone, artemisinin, lumefantrine and pyrimethamine). Blood-stages of both parasite lines were sensitive to all drugs tested, with IC50 values in the nanomolar range (**Fig. 5**). These results indicate that SNPs detected in *PfΔmei2* did not impact on the sensitivity of *PfΔmei2* blood-stages to the drugs tested in this study.



B

Strain	Z' value
WT *Pf*NF54HGL	0.78
NF54[1]	0.73
NF54[2]	0.62
PfΔmei2[1]	0.68
PfΔmei2[2]	0.82

C

	$IC_{50\,(nM)}$							
Strain	**DHA**	**CLQ**	**MEF**	**ATQ**	**ART**	**LUM**	**PYR**	
WT *Pf*NF54[1]	0.39	11.67	5.58	0.20	4.33	56.13	195.5	sensitive
WT *Pf*NF54[2]	0.41	9.74	3.92	0.09	4.24	54.53	186.3	sensitive
PfΔmei2[1]	0.40	9.86	5.57	0.44	4.50	74.79	194.5	sensitive
PfΔmei2[2]	0.77	15.43	7.10	0.59	9.23	64.42	222.7	sensitive

Figure 5. Sensitivity of *PfΔmei2* and WT *Pf*NF54 blood-stages to seven antimalarial drugs
A. Drug sensitivity of *PfΔmei2* (grey) and WT *Pf*NF54 (black) to seven antimalarial drugs was determined in an asexual Blood stage (ABS) SYBR Green drug-assay. The graph shows the IC50 value with standard error of the mean (SEM)(see **C** below for the exact values). DHA, dihydroartemisinin; CLQ, chloroquine; MEF, mefloquine; ATQ, atovaquone; ART, artemisinin; LUM, lumefantrine; PYR, pyrimethamine.
B. Z' values for each tested *Plasmodium falciparum* strain. The Table shows the Z' values for each of the plates tested in the ABS replication assay.
C. IC50 values for each culture for the drugs shown in **A**. IC50 was determined using a four-parameter non-linear regression model using least-squares to find the best fit. Error bars represent standard error of the mean.

Discussion

To generate *Pf* GAPs with a late liver-stage growth arrest, we selected four genes for deletion based on published phenotypes of rodent malaria parasite mutants, lacking the equivalent orthologous genes. In contrast to the rodent parasite data, three out of these four genes (*palm, hcs1, cbr*) appear to be essential for the formation of *Pf* sporozoites inside the oocyst. A comparable discrepancy in gene function between rodent malaria parasites and *Pf* has been reported for genes encoding apicoplast-located proteins involved in the transformation of Acetyl-CoA into Acyl-ACP for generation of fatty acids by the FASII pathway[18]. There is no proof that the three proteins selected, PALM, HCS1 and CBR, play a direct role in this (part of the) FASII-pathway, although CBR may operate in fatty acid elongation[30] and the rodent *Plasmodium* PALM localizes to the apicoplast, which would be compatible with a function in the FASII pathway[20]. However, additional studies are required to unravel the biochemical pathways in which these proteins take an indispensable role for formation of *Pf* sporozoites. In contrast to *palm, hcs1* and *cbr*, we found that *Pf* GA2 lacking *mei2* phenocopies *P.yoelii*[24,25] and *Pb mei2* gene-deletion mutants (this study). The rodent *mei2* knock-out malaria parasite mutants develop similarly to wild-type parasites in the blood, in the mosquito and during part of their intra-hepatic development process, but their growth is arrested during replication inside hepatocytes. Recently, a similar phenotype of late liver growth arrest has also been described for a *Pf mei2* gene-deletion mutant by Goswami et al.[31]. We found a highly similar development of the *Pf*Δ*mei2* parasites into replication-competent, late liver-stages and the absence of formation of merozoites that infect human red blood cells. The MEI2 (or PlasMei2) protein is a member of a family of RNA-binding proteins containing an RNA recognition motif and is only expressed in late-liver-stages where it has a granular cytoplasmic location[31]. Detailed observations on the development of *Pf* liver-stages lacking the MEI2 protein showed aberrant formation of cytomeres, structures formed in maturing schizonts during the formation of the multiple daughter merozoites, and impaired DNA replication and segregation[31]. Similar to what has been observed by Goswami *et al.*[31], we provide evidence that these schizonts do not produce merozoites that are capable of infecting RBC. In standardized experiments using FRG huHep mice transfused with human RBC[28,29], no evidence was found for development of blood-stage infections after injection of high numbers ($1x10^6$) of *Pf*Δ*mei2* sporozoites. These observations indicate a complete late-liver stage growth arrest of *Pf* parasites lacking the MEI2 protein. However, occasional break-through blood infections were found in mice injected with more than $2x10^5$ sporozoites of both *P.yoelii*[28] and *Pb mei2* gene-deletion mutants (this study). These occasional break-through blood infections of the rodent malaria mutants may be specific

for the rodent malaria species which has a short period of full liver-stage development of only two days compared to a period of more than six days for *Pf*. However, in the mouse studies with the rodent malaria parasites larger groups of mice were infected with sporozoites of the *mei2* gene-deletion mutants compared to the number of FRG huHep mice injected with *Pf*Δ*mei2* sporozoites. In addition, infectivity of *Pf* sporozoites may be lower in the FRG huHep mouse model than in humans and therefore formal proof of complete attenuation of *Pf*Δ*mei2*, parasites awaits controlled safety studies in humans[10].

The phenotype of growth arrest in the liver of *Pf*Δ*mei2* is highly similar to the phenotype of *Pf mei2* gene-deletion mutant (*Pf* mei2^{-}) reported by Goswami et al.[31]. Both mutants have been generated by CRISPR/Cas9-mediated gene deletion. However, differences exist in the plasmids used for deleting *mei2*, the deleted sequence and in drug-selection of the mutants. All phenotype analyses of the *Pf* mei2^{-} were performed with a mutant that still contains the *hdhfr* selectable-marker cassette in the *mei2*- locus and no data is reported on whole genome sequence analyses of the different *Pf mei2*$^{-}$ mutants with or without the *hdhfr* selectable marker. CRISPR/Cas9 modification can make off target gene mutations, not only at or near the target site, but also far from the target site[32,33], mutations that may affect the phenotype of *Pf* mutants, for example impacting virulence features or drug sensitivity.

To obtain regulatory approval for clinical evaluation of *Pf*Δ*mei2*, we performed detailed studies to investigate whether genetic modification procedures did not cause unwanted genetic alterations, possibly impacting the virulence of the genetically modified parasites. Our genotype analyses, including whole genome sequencing, did not show unwanted integration of (heterologous) plasmid DNA sequences or genome rearrangements in *Pf*Δ*mei2* that may impact other phenotypic/virulence characteristics of *Pf*Δ*mei2*, except for the intentional deletion of *mei2* from the *Pf*Δ*mei2* genome. In addition, the sensitivity of *Pf*Δ*mei2* blood-stages to seven antimalarial drugs showed IC50 values in the nanomolar range similar to those found for of the parent WT *Pf*NF54. Based on our observation, it can therefore be expected that the safety profile of growth-arrested *Pf*Δ*mei2* sporozoites in humans is highly similar to sporozoites of PfSPZ-GA1[10], *Pf*SPZ Vaccine[1] or WT *Pf*NF54 sporozoites administered under chloroquine prophylaxis[34].

The results of the preclinical evaluation reported in this study have led to the regulatory approval of *Pf*Δ*mei2* (named GA2) for use in human studies by the Gene Therapy Office of the Dutch Ministry of Infrastructure and the Environment in the Netherlands, licensed to the Leiden University Medical Centre (GGO IM-MV 20-018_000) and subsequent approval of the Central Committee on Research Involving Human Subjects (CCMO;

NL75577.000.21). Safety and subsequent efficacy studies using the CHMI model have currently been initiated to provide the answer to the key question whether attenuated parasites with a growth arrest late in the liver substantially increases potency compared to Wsp vaccines consisting of attenuated parasites with an early-growth arrest, similar to what has been found in rodent studies[17] and is expected based on studies using chemo-attenuated PfSPZ immunization[16]. Moreover, parallel assessment of immunological samples obtained from individuals exposed to EA-GAP (GA1 and PfSPZ Vaccine) or LA-GAP GA2 will provide unprecedented insight in the immunology of liver stage of human malaria, thus far a black box. Most importantly, the prospect of a highly efficacious malaria vaccine using a cutting-edge molecular technology provides hope for regaining control for malaria control programs struggling with resistance.

Materials and Methods

Experimental animals (ethics statement): Leiden, LUMC (The Netherlands)

Animal experiments were granted with a license DEC12042 and 14207 by Competent Authority after an advice on the ethical evaluation by the Animal Experiments Committee Leiden and were performed in accordance with the Experiments on Animals Act (Wod, 2014), the applicable legislation in the Netherlands in accordance with the European guidelines (EU directive no. 2010/63/EU). Experiments were executed in a licensed establishment for experimental animals. Mice were housed in ventilated cages with autoclaved aspen woodchip, fun tunnel, wood chew block and nestlets (12:12 hour light-dark cycle; 21 ± 2°C; relative humidity of 55 ± 10%) and fed with a commercially-prepared autoclaved, dry rodent diet pellets and provided with water, both available *ad libitum*. Female OF1 and C57BL/6 mice (6-7 weeks; Charles River Laboratories, France) were used. Experiments involving generation of mutant parasite lines and phenotype analyses were performed using highly standardized and approved protocols that have been developed to reduce the number of animals and minimize suffering and distress. Mice were killed (cardiac puncture under isoflurane anesthesia or CO_2) at a parasitemia of 2-5%, before malaria-associated symptoms occur. Humane endpoints: the animals/ body condition was thoroughly examined daily. Animals are humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/ or movement), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized. In all experiments no mice were

euthanized before termination of the experiment and no mice died before meeting criteria for euthanasia.

Mosquitoes

Mosquitoes from a colony of *Anopheles stephensi* (line Nijmegen SDA500) were used. Larval stages were reared in water trays (at 28 ± 1°C; relative humidity 80%). Adult females were transferred to incubators at 26 ± 0.2°C (relative humidity of 80%) and were fed with 5% filter-sterilized glucose solution. For the transmission experiments, three to five day-old mosquitoes were used. Following infection, the *Pb* and *Pf,* infected mosquitoes were maintained at 21°C and 26°C, respectively, at 80% relative humidity.

Parasites

For generation of the rodent malaria LA-GAP *Pb*Δ*mei2,* the *Pb* ANKA reference line 1868cl1 was used (line RMgm-1320; www.pberghei.eu) which contains the reporter genes *mCherry* and *luciferase* under control of the constitutive *hsp70* and *eef1α* promoters, respectively, integrated into the neutral *230p* gene locus (PBANKA_0306000). This line does not contain a drug-selectable marker. Production of *Pb*Δ*mei2* and characterization of these parasites throughout their life cycle, including mosquito transmission, was performed under GMO permits IG 17-230_II-k en IG 17-135_III.

Pf parasites NF54 strain[35] was used as wild type *Pf* parasites *(*WT *Pf*NF54). Parasites from the *Pf*NF54 strain, and its derivative *Pf*3D7, are the most commonly used *Pf* parasites in laboratory studies and in Controlled Human Malaria infections (CHMI)[36]. The complete genome sequences of *Pf*3D7 and *Pf*NF54 have been published[37,38]. The parasites of *Pf*NF54 and *Pf*3D7 have been deposited with the Malaria Research and Reference Reagent Resource Center (MR4; MRA-1000 and MRA-102), which was developed by the National Institute of Allergy and Infectious Diseases (NIAID) and is managed by the American Type Culture Collection (ATCC) (BEI Resources; https://www.beiresources.org/About/MR4.aspx). Parent parasites used for the generation of the *Pf*Δ*mei2* and the other gene-deletion mutants were obtained from a characterized good manufacturing process (GMP) produced working cell bank of the WT *Pf*NF54[35], produced by Sanaria Inc[39,40]. WT *Pf*NF54 is sensitive to the following antimalarial drugs: atovaquone/proguanil, arthemeter/lumefantrine and chloroquine[41].

For cultivation of *Pf* blood-stage parasites[42], Fresh human serum and human red blood cells (RBC) were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria

research and microbiology tested for safety). Production of genetically modified parasites and characterization of these parasites throughout their life cycle, including mosquito transmission, was performed under GMO permits IG 17-134_II-k en IG 17-135_III.

Generation and genotyping of *Pb PbΔmei2*

The *Pb mei2* (PBANKA_1122300) gene was deleted by standard methods of transfection [43] using a gene-deletion plasmid (PbGEM-300555, pL2206) obtained from PlasmoGEM (Wellcome Trust Sanger Institute, UK; http://plasmogem.sanger.ac.uk)[44]. This construct is designed to replace the *mei2* open reading frame (*orf*) by the h*dhfr::yfcu* selectable marker (SM) cassette by double cross-over homologous recombination. The SM cassette contains the h*dhfr::yfcu* flanked by the *Pb eef1α* promoter region and 3' terminal sequence of pb*dhfr*. Before transfection, the construct was linearized by digesting with *Not*I. Parasites of line 1868cl1 were transfected with construct pL2206 (exp. 2834) and transformed parasites selected by positive selection with pyrimethamine[43]. Selected parasites were cloned by limiting dilution and cloned lines 2834cl1 and 2834cl2 were used for genotype analysis. Line 2834cl2 was further used to generate the gene-deletion mutant which is SM free. To remove the h*dhfr::yfcu* SM cassette from the genome of 2834cl2, the parasites were selected (negative selection) by treatment of infected mice with 5-fluorocytosine (5-FC)[45]. This treatment selects for parasites that have undergone homologous recombination between the two 3'-UTR of pb*dhfr* untranslated regions present in the integrated construct pL2206, flanking the h*dhfr::yfcu* cassette and thereby removing the SM[46]. Selection and cloning of the parasites resulted in the SM-free gene-deletion line *PbΔmei2* (2834cl2m1cl1). Correct integration of the construct and deletion of the *mei2* gene were confirmed by Southern blot analyses of Pulsed Field Gel (PFG)-separated chromosomes and diagnostic PCR analysis[43]. To show integration of the PlasmoGem construct containing the h*dhfr::yfcu* SM or removal of the h*dhfr::yfcu* SM by negative selection, the PFG-separated chromosomes were hybridized with a mixture of two probes: a probe recognizing the h*dhfr* gene and a control probe recognizing gene PBANKA_0508000 on chromosome 5[47]. PCR primers for genotyping are listed in **Supplementary Table 1**.

Phenotyping of *Pb PbΔmei2*

The *in vivo* multiplication rate of asexual blood stages was determined during the cloning procedure of the different QC mutants[48]. For collection and counting of sporozoites from infected *An. stephensi* mosquitoes[49] mosquito salivary glands were manually dissected (21 days after feeding). Salivary glands were collected in RPMI medium, homogenized

and filtered (40 µm Falcon, Corning, NL). Free sporozoites were counted in a Bürker counting chamber using phase-contrast microscopy. The human-hepatoma cell line Huh7[50] was used for *in vitro* cultivation of liver-stages. Briefly, 5×10^4 isolated sporozoites were added to monolayers of Huh7 cells on coverslips in 24 well plates (with a confluency of 80–90%) in complete RPMI1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2% (vol/vol) penicillin-streptomycin, 1% (vol/vol) GlutaMAX (Invitrogen), and maintained at 37°C with 5% CO2. At 24, 48 and 72 hours post infection (p.i.) nuclei were stained with Hoechst-33342 at a final concentration of 10 µM and live imaging of mCherry-expressing parasites was performed using a Leica fluorescence MDR microscope (40x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: mCherry: 0.7 s and Hoechst 0.136 s (1x gain). Liver-stage parasite sizes were measured using Leica LAS X software by determining the area of the parasite at its greatest circumference using the mCherry-positive area (μm^2).

To determine the attenuation phenotype of *Pb*Δ*mei2*, C57BL/6 mice were infected with $5x10^3$, 5×10^4 or $2x10^5$ sporozoites of WT or *Pb*Δ*mei2*. Isolated sporozoites, suspended in RPMI-1640 medium, were intravenously injected into the tail vein (200 µl per mouse). Parasite liver-loads in live mice were quantified by real-time *in vivo* imaging[51]. Parasite liver-loads were visualized and quantified by measuring luciferase activity of parasites in whole bodies of mice at 44, 56 and 65 hours p.i using the IVIS Lumina II Imaging System (Perkin Elmer Life Sciences, Waltham, USA). D-luciferin was dissolved in PBS (100 mg/kg; Caliper Life Sciences, USA) and 60 µl injected subcutaneously in the neck. Measurements were performed within eight minutes after the injection of D-luciferin. Quantitative analysis of bioluminescence of whole bodies was performed by measuring the luminescence signal intensity (RLU; relative light units) using the ROI (region of interest) settings of the Living Image® 4.5.5 software. Mice were monitored for blood-stage infections by Giemsa-stained blood smears made at day 4 to 30 p.i. The prepatent period (measured in days after sporozoite challenge) is defined as the day when a blood-stage infection with a parasitemia of 0.5–2% is observed[47].

Generation and genotyping of four *Pf* gene-deletion mutants (see Supplementary Table 1 for primer sequences)

*Pf*Δ*palm:* The *palm* gene (PF3D7_0602300) was deleted using a CRISPR/Cas9 approach where first a plasmid was introduced into parasites as an episome containing the *cas9* gene[52], CRISPR/Cas9 system in Plasmodium falciparum using the centromere plasmid[53]. This plasmid, pLf0086, contains the h*dhfr* SM (with the *P. chabaudi dihydrofolate reductase*

thymidylate synthase gene promoter (*Pc*DT; PCHAS_0728300) and the *cas9* gene (with the *Pf heat shock protein 90* gene promoter the (PF3D7_0708400; *Pfhsp90*). To generate pLf0086, plasmid pLf0070 (pDC2-cam-Cas9-U6.2-hdhfr)[54,55] was digested with *BamH*I to remove the sgRNA/U6 cassette. Re-circularized plasmid in the *BamH*I site was termed pLf0086. Transfection of WT *Pf*NF54 parasites with plasmid pLf0086 was performed by the method of spontaneous plasmid uptake from plasmid-loaded RBC[56]. Transfected parasites were selected by treatment with the drug WR99210 (2.6 nM) for a period of two weeks (until parasites were detectable in Giemsa-stained thin blood films) to select for parasites containing the plasmid pLf0086 episomally (Exp.121). Subsequently, selected parasites were simultaneously transfected with two sgRNA/donor DNA plasmids, pLf0124, pLf0125. Both plasmids contain two homology regions (HR) targeting *palm*, a *blasticidin-S-deaminase* (*bsd*) SM cassette (with the *Pf hsp70* promoter; *Pfhsp70;* PF3D7_0818900) and a *palm* sgRNA cassette. Each plasmid contains a different sgRNA. To generate the *palm* targeting vectors, a basic plasmid pLf0103 was designed that contains the *bsd* SM cassette. To generate the *bsd* SM cassette, a gBlock was designed and ordered (https://eu.idtdna.com/pages), containing the *bsd* gene flanked by two 34 bp f*lippase recognition target* (*frt*) sequences (GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC[57]) (**Supplementary Fig. 7**). These sequences allow to recycle the SM cassette[57]. This fragment was cloned into the *Pb* transfection construct pL0034 (RMgm-687; www.pberghei.eu[46]) using the restriction enzymes *EcoR*I/*Hind*III resulting in intermediate plasmid SKK159. The *pfhsp70* promoter was obtained by PCR amplification (KOD Hot Start DNA Polymerase, Merck Millipore) using primers p1/p2 and cloned into the intermediate plasmid SKK159 using the restriction enzymes *Kpn*I/*Xho*I resulting in the intermediate plasmid SKK160. Finally, the *3'utr* of *Pf histidine rich protein 2 (Pfhrp2;* PF3D7_0831800) was obtained by PCR amplification (KOD Hot Start DNA Polymerase, Merck Millipore) using primers p3/p4 and cloned into SKK160 using the restriction enzymes *Not*I/*Avr*II resulting in the final basic plasmid pLf0103. This construct contains additional restriction sites for introducing homology/targeting sequences to target any gene of interest, such as *Nae*I/*Sac*II and *Apa*I/*Hind*III and for introducing the sgRNA/U6 cassettes, such as *Aat*II/ *BamH*I (see below). Plasmid pLf0039 with the *Pf u6* RNA promoter (PF3D7_1341100) containing the *BtgZ*I adaptor[58] was used two generate two sgRNA-expression cassettes for two intermediate plasmids containing sgRNA009 (pLf0110) and sgRNA010 (pLf0111). The guide sgRNA sequences for *palm* (sgRNA009 and sgRNA010) were identified using the Protospacer software (alpha version; https://sourceforge.net/projects/protospacerwb/files/Release/) and were amplified using the primers p5/p6 and p7/p8. These sgRNAs were selected based on the best off target hits score throughout the genome given by Protospacer and the total number of mismatches

of the sgRNA with respect to the Protospacer adjacent motif site (PAM). Two 20 bp primer guide sgRNAs, surrounded by 15 bp vector-specific DNA necessary for InFusion cloning (HD Cloning Kit; Clontech), were annealed and used to replace the *BtgZ*I adaptor[58], resulting in intermediate plasmids pLf0100, pLf0101, that were digested with *Bln*I/ *Nru*I for evaluation successful cloning and confirmed by Sanger sequencing using primers p9/p10. These constructs contain additional restriction sites for lifting the complete *u6* cassette including the sgRNA, such as *Aat*II/*BamH*I (see below). Next, two different sgRNA/donor DNA constructs, containing each of the sgRNA as well as the donor DNA sequences, were generated in multiple cloning steps resulting in pLf0124 and pLf0125. These constructs contain both the sgRNA expression cassettes and the *bsd* SM cassette. To generate the *palm* targeting vectors, plasmid pLf0103 was modified by introducing two HRs, HR1 and HR2, targeting *palm*. HR1 was amplified using primers P11/12 and HR2 with p13/p14 from WT *Pf*NF54 genomic DNA. HR2 was cloned into pLf0103 using restriction sites *Apa*I/*Hind*III, resulting in intermediate plasmid F171. Subsequently HR1 was cloned into F171 using *Nae*I/*Sac*II, resulting in intermediate plasmid pLf0110 (F177). These plasmids are used to introduce sgRNA/U6 cassette from the intermediate plasmids pLf0100 and pLf0101, containing sgRNA009 and sgRNA010 respectively (using restriction sites *Aat*II/ *BamH*I) to generate pLf0124 and pLf0125, respectively. Parasites of Exp.121 were transfected with the two plasmids pLf0124 and pLf0125 (a mixture of 50 µg of each circular plasmid in 200 µl cytomix) using standard transfection methods[59]. Selection of transfected parasites was performed by applying double-positive drug pressure from day 3 until day 9 after transfection using the drugs WR99210 (2.6 nM) and Blasticidin (BSD, 5 µg/ml). On day 9 drug pressure was removed and parasites were maintained in drug-free medium until parasites were detectable in thin blood-smears (day 15 after transfection). Selected parasites were then grown without both drugs until the parasitemia reached over 10%, followed by a second BSD selection (5 µg/ml) for a period of 7 days, resulting in parasite population Exp.167 (*PfΔpalm-1*) and Exp.169 (*PfΔpalm-2*). After drug selection, diagnostic PCR[42] was performed from material isolated from iRBC. Correct replacement of the *palm* gene with the *bsd* cassette in the parasites after the second BSD selection in *PfΔpalm-1* and *PfΔpalm-2* parasites was confirmed by long range PCR amplification (LR-PCR) (primers P15/P16) and standard PCR amplification of the *palm* open reading frame (primers p17/p18) and the *bsd* SM cassette (primers p19/p20). The PCR fragments were amplified using KOD Hot Start Polymerase (Merck Millipore) following standard conditions with annealing temperatures of 50.5 and 51°C for 25s and an elongation step of 68°C for three minutes.

*Pf*Δ*cbr*: The *Pfcbr* gene (PF3D7_1367500) was deleted in WT *Pf*NF54 parasites by standard methods of CRISPR/Cas9 transfection[58,59] using a sgRNA-expressing plasmid pLf0178, containing the *cas9* expression cassette, guide-RNA expression cassette and an h*dhfr* SM cassette, in combination with a donor DNA plasmid pLf0179 that contains a *bsd* SM cassette (linked to *gfp*, and separated with skip peptide 2A; *bsd*-2A-*gfp*) for positive selection and a y*fcu* SM cassette for negative selection. The sgRNA-expressing plasmid was generated as follows: pLf0070[54] was digested with *Bbs*I and the sgRNA067 was selected using the CHOPCHOP webtool (https://chopchop.cbu.uib.no/)[60] and subsequently cloned into the pLf0070 using primers p21/p22. In brief, the primers (100 µM each primer) were phosphorylated with T4 polynucleotide kinase (10 Units per reaction) during 30 minutes at 37 °C, followed by an annealing program of five minute incubation at 94°C and a ramp down to 25°C at 5°C per minute, and subsequently ligated into the *Bbs*I digested pLf0070 vector using T4 ligase (five units) resulting in the plasmid pLf0178.

The donor DNA plasmid pLf0179 was generated to replace the *Pfcbr* open reading frame with the *bsd* SM cassette linked to *gfp* (*bsd*-2A-gfp). For generation of pLf0179, the HR1 and HR2 regions of *Pfcbr* were amplified from WT *Pf*NF54 genomic DNA using primers p23/p24 and p25/p26. Fragments were digested with *Hind*III/*Apa*I and *Nhe*I/*BamH*I respectively and ligated into plasmid pLf0169 to obtain pLf0179. Plasmid pLf0169 was generated as follows: a new gBlock was designed and ordered (https://eu.idtdna.com/pages), containing the *bsd*-2A-*gfp* genes flanked by the two *frt* sequences. This fragment was cloned into the intermediate construct pL0f103 (see above) to replace the *bsd* cassette by *bsd*-2A-*gfp* using the restriction sites *Bsab*I/*Avr*II, resulting in the intermediate plasmid pLf0165. A second intermediate plasmid F213 was created amplifying the complete y*fcu* SM cassette controlled by the *pfhsp90* promoter and the 3' terminator sequence from *Pb dihydrofolate reductase thymidylate synthase* gene (Pb*dhfr/ts;* PBANKA_0719300*; 3'PcDT,* PCHAS_0728300), from the existing vector pLf0003 (pHHT-FRT-Pf36[57]) using the primers p27/p28. The complete cassette was cloned into the vector pJET1.2/blunt (thermo scientific) using the restriction enzyme *EcoR*V. Finally the y*fcu* SM cassete from the F213 plasmid was subcloned into the vector pLf0165 using the restriction sites *Stu*I for the pLf0165 and *Pvu*II/*Stu*I for the *yfcu* SM, resulting in the plasmid pLf0169.

Transfection of WT *Pf*NF54 parasites with plasmids pLf0178 and pLf0179 was performed by spontaneous plasmid uptake from plasmid-loaded red blood cells cultured[59]. Transgenic parasites were selected by applying 'double' positive selection 72 hours after transfection with the drugs WR99210 (2.6 nM) and BSD (5 µg/ml) during seven days. Subsequently, both drugs were removed from the cultures until thin blood-smears were parasite-positive, followed by applying negative selection by addition of 5-FC (1 µM) in order to eliminate

parasites that retained the Donor DNA construct as episomal plasmid. Negative drug pressure in the cultures was maintained until thin blood-smears were parasite-positive. After negative selection parasites (Exp. 236) were harvested for genotyping by diagnostic PCR and Southern analysis[58,59]. To confirm the integration and the presence of the *bsd*-2A-*gfp* cassette, *5'*-integration, *3'* integration and *bsd,* PCRs were performed using the primers p29/p30, p31/p32 and p20/p33 respectively. In addition the absence of the *pfcbr* open reading frame was confirmed using the primers p34/p35 . The PCR fragments were amplified using KOD Hot Start Polymerase (Merck Millipore) following standard conditions with annealing temperatures of 50, 55, 60°C for 10 s and an elongation step of 68°C. Southern blot analysis was performed with gDNA digested with *EcoR*I and *Nco*I (4 hrs at 37°C) in order to confirm the deletion of *Pfcbr*. Digested DNA was hybridized with a probe targeting the *Pfcbr HR2*, amplified from WT *Pf*NF54 genomic DNA by PCR using primers p25/p26 and the ampicillin probe (*amp*), was amplified using primers p36/p37.

Pf△*hcs1*: The *Pfhcs1* gene (PF3D7_1026900) was deleted in WT *Pf*NF54 parasites by standard methods of CRISPR/Cas9 transfection[58,59] using two different sgRNA-expressing plasmids, containing the *cas9* expression cassette, guide-RNA expression cassettes and h*dhfr* SM cassette, in combination with donor DNA plasmid pLf0191 that contains a *bsd*-2A-*gfp* SM cassette. The two different sgRNA-expressing plasmids were generated as follows: pLf0070[54] was digested with *Bbs*I and sgRNA074 and sgRNA075 (selected with CHOP-CHOP web tool) were cloned using primers p38/p39 and p40/p41, respectively, resulting in the plasmids pLf0193 and pLf0194. The donor DNA plasmid pLf0191 was generated to replace the *Pfhcs1 open reading frame* with a *bsd*-2A-*gfp*SM cassette flanked by two *frt* sequences. The HR1 and HR2 targeting regions of *Pfhcs1* were amplified from WT *Pf*NF54genomic DNA using the primers P42/P43 and P44/P45 respectively (**Supplementary Table 1**). Fragments were digested with *Hind*III/*Asc*I and *Sac*II/*Nhe*I and ligated into plasmid pLf0169 to obtain pLf0191. Transfection of WT *Pf*NF54 parasites with constructs pLf0191, pLf0193 and pLf0194 was performed by spontaneous plasmid uptake from plasmid-loaded red blood cells cultured[59] and selection of *Pf*Δ*hcs1* parasites was as described above for generation of *Pf*Δ*cbr*, resulting in the line *Pf*△*hcs1* (Exp. 252). For genotyping *Pf*△*hcs1* parasites, diagnostic PCR was performed. To confirm the integration and the presence of the *bsd*-2A-*gfp* cassette, *5'*-integration, *3'* integration PCRs were performed using the primers p46/p47, p48/p49 respectively, additionally the absence of the *pfhcs1* open reading frame was confirmed using the primers p50/p51. The PCR fragments were amplified using Phusion DNA Polymerase (NEB) following standard conditions with annealing temperatures of 58°C for 30s and an elongation step of 68°C.

*Pf*Δ*mei2*: The *mei2* gene (PF3D7_0623400) was deleted in WT *Pf*NF54 parasites by standard methods of CRISPR/Cas9 transfection[58] using a donor DNA plasmid pLf0105 and two different sgRNA-donor containing plasmids, pLf0080 and pLf0092, targeting the *mei2* gene. For generation of plasmid pLf0105, two homology regions targeting the *mei2* gene were introduced in pLf0103. HR1 was amplified from WT *Pf*NF54 genomic DNA using primers P52/P53 and HR2 with primers P54/P55. The PCR fragments were sequenced after TOPO TA (Invitrogen) subcloning and subsequently cloned into pLf0103 using restriction sites *Nae*I/*Sac*II and *Apa*I/*Hind*III, resulting in plasmid pLf0105. To generate the two sgRNA-donor containing vectors, plasmid pLf0070[54] was digested with *Bbs*I and sgRNA030 and sgRNA032 were cloned, as described above, using primers P56/P57 and P58/P59, respectively, resulting in the plasmids pLf0080 and pLf0092. Transfection of WT *Pf*NF54 (Leiden vial 9002 obtained from Nijmegen; NF54 54/329 4514) with constructs pLf0105, pLf0080 and pLf0092was performed by spontaneous plasmid uptake from plasmid-loaded RBC[59]. Selection of transfected WT *Pf*NF54 parasites was performed by applying double positive selection (as described for selecting for generation of *Pf*Δ*cbr)* for a period of 6-19 days. After this treatment period with two drugs, cultures were maintained in drug-free medium until parasites were detectable in Giemsa-stained thin blood smears (a period of three weeks). Subsequently, parasites were treated for one week with BSD (5µg/ml), resulting in parasite population Exp.151 (*Pf*Δ*mei2*a parasites; **Fig. 1**). Subsequently, selected parasites were cloned by limiting dilution. In order to remove the *bsd* SM cassette from the genome of *Pf*Δ*mei2*a, blood stage parasites of the uncloned population of *Pf*Δ*mei2*-a (Exp. 151) were transfected with plasmid pLf0120 that contains a *flpe* recombinase expression cassette (**Fig. 1**). To create plasmid pLf0120, we used plasmid pMV-FLPe[57] (pLf0038) that contains a *bsd* SM cassette and an *flpe* recombinase expression cassette. We first replaced the *bsd* SM cassette of plasmid pMV-FLPe with the h*dhfr-yfcu* SM cassette of plasmid pLf0039[58] (*Hind*III/*Kpn*I) to create pLf0120. The h*dhfr*-y*fcu* gene is flanked by the promoter of the *Pfhsp86* and the Pb*dhfr/ts* short (0,5kb) terminator sequences. The *flpe* gene is flanked by the promoter of the *Pfhsp90* from *Pf*Dd2 strain (PfDd2_070012600;) and Pb*dhfr/ts* long (1kb) terminator sequences. After transfection of *Pf*Δ*mei2*a blood stages with pLf0120 (as described above), cultures were treated for a period of six days (day 3-9) with WR99210 (2.6 nM), followed by a period of two weeks culture without WR99210 treatment. Subsequently, selected parasites were cloned by limiting dilution. DNA from iRBC was obtained from 10 ml cultures (parasitemia 3–10%). Diagnostic PCR was performed using primer pair (P60/P61). As a control, the gene *p47* (PF3D7_1346800) was PCR amplified using primers 8428/8756 (P62/P63). Southern blot analysis was performed with gDNA digested with *Xmn*I (4 h at 37°C) in order to confirm the deletion of *Pfmei2*. Digested DNA was hybridized with probes targeting the

Pfmei2 (a fragment of 539bp of the *mei2* coding sequence amplified using primers P64/P65, Pf*p47* gene (PF3D7_1346800, as a control fragment of 3910 bp, amplified using primers P62/P66), *ampicillin* gene (amp probe; amplified using primers P36/P37), the *bsd* SM (amplified using primers P67/P20), the *cas9* (amplified using primers P68/P69 and the *flpe* gene (amplified using primers P70/P71). In order to confirm the precise nature of the genetic deletion, a PCR product that encompass the *mei2* gene-deletion region of *Pf*Δ*mei2* was cloned and sequenced. This PCR product was obtained using primer pair P72/P73, cloned in pJET (Thermo Fisher Scientific) and sequenced using primers P74/P75.

Phenotyping of *Pf* mutants *PfΔpalm, PfΔhcs1* and *PfΔcbr*

The growth rate of asexual blood-stages (parasitemia) was monitored by determination of parasitemia in standard *in vitro* cultures (in a semi-automated shaker incubator system) for a period of four days with a starting parasitemia of 0.1%[42]. Parasitemia was determined by counting infected RBC in Giemsa-stained thin blood films in three independent experiments. Gametocyte production and exflagellation[54] were quantified in gametocyte cultures .

For analysis of mosquito stages (oocysts and sporozoites) *An. stephensi* mosquitoes were infected with day 14 gametocyte cultures using the standard membrane feeding assay (SMFA)[59,61]. Oocysts and salivary gland sporozoites were counted at days 9 and day 21 p.i., respectively. For counting sporozoites, salivary glands from 10 to 15 mosquitoes were dissected, collected in 100 µl of PBS and homogenized using a grinder. Sporozoites were counted using a Bürker cell counter using phase-contrast microscopy.

Oocysts were analyzed in manually dissected midguts using a Leica MZ16 FA stereo-fluorescent microscope. The midguts were imaged with a Leica MZ camera at 10X magnification using Leica LAS X software. Individual oocysts were observed under a Leica DM2500 light microscope and documented with at 100X using Leica DC500 digital camera using Leica LAS X software. Sg-sporozoite numbers were analyzed in infected mosquitoes at day 18-21 p.i. For counting sporozoites, salivary glands from 30-60 mosquitoes were dissected and homogenized using a grinder in 100 µl of RPMI-1640 medium (pH 7.2) and sporozoites were analyzed in a Bürker cell counter using phase-contrast microscopy[54].

Phenotyping of *Pf* mutant *PfΔmei2*

The growth rate of asexual blood-stages and analysis of mosquito stages (oocysts and sporozoites) in *An. stephensi* mosquitoes was performed as described in the previous section for the other three gene-deletion mutants.

BioIVT hepatocytes: Analysis of development of WT *Pf*NF54 and *Pf*Δ*mei2* parasites in primary human hepatocytes[58] was performed as follows. Liver-stages of WT *Pf*NF54 and *Pf*Δ*mei2* were cultured *in vitro* using cryopreserved primary human hepatocytes obtained from BioIVT (Belgium) and thawed according to the instructions of the manufacturer. Cells were seeded at a density of 60,000 cells/well in a collagen-coated 96-well clear-bottomed black plate for two days. Medium was refreshed daily (hepatocyte medium: Williams's E medium supplemented with 10% heat inactivated fetal bovine serum, 2% penicillin-streptomycin, 1% fungizone, 0.1 IU/ml insulin, 1.6 µM dexamethasone). Per well, 7 x 10^4 freshly dissected WT *Pf*NF54 *and Pf*Δ*mei2* sporozoites were added to the hepatocyte monolayer. After a quick spin (10 minutes at 1900 *g*), the plate was incubated at 37°C under 5% CO2. The medium was replaced with fresh hepatocyte culture medium three hours p.i., and daily for 9 days thereafter. At days 3, 5, 7 and 9 p.i., hepatocytes were fixed with 4% paraformaldehyde in 1X PBS during 30 minutes. After fixation the wells were washed three times with 1X PBS and permeabilized with 300 µl of 0.5% triton in 1X PBS during 1 hour and then blocked with 10% of FCS in 1X PBS for 1 hour. Fixed cells were washed with 1X PBS and standard IFA were performed using antibodies against 1) the cytoplasmic protein *Pf*HSP70 (PF3D7_0818900; rabbit anti-*Pf*HSP70-PE/ATTO 594 conjugated primary antibody; 1:200 dilution of 100 µg/ml stock solution StressMarq, Biosciences, NL); 2) the plasma membrane surface protein MSP1 (PF3D7_0930300; mouse monoclonal antibody 1:1000 of 4.0mg/ml stock solution obtained from The European Malaria Reagent Repository, Edinburgh, UK) 3) and the parasitophorous membrane protein EXP1 (PF3D7_1121600, mouse monoclonal antibody (1:200 of 4.0 mg/ml stock solution obtained from The European Malaria Reagent Repository, Edinburgh, UK). In addition, cells were stained with Hoechst-33342 for nuclear staining. Cells were mounted in Image-iT signal Enhancer (Invitrogen Thermofisher, USA) and examined with a SP8 Leica confocal microscope at 100x magnification. Number of infected hepatocytes, antibody staining and size of parasites were analysed using ImageJ.

LONZA hepatocytes: Cryopreserved primary human hepatocytes purchased from Lonza Bioscience were thawed and seeded in µ-Slide 18 Well ibiTreat coverslip (IBIDI, Gräfelfing, Germany), pre-coated with rat-tail collagen I (BD Bioscience, USA) in William's E medium (Gibco) supplemented with 10 % fetal clone III serum (FCS, Hyclone), 100 u/mL penicillin and 100 ug/mL streptomycin (Gibco), $5 \times 10{-}3$ g/L human insulin (Sigma-Merck), $5 \times 10{-}5$ M hydrocortisone (Upjohn Laboratories SERB, France) at 37°C in 5 % CO2. The next day, cells were overlaid with matrigel (Corning) and medium was then renewed every two days. Three days later, sporozoites were isolated by aseptic hand dissection of salivary glands of *Pf*Δ*mei2*- and WT PfNF54-infected mosquitoes. Matrigel was removed from

hepatocyte culture and 30,000 sporozoites were inoculated to cells before centrifugation at 560 xg for 10 minutes at RT and further incubation at 37°C, 5 % CO2. Three hours later, infected cultures were covered with matrigel prior to addition of fresh cell culture medium. Medium was renewed every day, until cell fixation at the chosen times. Infected cultures were fixed with 4% paraformaldehyde for 15 minutes at room temperature and liver stage parasites were immunostained with polyclonal anti-PfHSP70 murine serum prepared in the lab, anti-PfEXP1 (kindly provided by Pr Jude Przyborski) and revealed with anti-mouse IgG Alexa Fluor® 594 and anti-rabbit IgG Alexa-Fluor 488-conjugated, respectively (Invitrogen). DAPI was added to visualize nuclei. Parasite number and size were determined using a Cell Insight High Content Screening platform equipped with the Studio HCS software (Thermo Fisher Scientific) at the CELIS platform (ICM, La Pitié-Salpêtière, Paris). Graphs and statistical analysis were done using Prism 8.4.3 software. The areas of parasites were compared using the Mann–Whitney U-test.

Liver stage development of both *Pf*Δ*mei2* and WT *Pf*NF54 parasites were analysed in liver-chimeric humanized mice (FRG huHep mice) purchased from Yecuris Corporation (Tualatin, OR) and housed at Oregon Health and Science University (OHSU) as per manufacturer's recommendation. All studies were performed according to the regulations of the Institutional Animal Care and Use Committee (IACUC; protocol IP00002077). Female *An. stephensi* mosquitoes, aged 3-5 days, were infected with *Pf*Δ*mei2* and WT *Pf*NF54 at LUMC (Leiden, the Netherlands)[54,59]. 12 days after feeding, mosquitoes were shipped to OHSU. Sporozoites were isolated by salivary gland dissection from infected mosquitoes at day 16 p.i. at OHSU for infection of the FRG huHep mice. Isolated salivary gland sporozoite were run over glass wool to remove contaminating mosquito material and sporozoites enumerated by hemocytometer. FRG huHep mice (*Pf*NF54 WT n=4; *Pf*Δ*mei2* n=7) were infected by intravenous injection (retro-orbital injection) of 10^5 sporozoites in a 100 µl volume of phosphate buffered saline. Five days p.i., FRG huHep mice were injected intravenously with 400 µl freshly washed human type AB red blood cells (RBC; 70% hematocrit) supplemented with 0.035mg/mouse clodronate (Formumax CloLip) and penicillin/streptomycin antibiotic (50 units penicillin, 50 µg streptomycin/ml, Sigma). The following day, FRG huHep mice were injected intraperitoneally with 700 µl freshly washed human type AB RBC (70% hematocrit). At 7 and 9 p.i. 100 µl of blood was collected into 2 ml NucliSens buffer (Biomerieux) for qRT-PCR analysis. At day 9 p.i. mice were euthanized and blood collected for cryopreservation in glycerolyte. To analyse for the presence of blood stages in blood samples collected from the FRG huHep mice (day 7, 9) 18S qRT-PCR[62] quantification of blood-stage parasites was performed . In addition to the qRT-PCR analyses, the cryopreserved blood of all FRG huHep mice

(collected at day 9) was used for *in vitro* cultivation of parasites, performed at the LUMC (Leiden, the Netherlands), to assess the presence/absence of blood stage parasites. For these experiments a standard protocol for *in vitro* cultivation of blood stages was used[58,59]. Cultures, maintained in a semi-automated shaker system, were monitored for blood stage parasites for a period of 28 days by analyzing Giemsa stained thin and thick blood smears and 18S qRT-PCR and fresh RBC (100 µl packed RBC in 1 ml culture medium) were added to the cultures weekly. Briefly, total DNA was isolated from blood samples using QIAamp DNA Blood Mini Kit (Qiagen, NL) in accordance with the manufacturer's instructions, and a one-step q-PCR Hotstar mastermix (Qiagen, NL) assay specific for the 18S ribosomal subunit of *Pf* was used to quantify the presence of parasites (forward primer: 5'- CCGACTAGGTGTTGGATGAAAGTGTTAA -3'; reverse primer: 5'- AACCCAAAGACTTTGATTTCTCATAA -3'; Probe: 5'-FAM-CTTTCGAGGTGACTTTTAGAT- MGB (quencher)[63]; cycling profile: 15 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 20 seconds at 60°C and 30 seconds at 72°C. Resulting CT values were compared to a standard curve of known quantity of 18S plasmid spanning 100 to 10(8) copies. To determine positive/negative parasite samples, the same cutoff as is used in controlled human malaria infection (CHMI) of five parasites/ml, assuming 7400 18s copies/parasite (at OHSU) and five parasites/ml, assuming 4252 18s copies/parasite.

Uncropped and unprocessed scans of gels and blots are shown in the **Supplementary Fig. 8 to 13**.

Whole genome sequencing of *PfΔmei2*

Genomic DNA of *PfΔmei2* was isolated from blood stage-infected RBC obtained from 10 ml cultures (parasitemia 3–10%). RBC were pelleted by centrifugation (1150 g; five minutes) and subsequently lysed with 5–10 ml of cold (4°C) erythrocyte lysis buffer (10x stock solution 1.5 M NH4Cl, 0.1 M KHCO3, 0.01 M Na2EDTA; pH 7.4). Pelleted parasites were treated with RNAse and proteinase-K before genomic DNA isolation by standard phenol-chloroform methods. Whole genome sequencing was performed at the King Abdullah University of Science and Technology (KAUST, Thuwal, Saudi Arabia Prof. Arnab Pain). Genomic DNA quantification was carried out using Qubit based colorimetric assay (Invitrogen). A total of 200 ng of DNA was used for DNA library preparation using NebNext Ultra II DNA library prep kit for for Illumina (NEB). Upon library quantification and size verification, DNA library sequencing was carried out on a MiSeq platform (Illumina) that produced 2x150 bp paired-end reads. The quality of the raw reads was assessed using FATSQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Low-quality

reads and Illumina adaptors sequences from the end of the reads were removed using Trimmomatic [64]. Quality trimmed reads were mapped to *Pf* 3D7 reference genome (release 40 in PlasmoDB- http://www.plasmoddb.org) using BWA (version 0.7.17)[65]. Read pairing information, flag and duplicate reads were removed using Picard's CleanSam, FixMateInformation, and MarkDuplicates tools. The full genome sequence has been uploaded in the European Nucleotide Archive. Project ID has been created in ENA with the study accession number PRJEB40003 (Public release date set to 25/08.2021). SNPs were called using the genome analysis tool kit (GATK) best practices pipeline[66]. Identified SNPs were filtered using vcftools to keep high-quality SNP with the quality score (Q) ≥ 30 and depth (d) ≥ 50. A total of 105 high-quality SNPs were identified. SNPs were annotated and their effect on coding sequences of genes was done via snpEFF. For insertion and deletion (InDel) identification, raw gapped alignment were realigned was using GATK RealignerTargetCreator and IndelRealigner tools. Insertion between 20-2000 bp and deletion between 20-2000 bp were identified using GATK's SelectVariants tool. Variants were tagged with quality score (Q) ≥ 30 and depth (d) ≥ 50 tagged using vcfannotate option and only InDels with a quality score (Q) ≥ 30 and depth (d) ≥ 100 were filtered for further analysis. Confirmation of absence of the gene sequences of *cas9*, *ampicillin*, *blasticidin*, *hdhfr*, *yfcu*, *flpe recombinase* was determined by concatenating the DNA sequence of these genes into a single fasta file and mapping the quality trimmed reads to the indexed sequences using HISAT2 (V 2.1.0)[67]. Read coverage was visualized using Integrative Genome Viewer browser [68].

Drug-sensitivity testing of *PfΔmei2*

Drug sensitivity was established in standard drug-sensitivity assays[69]. Comparative drug sensitivity testing was performed for three *Pf* lines (NF54-HGL, WT *Pf*NF54 and *PfΔmei2*) with 7 different compounds in the asexual blood stage (ABS) replication assay. As assay reference, NF54-ΔPf47-5′ hsp70-GFP-Luc [69] (hereafter: NF54-HGL), a transgenic strain of *Pf* that stably expresses a GFP-Luciferase fusion protein under control of the constitutive *Pf hsp70* promoter, was used. In the assay parasites from asynchronous asexual blood stage cultures were diluted to achieve a parasitemia of 0.8% in 3% hematocrit in RPMI1640 medium supplemented with 10% human serum. 30 µl of diluted parasites were combined with 30 µl of diluted compound in a 384 well microtiter plate. Following 72 hour incubation at 37°C, 3% O2, 4% CO2, relative parasitemia was determined through a SYBR Green assay. Compounds were dissolved in DMSO to a stock concentration of 10 mM and diluted in DMSO and then in RPMI1640 medium to reach a final DMSO concentration of 0.1% for all conditions tested. Compounds were tested at nine dilutions

in 0.5 log steps from 10 µM to 1 nM with duplicate wells on each plate. Dihydroartemisinin (DHA) was taken along as a reference compound from 100 pM to 1 µM. Plate controls included MAX (0.1% DMSO) and MIN (1 µM DHA) with 14 replicates each per plate. Relative parasitemia was normalized to the MIN and MAX controls. IC50 values were determined using a four parameter non-linear regression model using least-squares to find the best fit using the Prism software package (GraphPad Software, San Diego). For each of the plates, Z' values were calculated based on the MIN and MAX controls.

Statistical analysis

Statistical analyses were performed using Mann Whitney test with the GraphPad Prism software package 9 (GraphPad Software, Inc). A p-value less than 0.05 was considered significant.

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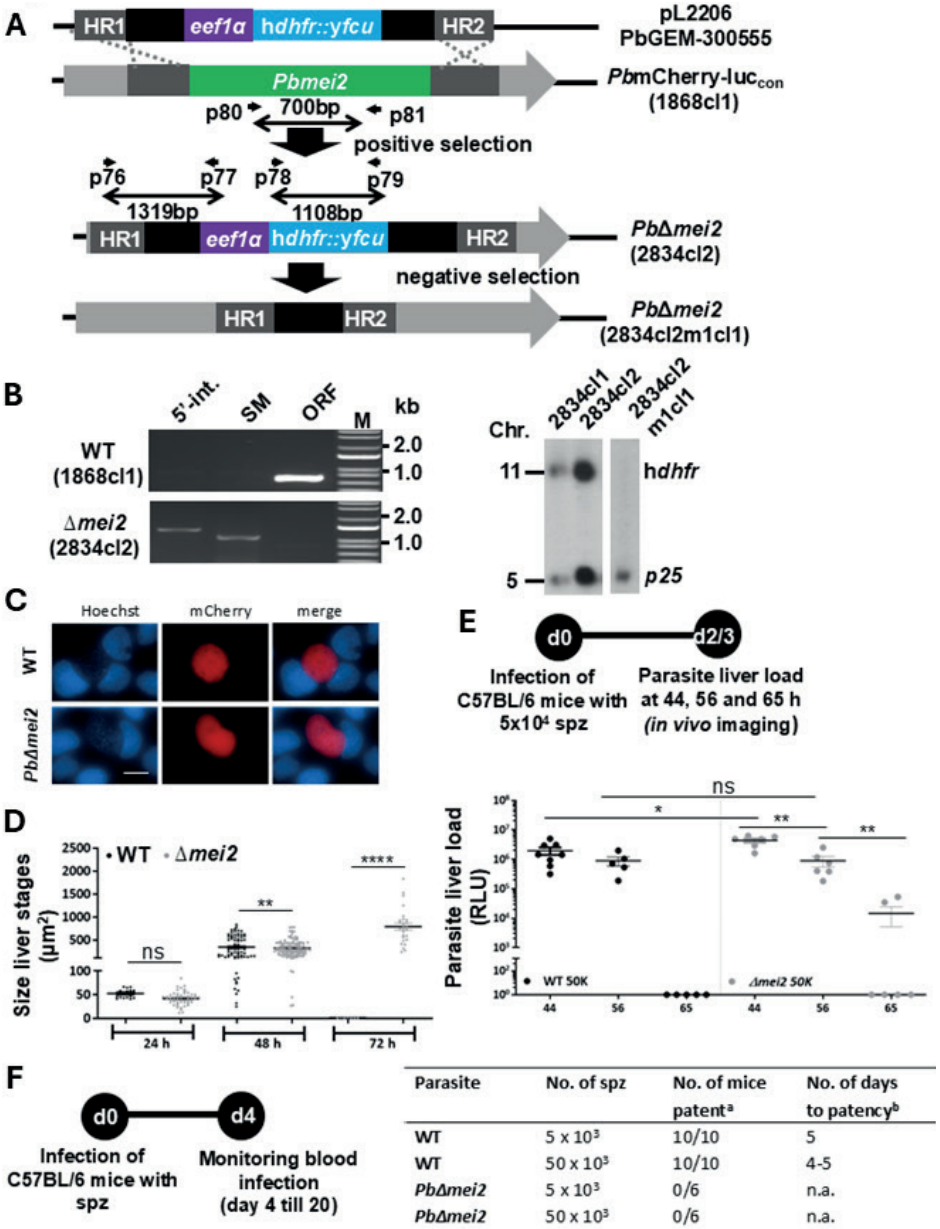
Supplementary appendix

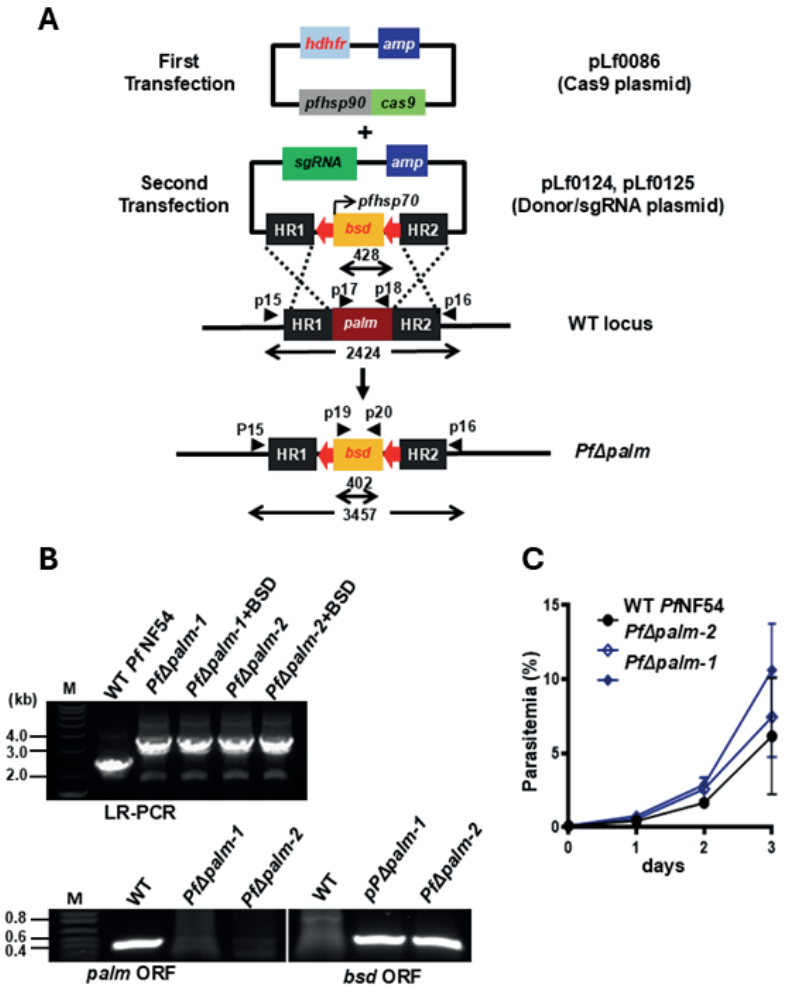
Supplementary figures



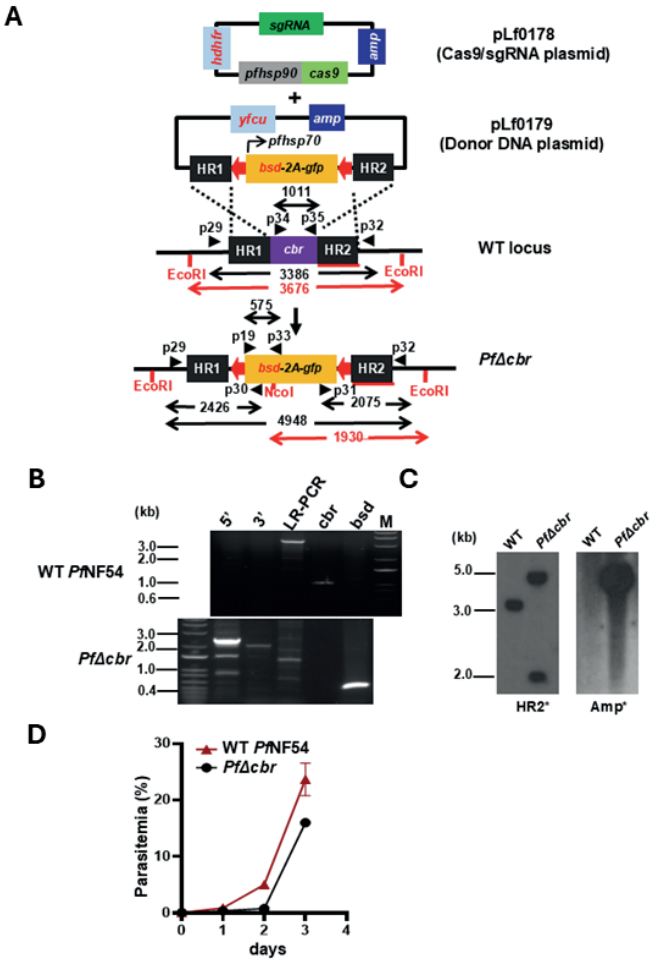
Parasite	No. of spz	No. of mice patent[a]	No. of days to patency[b]
WT	5 x 10^3	10/10	5
WT	50 x 10^3	10/10	4-5
PbΔmei2	5 x 10^3	0/6	n.a.
PbΔmei2	50 x 10^3	0/6	n.a.
PbΔmei2	200 x 10^3	3/10	9-10
PbΔmei2-b-a	200 x 10^3	5/11	9-10

[a] The number of mice that developed a blood stage infection
[b] The time of blood stage parasitemia between 0.5-2%
WT: wild type; n.a. not applicable

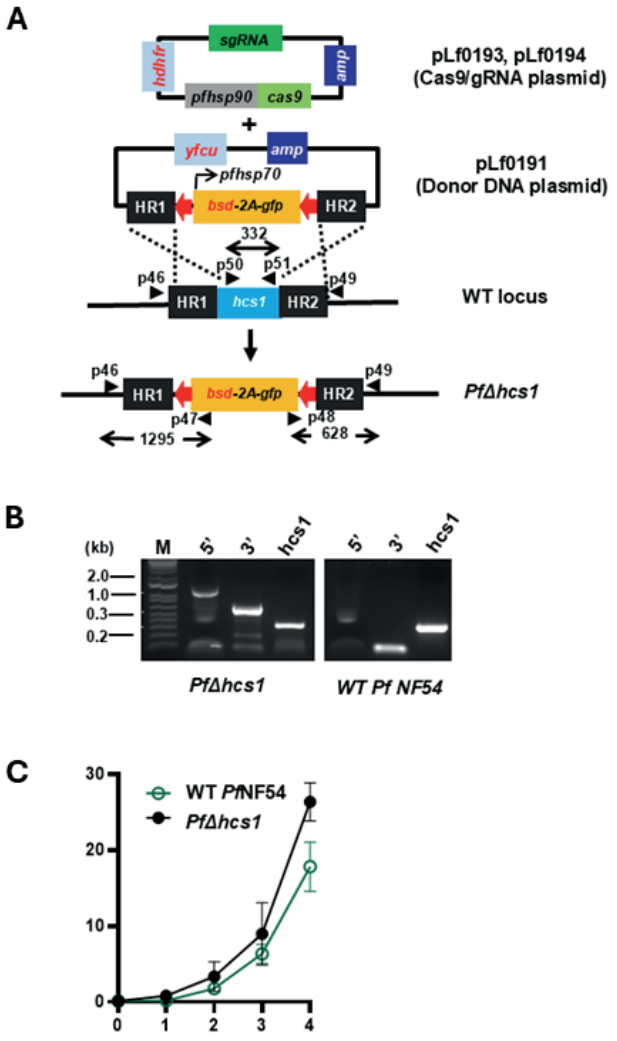
Supplementary Figure 1. Generation and genotype and phenotype analysis of the drug-selectable marker free *P. berghei* ANKA gene-deletion mutant *Pb*Δ*mei2*.
A. Schematic representation of the introduction of the h*dhfr*-y*fcu* selectable marker (SM) cassette into the *Pbmei2* gene locus of the parent *P. berghei* parasite (line 1868cl1). Construct pL2206 contains the SM flanked by the *eef1a* promoter region and the 3'-*utr* of pb*dhfr* and integrates into the *mei2* locus by double cross-over homologous recombination at the *mei2* homology regions (HR1, HR2). Positive selection with pyrimethamine selects for parasites that have the *mei2* coding sequence replaced by the SM cassette, resulting in parasite line 2834cl2. To remove the SM cassette from the genome of 2834cl2, blood stage parasites of 2834cl2 were selected (negative selection) by treatment of infected mice with 5-Fluorocytosine (5-FC), selecting for parasites that have undergone homologous recombination between the two 3'-*utr* of pb*dhfr* untranslated regions present in the integrated construct pL2206, flanking the SM cassette and thereby removing the SM. This creates the SM free *Pb*Δ*mei2* gene-deletion mutant (2834cl2m1cl1). Location of primers (p) used for PCR analysis and PCR product sizes are shown. Primer details in **Supplementary Table 1**.
B. Diagnostic PCR (left panel) and Southern analysis of PFG-separated chromosomes (right panel) confirm correct integration of construct pL2206 in parasites of line 2834cl2. PCR shows the presence of the h*dhfr*::y*fcu* SM (primers **p78/p79**); 5' integration PCR (5'-int; primers **p76/p77**); *mei2* open reading frame PCR (ORF; primers **p80/p81**). Primer locations and product sizes are shown in **A** and primer details in **Supplementary Table 1**. Hybridization of PFG-separated chromosomes (chr.) (right panel) with a mixture of two probes, h*dhfr* probe and a control probe *p25* (PBANKA_0515000; chromosome 5) shows the presence of the SM in the *mei2* locus on chromosome 11 in two clones of the 2834 line and the absence of the SM in the *mei2* locus on chromosome 11 in SM free *Pb*Δ*mei2* gene-deletion mutant (2834cl2m1cl1). M, molecular weight marker; 1kb DNA ladder (Invitrogen).
C. Representative fluorescence-microscopy images of live parasites at 48 hours after infection of cultures Huh7 hepatocytes with sporozoites of WT and *Pb*Δ*mei2*. Liver-stages express cytoplasmic mCherry under control of the constitutive *hsp70* promoter. Scale bar, 10μm.
D. Size of WT (black) and *Pb*Δ*mei2* (grey) liver-stages at different time points after infection of cultured hepatocytes. The size of parasites (n=20-70) was measured at each time point by determining the area of the parasite's cytoplasm at its greatest circumference using the mCherry-positive area (μm2). Nuclei of parasites and hepatocytes were stained with Hoechst (blue), scale bar: 10 μm. Significance values (unpaired Mann Whitney test): $^{**}p < 0.01$; $^{****}p < 0.0001$; n.s. – not significant.
E. The time line showing infection of C57BL/6 mice by intravenous injection of 5×10^4 of WT (black) and Pb*Δmei2* (grey) sporozoites, followed by determination of parasite liver loads in mice (n=6) by *in vivo* imaging. Parasite liver loads as determined by measuring *in vivo* luciferase activity and depicted as relative light units (RLU). Significance values (unpaired Mann Whitney test): $^{*}p < 0.05$; $^{**}p < 0.01$; n.s. – not significant.
F. The time-line shows infection of C57BL/6 mice with sporozoites and determination of the prepatency of blood infection. The table shows the time to blood-stage patency in C57BL/6 mice infected by intravenous injection of sporozoites.
Error bars represent standard error of the mean.



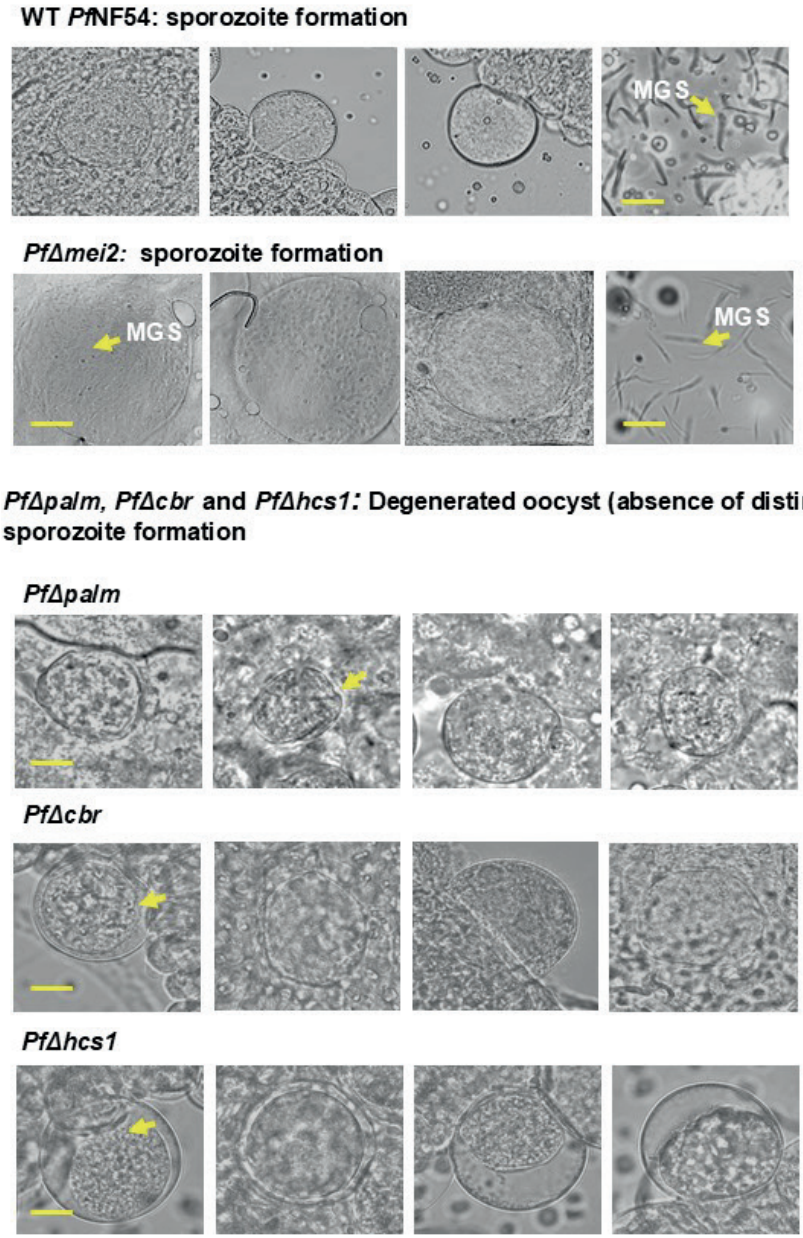
Supplementary Figure 2. Generation, genotyping and blood-stage growth of *PfΔpalm*
A. Schematic representation (not drawn to scale) of the Cas9 (pLf0086) and the two Donor/gRNA plasmids (pLf0124 and pLf0125) constructs used to generate the two *PfΔpalm* lines (*PfΔpalm*-1, exp. 167 and *PfΔpalm*-2 exp.169). The *Pfpalm* homology regions (HR1, HR2), *blasticidin-S-deaminase* (*bsd*) selectable marker (SM) cassette, location of primers (black arrows) and PCR amplicons (black) are indicated. Primer sequences (in black) are shown in **Supplementary Table 1**. WT - wild type *Pf*NF54; human *dihydrofolate reductase-thymidylate synthase* (h*dhfr*) SM cassette; yeast cytosine deaminase uridyl phosphoribosyl transferase (y*fcu*) SM cassette; 5' *utr* heat shock protein 90 (*pfhsp90*), *5' utr* heat shock protein 70 (*pfhsp70*), *ampicillin (amp)*, single guide RNA (sgRNA).
B. Diagnostic PCR confirms correct integration of the donor/gRNA construct by LR-PCR (primers p15/p16), presence of the *bsd* SM (primers p19/p20) and absence of the *Pfpalm* coding sequence in *PfΔpalm*-1 and *PfΔpalm*-2 parasites (primers p17/p18). Primer locations and product sizes are shown in **(A)** and primer details in **Supplementary Table 1**.
M: molecular weight marker; 1kb DNA ladder (Invitrogen).
C. *In vitro* growth rate of *PfΔpalm*-1, *PfΔpalm*-2 and WT *Pf* NF54 asexual blood-stages. Parasitemia (%) during a three day culture period (mean and s.d. of three cultures). Error bars represent standard deviation.



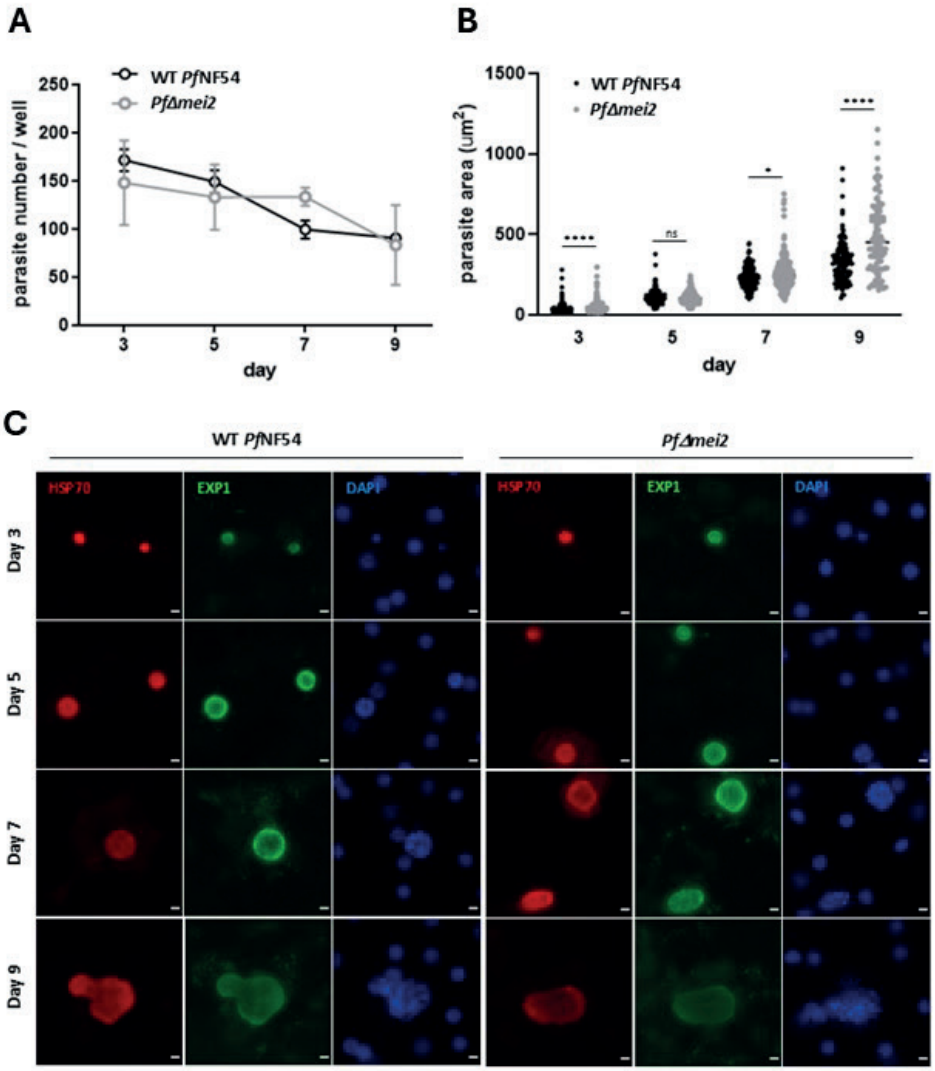
Supplementary Figure 3. Generation, genotyping and blood-stage growth of *PfΔcbr*
A. Schematic representation (not drawn to scale) of the Cas9/gRNA plasmid (pLf0178) and the Donor DNA plasmid (pLf0179) construct used to generate the *PfΔcbr* line (exp.236). The *Pfcbr* homology regions (HR1, HR2), the *blasticidin-S-deaminase* (*bsd*) selectable marker (SM) linked to *gfp* through the 2A peptide, location of primers (black arrows) and PCR amplicons (black) are indicated. Primer sequences (in black) are shown in **Supplementary Table 1**. WT - wild type WT *Pf*NF54; human *dihydrofolate reductase-thymidylate synthase* (h*dhfr*) SM cassette; yeast cytosine deaminase uridyl phosphoribosyl transferase y*fcu*) SM cassette; 5' *utr* heat shock protein 90 (*pfhsp90*), *5' utr* heat shock protein 70 (*pfhsp70*), *ampicillin (amp*), single guide RNA (sgRNA).
B. Diagnostic PCR confirms correct integration of the donor/gRNA construct by 5' (primers p29/p30) and 3'integration (primers **p31/p32**), presence of the *bsd* SM (primers **p19/p33**) and absence of the *Pfcbr* open reading frame (primers p34/p35) in *PfΔcbr* parasites. Primer locations and product sizes are shown in **A** and primer details in **Supplementary Table 1**. M: molecular weight marker; 1kb DNA ladder (Invitrogen).
C. Southern analysis of digested DNA of WT *Pf*NF54 and *PfΔcbr* confirms *Pfcbr* gene deletion. DNA was hybridized with a probe targeting the HR2 of *Pfcbr* (primers **p31/p32**) and control probe targeting *ampicilin* (*amp*, primers **p36/p37**). The hybridization fragments of WT *Pf*NF54 (3.6 kb) are absent in the *PfΔcbr*, and the two fragments (1.9 and 5.0 kb) of the single crossing-over integration were present in *PfΔcbr*, indicating removal the *Pfcbr* coding sequence by single cross-over integration. Product sizes are shown in **A.** Primer details in **Supplementary Table 1**.
D. *In vitro* growth rate of *PfΔcbr* and WT *Pf* NF154 asexual blood-stages. Parasitemia (%) during a 4-day culture period (mean and s.d. of 3 cultures). Error bars represent standard deviation.



Supplementary Figure 4. Generation, genotyping and blood-stage growth *PfΔhcs1*
A. Schematic representation (not drawn to scale) of the Cas9/gRNA plasmids (pLf0193, pLf0194) and the Donor DNA plasmid (pLf0191) construct used to generate the *PfΔhcs1* line (exp.236). The *Pfhcs1* homology regions (HR1, HR2), the *blasticidin-S-deaminase* (*bsd*) selectable marker (SM) linked to *gfp* through the 2A peptide, location of primers (black arrows) and PCR amplicons (black) are indicated. Primer sequences (in black) in **Supplementary Table 1**. WT - wild type WT *Pf*NF54; human *dihydrofolate reductase-thymidylate synthase* (h*dhfr*) SM cassette; yeast cytosine deaminase uridyl phosphoribosyl transferase y*fcu*) SM cassette; 5' *utr* heat shock protein 90 (*pfhsp90*), *5' utr* heat shock protein 70 (*pfhsp70*), *ampicillin (amp*), single guide RNA (sgRNA).
B. Diagnostic PCR confirms correct integration of the donor/gRNA construct by 5' (primers **p15/p16**) and 3' integration (primers **p46/p47**), presence of the *bsd* SM (primers **p48/p49**) and absence of the *Pfhcs1* open reading frame in *PfΔhcs1* parasites (primers **p50/p51**). Primer locations and product sizes are shown in **A** and primer details in **Supplementary Table 1**.
M: molecular weight marker; 1kb DNA ladder (Invitrogen).
C. *In vitro* growth rate of *PfΔhcs1* and WT *Pf* NF154 asexual blood-stages. Parasitemia (%) during a four day culture period (mean and s.d. of 3 cultures). Error bars represent standard deviation.



Supplementary Figure 5. Oocyst of WT *Pf*NF54, *PfΔmei2, PfΔpalm, PfΔcbr* and *PfΔhcs1* parasites
Light microscope pictures of oocysts from days 9-12 after feeding gametocytes to *An. stephensi* mosquitoes. Sporozoite formation in oocysts from WT *Pf*NF54 and *PfΔmei2* while no sporozoite formation observed in *PfΔpalm, PfΔcbr and PfΔhcs1* oocysts. These oocysts are classified as degenerate based on the absence of distinct sporozoite formation and/or vacuolated cytoplasm. Midgut sporozoites (MGS). Scale bar, 10μm.

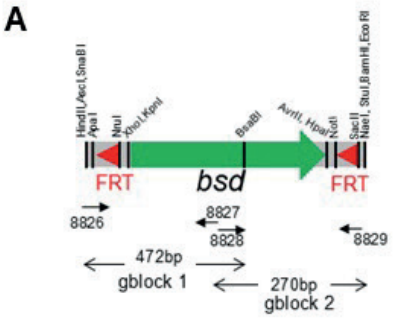


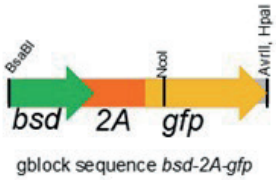
Supplementary Figure 6. *Pf*Δ*mei2* liver-stage development in cultured human primary hepatocytes (LONZA)

A. Number of WT *Pf*NF54 and *Pf*Δ*mei2* parasites per well at day3, 5, 7 and 9 p.i. Error bars represent standard error of the mean.

B. Liver-stage size on day 3, 5, 7 and 9 p.i. (99-245 parasites measured in 2 wells). The average of the parasite's cytoplasm at its greatest circumference using HSP70-positive area (µm²), s.d. and significances values are shown (unpaired Mann Whitney test: *p < 0.05; ****p < 0.0001; ns: not significant).

C. Representative confocal microscopy images of liver-stages on days 3, 5, 7 and 9 p.i. Left panel WT *Pf*NF54; right panel *Pf*Δmei2. Fixed hepatocytes were stained with the following antibodies: mouse anti-*Pf*HSP70 (α hsp70) and rabbit anti-*Pf*EXP1 (α exp1). Nuclei stained with Dapi. All pictures were analyzed using a Cell Insight High Content Screening platform equipped with the Studio HCS software; Alexa Fluor® 488 (green); anti-IgG Alexa Fluor® 594 (red); Dapi (blue). Scale bar, 5µm.





C **gblock sequence *bsd***

gblock sequence 1

HindIII

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attctctagaaagtataggaacttcTCGCGAgtcagctaCTCGAGgtcagctaGGTACCgtcagctaATGCATGCCAAGCCTTTGT
CTCAAGAAGAATCCACCCTCATTGAAAGAGCAACGGCTACAATCAACAGCATCCCCATCTCTGAAGACTAC
AGCGTCGCCAGCGCAGCTCTCTCTAGCGACGGCCGCATCTTCACTGGTGTCAATGTATATCATTTTACTG
GGGGACCTTGTGCAGAACTCGTGGTGCTGGGCACTGCTGCTGCTGCGGCAGCTGGCAACCTGACTTGTA
TCGTAGCGATCGGAAATGAGAACAGGGGCATCTTGAGCCCCTGCGGACGGTGCCGACAGGTGCTTCTCG
ATCTGCATCCTGGGATCAA

BsaB1

gblock sequence 2

BsaB1

GATCTGCATCCTGGGATCAAAGCCATAGTGAAGGACAGTGATGGACAGCCGACGGCAGTTGGGATTCGT
GAATTGCTGCCCTCTGGTTATGTGTGGGAGGGCTAAGTTAACgtcagctaCCTAGGgtcagctaGCGGCCGCgtca
gctaGaagttcctattctctagaaagtatagGaacttcgtcaCCGCGGgtcagctaGCCGGCgtcagctaAGGCCTgtcagctaGGATCCgt
cagcta***GAATTC***TTCTTCAATTCACTGGCCGTC

EcoRI

D

gblock sequence *bsd-2a-gfp*

BsaB1

GATCTGCATCCTGGGATCAAAGCCATAGTGAAGGACAGTGATGGACAGCCGACGGCAGTTGGGATTCGTG
AATTGCTGCCCTCTGGTTATGTGTGGGAGGGCGGAGAAGGAAGAGGAAGTTTATTAACATGTGGAGATGTA
GAAGAAAATCCAGGACCAATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAG
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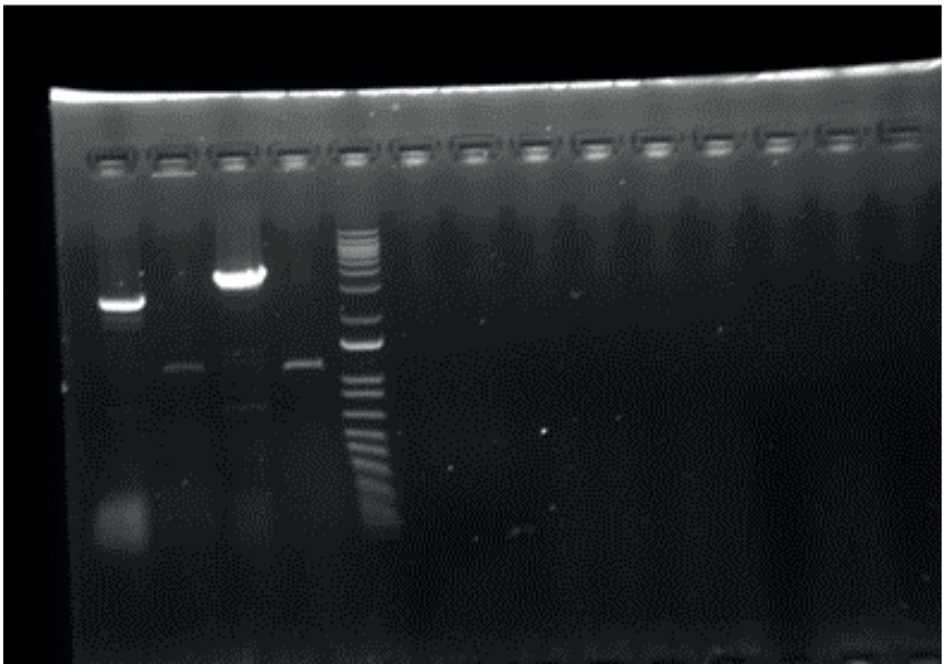
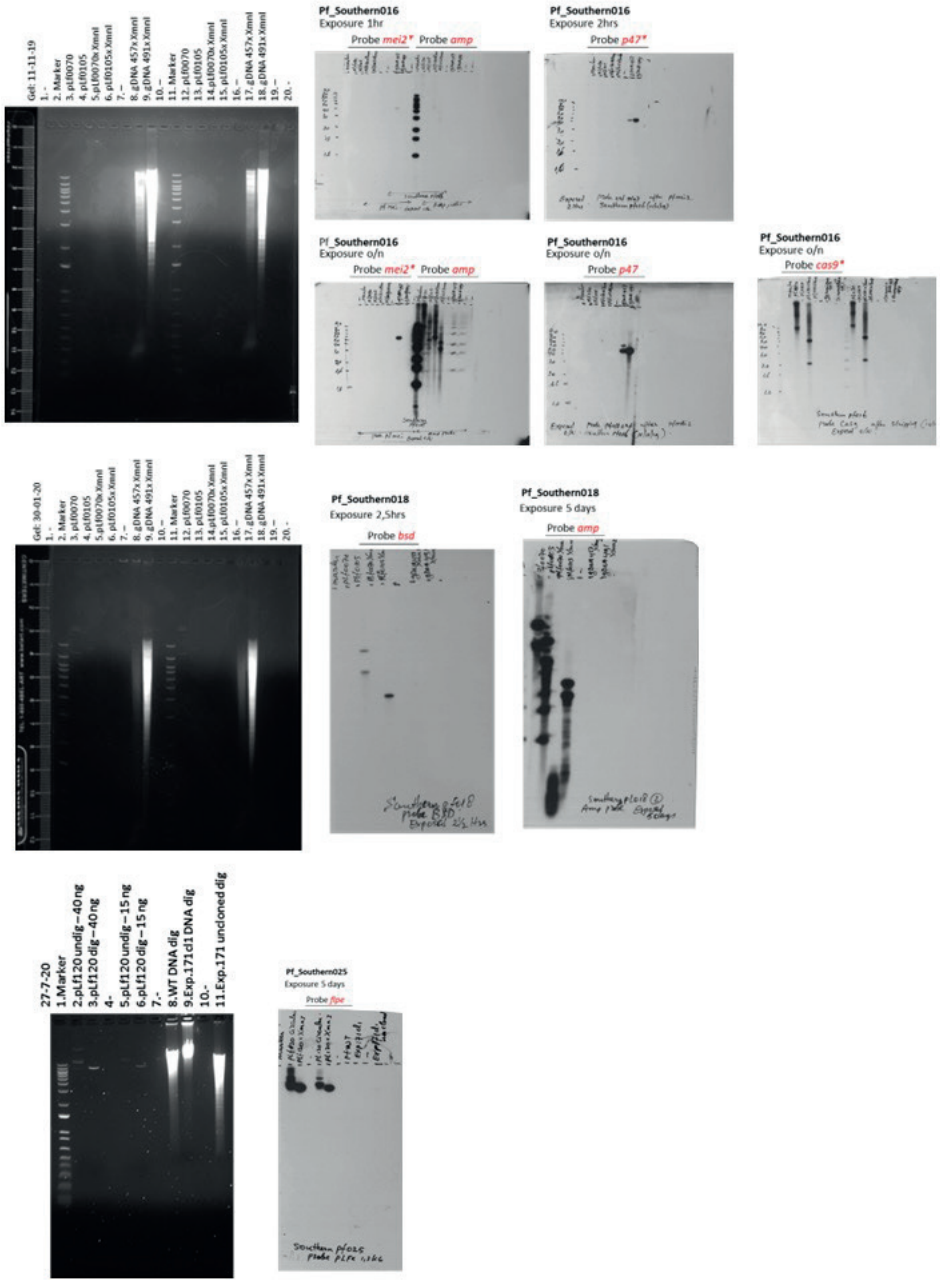
ATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAGTTAAT
TTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATT
GGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAA
CGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAG
CTCTACAAA***CCTAGG***ATT***GTTAACG***TC

AvrII *HpaI*

Supplementary Figure 7. Generation of the *bsd* selectable marker cassette of plasmid pLf0103
A. Schematic representation of the *blasticidin-S-deaminase* (*bsd*) selectable marker (SM, in green), flanked by 2 *frt* sites (in red) and restriction sites generated by ligating two gblocks. Primers and PCR fragment are indicated (primer details in **Supplementary Table 1**).
B. Schematic representation of the *bsd*-2A-*gfp* expression cassette (*bsd* in green, 2A in orange and *gfp* in yellow). Restriction sites are indicated.
C. Sequences of the 2 gblocks used to generate the *bsd* SM (in green), flanked by 2 *frt* sites.
D. Sequence of the gblock used to generate the *bsd*-2A-*gfp* expression cassette (*bsd* in green, 2A in orange and *gfp* in yellow).

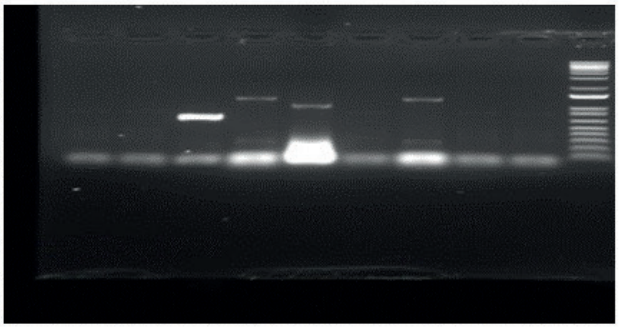


Supplementary Figure 8. Raw original images for Figure 1B

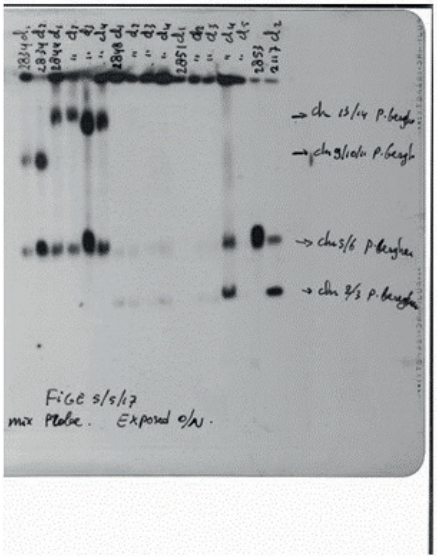


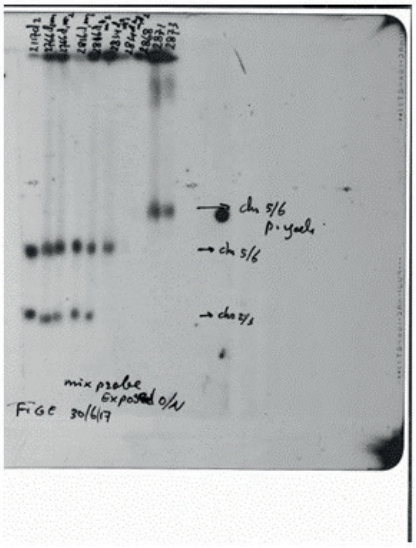
Supplementary Figure 9. Raw original images for Figure 1C

PCR gel for Supplementary Figure 1B

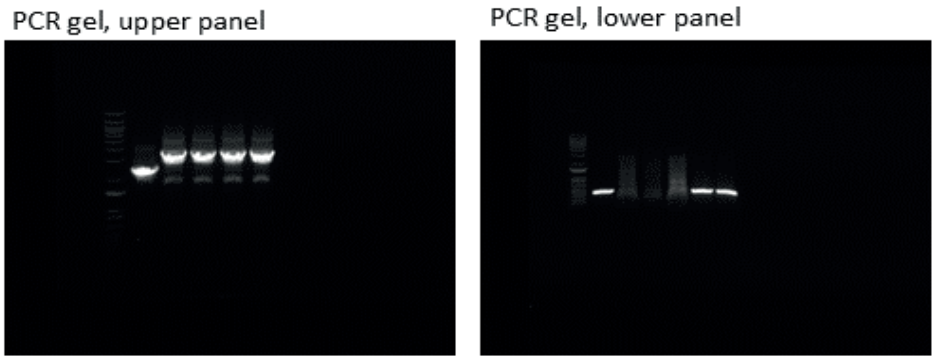


Blots of FIGE gels for Supplementary Figure 1B



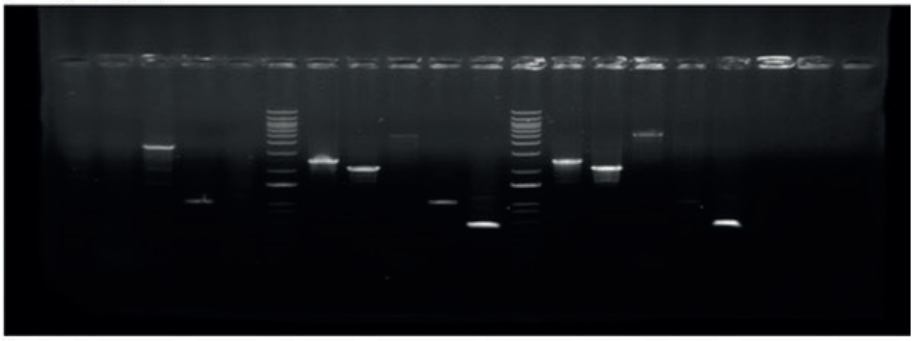


Supplementary Figure 10. Raw original images for Supplementary Figure 1B

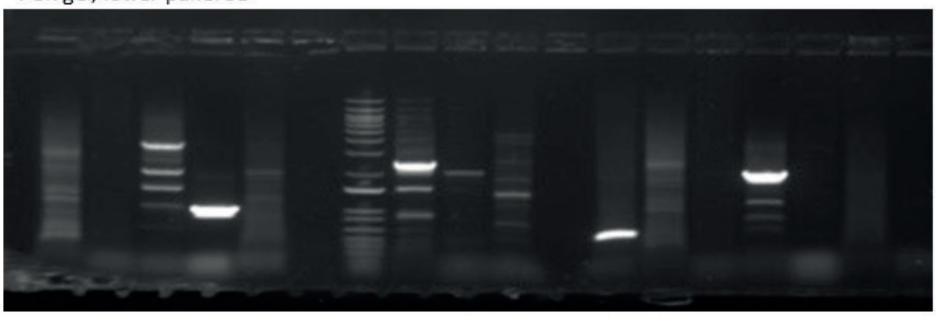


Supplementary Figure 11. Raw original images for Supplementary Figure 2B

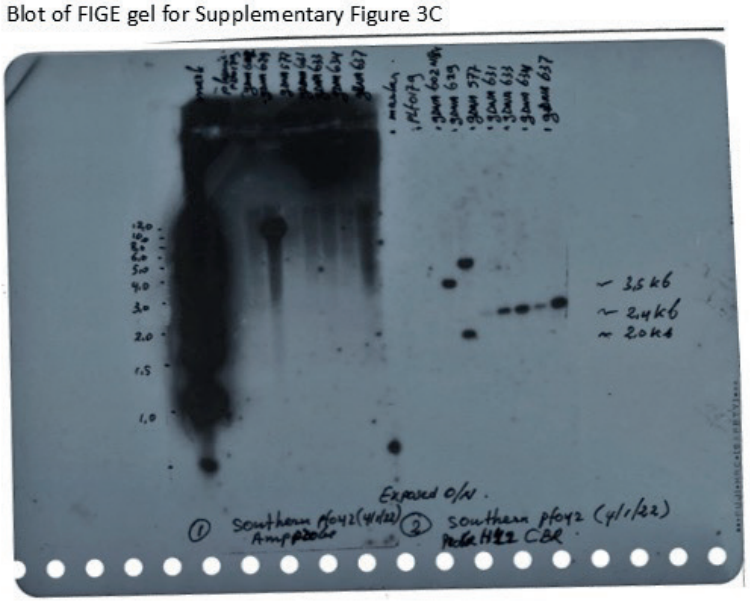
PCR gel, upper panel 3B



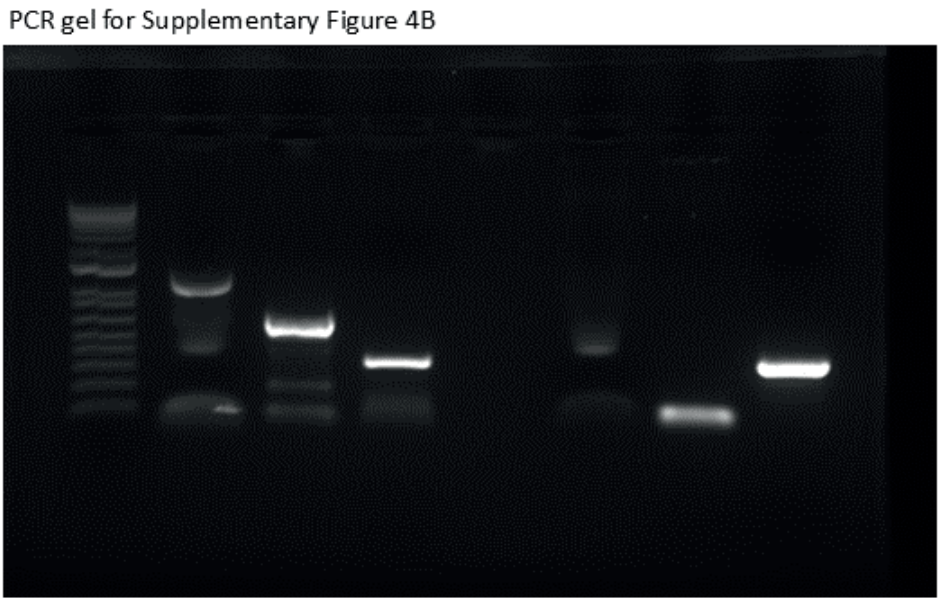
PCR gel, lower panel 3B



Blot of FIGE gel for Supplementary Figure 3C



Supplementary Figure 12. Raw original images for Supplementary Figure 3B and 3C



Supplementary Figure 13. Raw original images for Supplementary Figure 4B

Supplementary tables

Supplementary Table 1: primers used in this study

Primer name	Primer ID	Leiden code	Gene ID	Sequence	Enzyme	Description
Generation and genotyping of Plasmodium falciparum mutants						
pfhsp70 5' promoter forward	p1	8801	PF3D7_0818900	ctctcctcgagCGCATAAATATCTGGTGAAATACAA	*Xho* I	For amplification of *pfhsp70* 5' promoter region
pfhsp70 5' promoter reverse	p2	8802	PF3D7_0818900	ggtggtGGTACCGAACCTTTTGCACTAGCCAATTTTT	*Kpn* I	For amplification of *pfhsp70* 5' promoter region
pfhrp2 3' terminator forward	p3	8803	PF3D7_0831800	cctcctCCTAGGATTTAATAATAGATTAAAAATATTA	*Avr* II	For amplification of *pfhrp2* 3' terminator region
pfhrp2 3' terminator reverse	p4	8804	PF3D7_0831800	gcggtgcggGCGGCCGCTTTAATAAATATGTTCTTATATATA	*Not* I	For amplification of *pfhrp2* 3' terminator region
pfpalm gRNA009 forward	p5	8700	PF3D7_0602300	TATTAGTGAGACATGTCATGAATA	*Bbs* I	For *pfpalm* gRNA009 hybridization
pfpalm gRNA009 reverse	p6	8701	PF3D7_0602300	AAACTATTCATGACATGTCTCACT	*Bbs* I	For *pfpalm* gRNA009 hybridization
pfpalm gRNA009 forward	p7	8702	PF3D7_0602300	TATTTGAAAACAGAACAGATTTCG	*Bbs* I	For *pfpalm* gRNA010 hybridization
pfpalm gRNA009 reverse	p8	8703	PF3D7_0602300	AAACCGAAATCTGTTCTGTTTTCA	*Bbs* I	For *pfpalm* gRNA010 hybridization
	p9					For gRNA sequencing
	p10					For gRNA sequencing
pfpalm HR1 forward	p11	8782	PF3D7_0602300	aagaagaagcttAGTGAAATAAAATATCCACAAAAGG	*Nae* I	For amplification of *pfpalm* homology region 1
pfpalm HR1 reverse	p12	8783	PF3D7_0602300	GTTAAATCTTACTTTGTATACACATAAGAG	*Sac* II	For amplification of *pfpalm* homology region 1
pfpalm HR2 forward	p13	8788	PF3D7_0602300	GATTCATTAATGCAGAGTGAAATAAAATATCCACAAAAGG	*Apa* I	For amplification of *pfpalm* homology region 2
pfpalm HR2 reverse	p14	8789	PF3D7_0602300	ATCAGGCGCCAAGCTGTTAAATCTTACTTTGTATACACAT	*Hind* III	For amplification of *pfpalm* homology region 2
pfΔpalm LRPCR forward	p15	8920	PF3D7_0602300	TATATATATTTATTTATTTATTTATTTATTT		For long range PCR for *pfΔpalm* locus
pfΔpalm LRPCR reverse	p16	8921	PF3D7_0602300	TTTTTTTTTCTTTTAGGTAATTATACTATG		For long range PCR for *pfΔpalm* locus
pfpalm ORF forward	p17	9176	PF3D7_0602300	ATTATTAAAAGCATGTTTGGCTTCT		For amplification of *pfpalm* open reading frame
pfpalm ORF reverse	p18	9177	PF3D7_0602300	AATGTACAAATACTTGTCAACAATTTT		For amplification of *pfpalm* open reading frame
bsd SM forward	p19	9180	PF3D7_0602300	ATGCATGCCAAGCCTTTGTC		For amplification of *blasticidin* selectable marker
bsd SM reverse	p20	9181	PF3D7_0602300	TTAGCCCTCCCACACATAAC		For amplification of *blasticidin* selectable marker
pfcbr gRNA067 forward	p21	9460	PF3D7_1367500	TATTGATAATAAAATTGACACCAA	*Bbs* I	For *pfcbr* gRNA067 hybridization
pfcbr gRNA067 reverse	p22	9461	PF3D7_1367500	AAACTTGGTGTCAATTTTATTATC	*Bbs* I	For *pfcbr* gRNA067 hybridization
pfcbr HR1 forward	p23	9456	PF3D7_1367500	TCCTTAAGCTTGTTAACGAGTTTTGTGCTTATACATGTG	*Hind* III	For amplification of *pfcbr* homology region 1
pfcbr HR1 reverse	p24	9457	PF3D7_1367500	TTATTGGGCCCACTAGTGAATTACACTAAACAAAAAATCTTTAC	*Apa* I	For amplification of *pfcbr* homology region 1
pfcbr HR2 forward	p25	9458	PF3D7_1367500	TTCCTCCGCGGACTAGTGTATTCTAAACTATAGAGACATTC	*Nhe* I	For amplification of *pfcbr* homology region 2
pfcbr HR2 reverse	p26	9459	PF3D7_1367500	TAATTGCCGGCGCTAGCGTAAGAATTTTTTAAATTTATTCTTTCC	*Bam*H1	For amplification of *pfcbr* homology region 2
yfcu complete cassette forward	p27	7876		TATTAAGGCCTGGAAAGGGGCCATTGG		For amplification of *yfcu* selectable marker cassette
yfcu complete cassette reverse	p28	8760		GAGTCAGTGAGCGAGGAAGC		For amplification of *yfcu* selectable marker cassette
5' integration *pfΔcbr* forward	p29	9572	PF3D7_1367500	GACAACGACAATGGTTTTATG		For amplification of 5' integration *pfΔcbr* locus
5' integration *pfΔcbr* reverse	p30	7734	PF3D7_1367500	AAATTCTCGAGGAACCTTTTGCACTAGCCAATTTTTC		For amplification of 5' integration *pfΔcbr* locus
3' integration *pfΔcbr* forward	p31	6761	PF3D7_1367500	TGCCCGAAGGTTATGTACAG		For amplification of 3' integration *pfΔcbr* locus
3' integration *pfΔcbr* reverse	p32	9575	PF3D7_1367500	GATGGCCCATTAAGCGTTC		For amplification of 3' integration *pfΔcbr* locus
bsd SM reverse	p33	L1753		CCGTATGTTGCATCACCTTCACCC		For amplification of *blasticidin* selectable marker
pfcbr ORF forward	p34	9576	PF3D7_1367500	GATCATTCAGATCCATGCTTAG		For amplification of the *pfcbr* open reading frame
pfcbr ORF reverse	p35	9577	PF3D7_1367500	GCCCATTTGGTTAGTATTC		For amplification of the *pfcbr* open reading frame
amp probe forward	p36	9172		ATGAGTATTCAACATTTCCGTGT		For amplification of *ampicillin* probe
amp probe reverse	p37	9275		TCGTTGTCAGAAGTAAGTTGGC		For amplification of *ampicillin* probe
pfhcs1 gRNA074 forward	p38	9629	PF3D7_1026900	TATTTCTTATACAATATTGTTGTG	*Bbs* I	For *pfhcs1* gRNA074 hybridization
pfhcs1 gRNA074 reverse	p39	9630	PF3D7_1026900	AAACCACAACAATATTGTATAAGA	*Bbs* I	For *pfhcs1* gRNA074 hybridization
pfhcs1 gRNA075 forward	p40	9631	PF3D7_1026900	TATTAAATTTATTAACGTGCACAC	*Bbs* I	For *pfhcs1* gRNA075 hybridization
pfhcs1 gRNA075 reverse	p41	9632	PF3D7_1026900	AAACGTGTGCACGTTAATAAATTT	*Bbs* I	For *pfhcs1* gRNA075 hybridization
pfhcs1 HR1 forward	p42	9633	PF3D7_1026900	aagaagAAGCTTTTCCAGAAATATATTAACAAGGAAAATATA	*Hind* III	For amplification of *pfhcs1* homology region 1
pfhcs1 HR1 reverse	p43	9634	PF3D7_1026900	ggcggacgGGCGCGCCTGTACATTAAATTATATTGTAACAAATTGTA	*Asc* I	For amplification of *pfhcs1* homology region 1
pfhcs1 HR2 forward	p44	9635	PF3D7_1026900	ccgccgCCGCCGGAATATGGACACACAAATATAAATAAATAAA	*Sac* II	For amplification of *pfhcs1* homology region 2
pfhcs1 HR2 reverse 2	p45	9636	PF3D7_1026900	gctgctGCTAGCCATATTTGCATATTTACATATTTAAATGAAA	*Nhe* I	For amplification of *pfhcs1* homology region 2
5' integration *pfΔhcs1* forward	p46	9799	PF3D7_1026900	GGAAAAATCCAATAGCCGCTGAAGAG		For amplification of 5' integration *pfΔhcs1* locus
5' integration *pfΔhcs1* reverse	p47	9397	PF3D7_1026900	GTTTGTATTTCACCAGATATTTATGCG		For amplification of 5' integration *pfΔhcs1* locus
3' integration *pfΔhcs1* forward	p48	9175	PF3D7_1026900	ATGAACATAAAGTACAACATTAATATATAGC		For amplification of 3' integration *pfΔhcs1* locus
3' integration *pfΔhcs1* reverse	p49	9798	PF3D7_1026900	GGAGACGATATCTATTATACGAGCCTCTATG		For amplification of 3' integration *pfΔhcs1* locus
pfhcs1 ORF forward	p50	9627	PF3D7_1026900	gcggtcggGCGGCCGCtaaATTTTTGAAAGGTTTTACGAAGAT		For amplification of the *pfhcs1* open reading frame
pfhcs1 ORF reverse	p51	9628	PF3D7_1026900	acgacgACGCGTAGTACATGTCTGTGTTAATAATTTC		For amplification of the *pfhcs1* open reading frame
pfmei2 HR1 forward	p52	8761	PF3D7_0623400	TAATTAGGCCTGTTGCCGGCGGCATCTTGTGCTTACATATAAC		For amplification of *pfmei2* homology region 1
pfmei2 HR1 reverse	p53	8813	PF3D7_0623400	TTCCTCCGCGGGATATCTTTACATGAATAATTCTAGTGCC	SacII	For amplification of *pfmei2* homology region 1
pfmei2 HR2 forward	p54	8723	PF3D7_0623400	TTATTGGGCCCGTCGACCGTGTATAATTTACTATTCATCTTATC	ApaI	For amplification of *pfmei2* homology region 2
pfmei2 HR2 reverse	p55	8724	PF3D7_0623400	TCCTTAAGCTTTACGTAATATTAACCTTAAACATGTCATGAG	HindIII	For amplification of *pfmei2* homology region 2
pfmei2 gRNA030 forward	p56	8649	PF3D7_0623400	TATTGTTTCGTGGCCTGAATCAGA		For *pfmei2* gRNA030 hybridization
pfmei2 gRNA030 reverse	p57	8650	PF3D7_0623400	AAACTCTGATTCAGGCCACGAAAC		For *pfmei2* gRNA030 hybridization
pfmei2 gRNA032 forward	p58	8706	PF3D7_0623400	TATTGATCAATAATGTTAATAATA		For *pfmei2* gRNA032 hybridization
pfmei2 gRNA032 reverse	p59	8707	PF3D7_0623400	AAACTATTATTAACATTATTGATC		For *pfmei2* gRNA032 hybridization
pfΔmei2 LRPCR forward	p60	9131	PF3D7_0623400	GCCAATTATAATTTGTCCATTTC		For long range PCR for *pfΔmei2* locus
pfΔmei2 LRPCR reverse	p61	9132	PF3D7_0623400	CTTATATCAGAATATCTAATCAAATATG		For long range PCR for *pfΔmei2* locus
pfp47 control forward	p62	8428	PF3D7_1346800	AACTATTAAGCTCAACACAATACG		For amplification of *pfp47* control gene
pfp47 control reverse	p63	8756	PF3D7_1346800	atatGCGGCCGCCCACCTTGTTCACAAATACATC		For amplification of *pfp47* control gene
pfmei2 ORF forward	p64	8906	PF3D7_0623400	GCACAAGTCAGGTAAATTTGAG		For amplification of the *pfmei2* open reading frame
pfmei2 ORF reverse	p65	8907	PF3D7_0623400	TCTTCCCCAAGTAACAGAACAG		For amplification of the *pfmei2* open reading frame
pfp47 control reverse 2	p66	8889	PF3D7_1346800	TTCCACCTTGTTCACAAATACATC		For amplification of *pfp47* control gene
bsd SM reverse 3	p67	9182		TCCACCCTCATTGAAAGACAA		For amplification of *blasticidin* selectable marker
cas9 probe forward	p68	9019		AAGTTTGCATCAGCAAGTATAA		For amplification of *cas9* probe
cas9 probe reverse	p69	9622		CAGGGAGAACCAAACCACCC		For amplification of *cas9* probe
flpe probe forward	p70	5723		GTCAGGATCCATGGCTCCCAAGAAGAAGAGGAAGGTGATGAG		For amplification of *flpe* probe
flpe probe reverse	p71	3823		TATGTTGCGGCCGCTTATATGCGTCTATTTATGTAGG		For amplification of *flpe* probe
pfΔmei2 confirmation forward	p72	9736	PF3D7_0623400	GAATAATGTAAAAGGCACTAG		For confirmation of the *pfΔmei2* deletion
pfΔmei2 confirmation reverse	p73	9781	PF3D7_0623400	GATGAATAGTAAATTATACACGGTC		For confirmation of the *pfΔmei2* deletion
pfΔmei2 sequencing 1	p74	8791	pJet	CGACTCACTATAGGGAGAGCGGC		For confirmation of the *pfΔmei2* deletion
pfΔmei2 sequencing 2	p75	8792	pJet	AAGAACATCGATTTTCCATGGCAG		For confirmation of the *pfΔmei2* deletion
Generation and genotyping of Plasmodium berghei mutant						
5' *Pb*Δ*mei2* integration forward	p76	8440	PBANKA_1122300	ACCGACCTGGTCAAAGAGGCACCAGTTTAAG		For integration PCR of the *pbΔmei2* deletion
5' *Pb*Δ*mei2* integration reverse	p77	7289	PBANKA_1122300	TAAAGCACAATATCTAGGATACTAC		For integration PCR of the *pbΔmei2* deletion
hdhfr::fcu forward	p78	4698		GTTCGCTAAACTGCATCGTC		For amplification of *hdhfr* selectable marker cassette
hdhfr::fcu reverse	p79	4699		GTTTGAGGTAGCAAGTAGACG		For amplification of *hdhfr* selectable marker cassette
pbmei2 ORF forward	p80	8768	PBANKA_1122300	TAGGCTTATATTGAGGCACAG		For confirmation of the *pbΔmei2* deletion
pbmei2 ORF reverse	p81	8769	PBANKA_1122300	GGATGCTCTATTAAGTCCTTATG		For confirmation of the *pbΔmei2* deletion
p25 ORF forward	p82	1462	PBANKA_0515000	CATGCCATGGATGAATACTTATTACAGTG		For amplification of *pb25* probe
p25 ORF reverse	p83	1463	PBANKA_0515000	CCGGAATTCTTAAATGATATTTGAAAATATTAG		For amplification of *pb25* probe
hdhfr ORF forward	p84	886		GGAAGATCTATGGTTGGTTCGCTAAACTGCATCG		For amplification of *hdhfr* probe
hdhfr ORF forward	p85	887		GGAAGATCTTTAATCATTCTTCTCATATACTTC		For amplification of *hdhfr* probe

6

Chapter 7

Single immunization with genetically attenuated *PfΔmei2* (GA2) parasites by mosquito bite in controlled human malaria infection: A placebo-controlled randomized trial

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Published: Nature Medicine, 2025
DOI: 10.1038/s41591-024-03347-2

Abstract

Malaria vaccines consisting of metabolically active *Plasmodium falciparum* (*Pf*) sporozoites can offer improved protection compared with currently deployed subunit vaccines. In a previous study, we demonstrated the superior protective efficacy of a three-dose regimen of late-arresting genetically attenuated parasites administered by mosquito bite (GA2-MB) compared with early-arresting counterparts (GA1-MB) against a homologous controlled human malaria infection. Encouraged by these results, we explored the potency of a single GA2-MB immunization in a placebo-controlled randomized trial. Primary outcomes were safety and tolerability, time-to-parasitemia and protective efficacy. Humoral and cellular immunological results were considered secondary outcomes. Here we report the safe administration of GA2-MB with no breakthrough malaria and sterile protection in nine of ten participants at six weeks after a single immunization with 50 GA2-infected mosquitoes, compared with none of five mock-immunized participants, against a homologous controlled human malaria infection. Immunization increased circulating *Pf*-specific polyfunctional effector memory CD4+ T cells coexpressing tumor necrosis factor and interleukin-2. This unprecedented 90% protective efficacy after a single low-dose immunization holds great promise for the potency of GA2 immunization. Future studies should demonstrate whether GA2 is similarly efficacious in pre-exposed populations and whether the favorable safety profile reported here holds up in larger groups.

Clinical trials registration: NCT05468606.

Introduction

Each year more than 600000 people die from malaria, mainly children under the age of five[1], making it the fifth leading cause of child mortality[2]. Although the widespread deployment of both RTS,S and R21 subunit vaccines in regions with moderate-to-high transmission marks tremendous progress in reducing malaria-related morbidity and mortality[3,4], their limited efficacy and the need for boosters to sustain protection call for ongoing effort into the development of improved vaccines with increased potency to achieve high-level durable protection that can ultimately break transmission[5]. Immunization strategies based on the use of whole *Plasmodium falciparum* (*Pf*) sporozoites have the potential to provide this much sought after high-level protection, particularly late liver stage-arresting parasites that are attenuated genetically through the knockout of genes crucial for the development of blood-stage disease[6-8].

Previously, we demonstrated that genetically attenuated *Pf* sporozoites can be safely administered to humans by injection and mosquito bite[9,10]. Through targeted gene deletion, we created two different parasite lines based on the *PfNF54* strain: early-arresting *PfΔb9Δslarp* (GA1)[11] and late-arresting *PfΔmei2* (GA2)[8]. We demonstrated with three immunizations through mosquito bites that parasites arresting development late in the liver at day 6 post-infection are much more potent at inducing protection[12] than early-arresting counterparts (89% versus 13% protection in a controlled human malaria infection (CHMI)[10]. The high-level protection was accompanied by potent circulating cellular memory responses, potentially against late liver-stage antigens[10].

In previous studies involving immunization with sporozoites under chemoprophylaxis, detailed parasite detection by quantitative polymerase chain reaction analysis for *Pf* (*Pf*qPCR) during immunization regimens indicated that 10–90% of previously malaria-naive participants become parasitemic after the second immunization[13-18]. This result suggests that single immunization with parasites that reach the late liver stage can provide varying levels of immunity. We hypothesized that GA2 might also have that potential and we decided to assess the efficacy of a single GA2-immunization regimen against a homologous CHMI.

Results

We enrolled 15 participants in a randomized double-blind placebo-controlled trial. Details on recruitment and participant characteristics can be found in **Fig. 1** and **Table 1**. Participants were exposed to the bites of 50 (±5) GA2-infected or uninfected *Anopheles stephensi* mosquitoes (GA2-MB or placebo, respectively) on 12 April 2023 (**Fig. 2 A**).

For one participant in the placebo group, the target dose of 45–55 blood-fed mosquitoes was not reached at immunization (**Fig. 2 B**). Six weeks later, all participants underwent CHMI through the bites of five mosquitoes infected with unattenuated homologous wild-type *Pf* parasites. After CHMI, blood feedings were confirmed in either five infected mosquitoes (six of 10 GA2-MB participants and four of five in the placebo group) or four infected mosquitoes (four of 10 GA2-MB participants and one of five in the placebo group) (**Supplementary Fig.1 A**).

Study visits were held on the day before immunization, on days 6, 9 and 14 post-immunization, on the day before CHMI, daily from day 6 to day 21 post-CHMI, and on days 28, 31 and 35 post-CHMI. During these visits, adverse events (AEs) were collected, safety was assessed and a highly sensitive *Pf*qPCR analysis in whole blood was performed. The lowest limit of detection for the *Pf*qPCR was 50 parasites per ml of blood. Escape treatment (three-day regimen of atovaquone–proguanil) was provided at a concentration of >100 parasites per ml or at day 28 after CHMI.

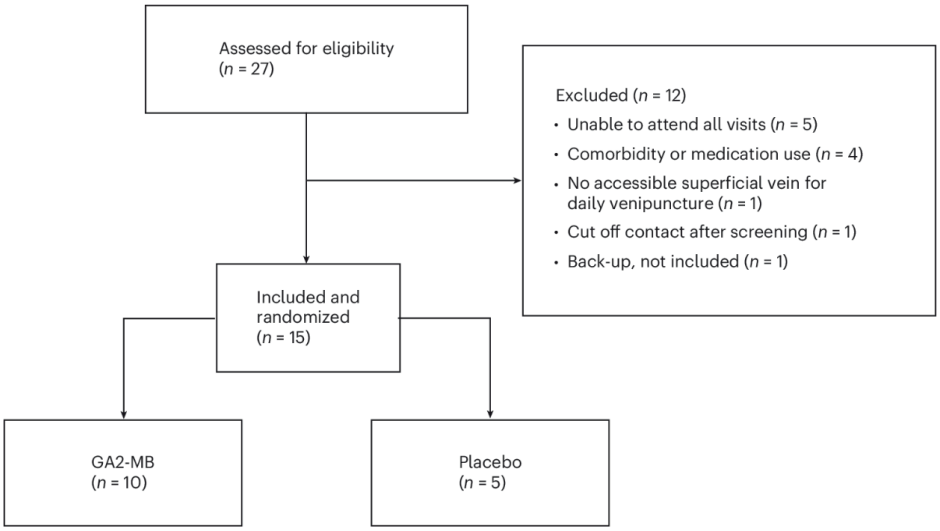

Figure 1. CONSORT diagram on recruitment and inclusions
Between 13 February 2023 and 17 March 2023, 27 persons were screened, of whom 15 were included, randomized, (mock-)immunized and infected with wild-type malaria parasites in a CHMI.

Table 1. Baseline characteristics of participants at screening

	GA2 (n=10)	Placebo (n=5)	Total (n=15)
Age			
Mean (SD)	24 (3)	24 (5)	24 (4)
Median (range)	23 (21-30)	25 (19-31)	23 (19-31)
Gender			
Male (%)	4 (40.0)	3 (60.0)	7 (46.7)
Female (%)	6 (60.0)	2 (40.0)	8 (53.3)
BMI			
Mean (SD)	23.9 (3.3)	22.7 (2.1)	23.5 (2.9)
Median (range)	23.8 (19.4-28.6)	23.7 (20.3-25.0)	23.7 (19.4-28.6)

BMI: body mass index; SD: standard deviation.

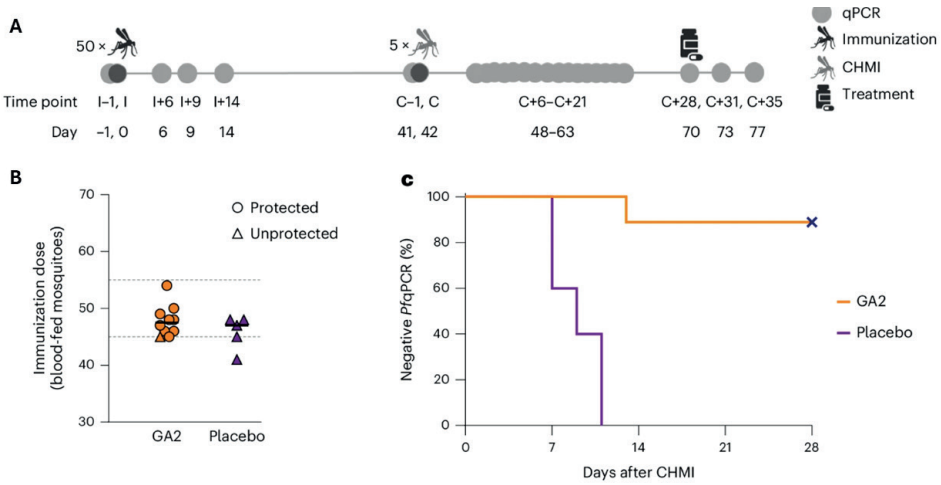

Figure 2. Study design, immunization dose and protection against CHMI
A. Schematic overview of study design.
B. Number of blood-fed mosquitoes per participant at immunization. Black horizontal lines represent the median. The dashed horizontal line represents the target dose (45–55 blood-fed mosquitoes).
C. Kaplan–Meier curve of percentage of participants that had a negative *Pf*qPCR (<100 parasites per ml) in peripheral blood after CHMI. X, censored; log-rank test, $p<0.0001$.

Primary outcomes

Single immunization with GA2-MB was safe and well tolerated with no study-related serious AEs or breakthrough malaria. Neither were parasite concentrations ≥50 parasites per ml detected in any blood sample after immunization. The mosquito bites led to a severe itch in one participant and moderate swelling and mild blistering in another participant, for which topical corticosteroids were prescribed. All other AEs after immunization were mild to moderate. Further details on safety and tolerability can be found in **Supplementary Table 1**.

We found that nine of 10 (90%) participants in the GA2-MB group were fully protected against *Pf* malaria and remained *Pf*qPCR-negative until day 28 post-CHMI (**Fig. 2 C**). By contrast, all participants in the placebo group became parasitemic (log-rank test $p < 0.0001$). Although the median time-to-parasitemia in the placebo group was 9 days (range 7–11 days), detection of parasitemia in the one unprotected GA2-MB participant was considerably delayed to day 13 (**Supplementary Fig. 1 B**). When assessing protection solely in the participants that received a dose of five infected blood-fed mosquitoes at CHMI (not a prespecified analysis in our protocol), we found a protective efficacy of 83% (5 of 6 GA2-MB participants protected versus zero of four placebo participants).

Secondary outcomes

We assessed antibody responses one day before CHMI (C−1) and detected significantly higher levels of antibodies targeting *Pf* circumsporozoite protein, but not the key late liver-stage and blood-stage antigens *Pf* apical membrane antigen-1 and *Pf* merozoite surface protein-1 in GA2-MB participants compared with placebo (**Fig. 3 A**). *Pf*-specific cellular immunity in GA2-MB participants assessed by stimulation of peripheral blood mononuclear cells with *Pf*-infected red blood cells (*Pf*RBC), a surrogate for late liver-stage antigens, and uninfected RBCs (unRBCs) showed a strong type-1 proinflammatory (interferon-γ (IFNγ), tumor necrosis factor (TNF) and interleukin-2 (IL-2)), and a moderate type-2 anti-inflammatory (IL-4, IL-5 and IL-13) and regulatory (IL-10) profile in $CD4^+$ and $V\delta2^+$ γδ T cells, but not in $CD8^+$, Vδ2– γδ and natural killer T cells (**Fig. 3B** and **Supplementary Fig. 2–4**). GA2-MB elicited higher frequencies of polyfunctional $CD4^+$ and $V\delta2^+$ γδ T cells expressing more than one type-1 cytokine, in comparison with placebo (**Fig. 3 C**). Whereas $CD4^+$ T cells preferentially co-expressed TNF and IL-2 with or without IFNγ, $V\delta2^+$ γδ T cells co-expressed high levels of IFNγ and TNF with or without IL-2. We observed a relatively minor proportion of type-1 polyfunctional $CD4^+$ and $V\delta2^+$ γδ T cells co-expressing type-2 cytokines (**Supplementary Fig. 5**). Polyfunctional CD4 T cells, but not $V\delta2^+$ γδ T cells, were enriched among memory T cells (CD3+CD45RA−), indicating the capacity of single GA2-MB immunization to form *Pf*-specific cellular memory (**Fig. 3 D** and **Supplementary Fig. 6 A–F**). These GA2-induced memory T cells preferentially acquired effector memory phenotype as early as 2 weeks post-immunization and remained high during the post-CHMI time point, whereas central memory T cells were induced at much lower frequency (**Fig. 3 E** and **Supplementary Fig. 6 G,H**).

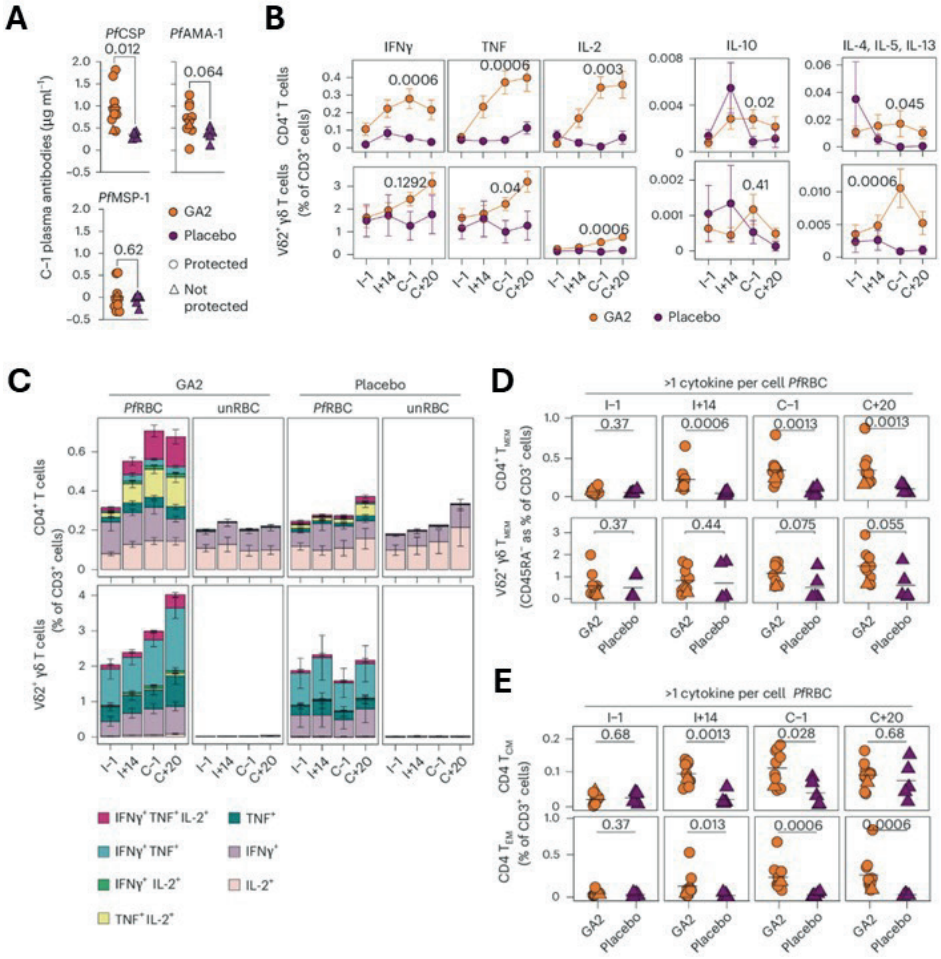

Figure 3. Prominent polyfunctional CD4⁺ memory T cell response in GA2 single-immunized participants

A. Plasma antibody levels against the indicated *Pf* antigens on the day before CHMI (C–1). Values are $\log_{10}$ transformed.

B. Frequency of CD4⁺ and Vδ2⁺ γδ T cells expressing the indicated cytokines upon stimulation with *Pf*RBC corrected for unRBC stimulation.

C. Frequency of CD4⁺ and Vδ2⁺ γδ T cells expressing single or more than one of the indicated cytokines per cell upon *Pf*RBC and unRBC stimulation.

D. Frequency of CD45RA⁻ CD4⁺ (upper) and Vδ2⁺ γδ (lower) memory T cells (T_{MEM}) among polyfunctional cells at the indicated time points upon *Pf*RBC stimulation.

E. Frequency of central (T_{CM}) (upper) and effector (T_{EM}) (lower) memory cells among polyfunctional CD4⁺ T cells.

Filled circles and triangles indicate the data from individual participants and the horizontal black line indicates the arithmetic mean (**A, D, E**). Filled circles and error bars indicate the arithmetic mean and s.e.m., respectively (**B**). Bar charts represent arithmetic means and error bars represent s.e.m. (**C**). Two-tailed Mann–Whitney test **(A, B** (at C–1), **D, E**). *Pf*AMA-1, *Pf* apical membrane antigen-1 ; *Pf*CSP, *Pf* circumsporozoite protein; *Pf*MSP-1, *Pf* merozoite surface protein-1.

Discussion

In this study, we demonstrated the capacity of a single immunization with 50 GA2-infected mosquitoes to protect 90% of malaria-naive individuals against a homologous CHMI. Until now, malaria vaccines have always been tested in regimens of two or more immunizations, but single immunization has important potential advantages over multiple immunizations with regards to implementation in endemic settings as well as for travellers' vaccinations. Although high-level protective efficacy after immunization with sporozoites has been observed previously[10,13-17,19], never has protective efficacy been demonstrated in a CHMI after one immunization. Surprisingly, this high level of protective efficacy seems to be similar to three immunizations with GA2-infected mosquito bites[10], suggesting that the boosting effect of additional immunizations is limited. Further research into the dynamics of immune components in a larger cohort of participants is needed to understand changes on an individual basis and to evaluate how these changes relate to protection. In addition, the longevity of the immune response and protective efficacy after both single and triple GA2-MB immunization needs to be further evaluated and compared. Blood-stage breakthrough infections have been observed after immunizing mice with *Plasmodium yoelli Δmei2*[20]. When taking the current and our previous GA2-MB study[10] together, a total of 50 participants have now been exposed to GA2-MB: 15 participants to 15 GA2-MB and 35 participants to 50 GA2-MB (nine of whom underwent three exposures). None of these participants have developed breakthrough malaria. This is in line with results in mice with humanized livers that were exposed to *PfΔmei2* and did not develop breakthrough blood infections either[8]. Future studies should demonstrate whether this favorable safety profile of GA2 holds in larger populations or whether genetically attenuated parasites with more gene knockouts in addition to *mei2* are warranted to eliminate the risk of breakthrough infections after immunizations[21]. Because of the lack of knowledge on immunogenic late liver-stage antigens and the technical limitations of generating large numbers of infected hepatocytes suitable for in vitro stimulation, *Pf*RBCs were used as a surrogate antigenic source in this study, similar to previously published clinical studies[9,17,22]. Furthermore, the preferential liver resident capacity of *Pf*-specific $CD8^+$ T cells may have thwarted our attempts to detect them in peripheral blood samples. In previous sporozoite immunization studies, polyfunctional $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells expressing proinflammatory cytokines, particularly IFNγ, were associated with protection[10,17,22,23]. However, after single GA2-MB immunization we find the production of proinflammatory cytokines other than IFNγ (notably TNF and IL-2) by $CD4^+$ T cells to be more pronounced, in addition to an increase in effector rather than central memory phenotypes, which differentiate early after immunization and persist throughout the CHMI follow-up period.

A limitation of our study is the small sample size of healthy malaria-naive participants who do not adequately represent the target population for malaria vaccines in endemic areas. In addition, administration of GA2 through mosquito bites is not a feasible method for large-scale immunization campaigns. To translate the high-level protective efficacy of GA2-MB to an amenable way of vaccine administration through parenteral immunization, future studies need to assess whether aseptically purified, vialed and cryopreserved sporozoites with the *mei2* deletion are as safe and as efficacious as GA2-MB in this study and similarly potent in malaria endemic areas.

Nonetheless, our finding that a single immunization with GA2-MB can induce high-level protection against a homologous CHMI provides strong support for the further clinical development of potentially highly potent next generation single-immunization malaria vaccines based on late-arresting genetically attenuated sporozoites.

Materials and methods

Study design and recruitment

A randomized, double-blind, placebo-controlled trial with a CHMI was conducted from February to November 2023 at Leiden University Medical Center, Leiden, the Netherlands. Fifteen malaria-naive participants aged 15–30 years were included after a health assessment including medical history, physical examination, a general laboratory evaluation including hematology and biochemistry assessment, a drugs test to exclude cocaine and amphetamine use and electrocardiography. Female participants were counselled to use adequate contraception throughout the study and were tested for pregnancy with a serum beta-human chorionic gonadotropin test on both the day before immunization (I−1) and the day before CHMI (C−1). All participants provided written informed consent. Ten participants were immunized with the bites of 45–55 GA2-infected mosquitoes and five participants received a mock- immunization with uninfected mosquitoes as a placebo. Six weeks after immunization, all 15 participants underwent a homologous CHMI with the bites of five wild-type *Pf3D7*-infected mosquitoes. From day 6 to day 21 after CHMI (C+6 to C+21), participants were closely followed with daily ambulatory visits for the collection of AEs, safety assessment, blood sampling and highly sensitive *Pf*qPCR analysis in whole blood as previously described (the lowest limit of detection was 50 parasites per ml)[24]. Participants were treated with a three-day regimen of atovaquone–proguanil when they exhibited parasitemia (*Pf*qPCR > 100 parasites per ml) or at day 28 after CHMI (C+28). AEs were recorded by participants in a diary. AEs were graded in four categories (mild, moderate, severe and serious) that were prespecified per

protocol. Both participants and investigators were blinded to intervention. Mosquito cages were prepared by technicians independent from the clinical investigators. Randomization was carried out by an independent member of the study team. Safety, time-to-parasitemia and protective efficacy were the primary outcomes. Secondary study outcomes were humoral and cellular immunology results. Data capture was done using an electronic case report form (Castor CDMS v.2023.1.x.x). The trial protocol was approved by the Dutch Central Committee for Research involving Human Subjects (CCMO, file number NL82130.000.22) and registered at ClinicalTrials.gov (NCT05468606) and EudraCT (2022-002646-40).

Parasite culturing, mosquito rearing and exposures

The characterization of GA2 and its generation from *PfNF54*, its genetic backbone, have been described previously[8]. The wild-type parasite used for the CHMI (*Pf3D7*) is a clone of the *PfNF54* parasite strain. Parasites were cultured in standard conditions using semi-automated shaker culture systems[25] and subsequently fed to female *Anopheles stephensi* mosquitoes by standard membrane feeding[26]. Mosquitoes were reared and infected following standard procedures at the insectary of Leiden University Medical Center following established methods[26]. Production of the parasites and mosquitoes underwent strict quality control before release by a qualified person. Fourteen days after feeding the parasites to the mosquitoes, a sample of 20 mosquitoes was taken from every mosquito batch (consisting of 200–500 mosquitoes) to assess sporozoite yield in the mosquito salivary glands. Only batches that had an average yield of at least 1,000 sporozoites per mosquito were used for exposure to participants. For the immunization, the average yield of the batches was 34,000 and 67,000 sporozoites per mosquito and for the challenge the yield of the batches ranged from 11,300 to 35,300 sporozoites per mosquito. Exposure of mosquitoes to participants was done using small cages with mesh-covered openings that were applied for 15 min to the deltoid region (immunization) or inner lower arm (CHMI). After exposure, mosquitoes were dissected to confirm feeding. In addition, after CHMI exposures, salivary glands were dissected and microscopically assessed for the presence of sporozoites. At immunization, exposures were repeated until the target dose of 45–55 mosquitoes was reached or up to a maximum of three times. For CHMI, the procedure was repeated until five infected mosquitoes had taken a blood meal or up to a maximum of four exposures. *Pf* antigen-binding antibody measurements in ELISA were performed as described previously[10]. In brief, half-area 96-well high-binding plates were coated overnight at 4 °C with 1 μg ml−1 of antigen at 25 μl per well in 0.1 M sodium carbonate buffer (pH 9.6). Upon blocking with 5% skim milk in phosphate-buffered saline for 2 h,

serially diluted plasma samples (starting dilution of 1:500 serially diluted in eight steps each by 1:2.5) were incubated for two hours. Bound antibodies were detected with 450 nm absorbance using goat anti-human immunoglobulin G conjugated with horseradish per oxidase and 3,3′,5,5′-tetramethylbenzidine substrate development stopped with 10% sulfuric acid. A standard curve developed using polyclonal immunoglobulin G of a known concentration was used for normalization. Measures from at least two independent experiments with a coefficient of variance below 30% were considered for analysis.

T cell response measurement using flow cytometry

Cellular response using *Pf*RBC stimulation was performed as described previously[27]. In brief, peripheral blood mononuclear cells were stimulated with RBCs from a healthy blood donor, either as unRBC or *Pf*RBC, for 24 h during which 10 µg ml−1 Brefeldin A (Sigma) was added at 4 hours post stimulation. Cells were stained with a panel of antibodies (**Supplementary Table 2**) to identify T cell subsets (CD56, γδ Vδ2 T cell receptor, CCR7, CD3, CD4, CD8, CD25 and CD11c), cytokine expression (IFNγ, TNF, IL-10, IL-2, IL-4, IL-5, IL-13) and phenotype (CD45RA and CCR7). For fixation and intracellular staining an Intracellular Fixation & Permeabilization Buffer Set (Invitrogen) was used. To stain dead cells Aqua Live/Dead dye (Invitrogen) was used. Cells were acquired on the three-laser spectral analyzer Aurora (configuration 16V-14B-8R) and analyzed using FlowJo v.10.8.2 as described in **Supplementary Fig. 2 and 3**. The frequency of *Pf*-specific cytokine-positive cells in *Pf*RBC-stimulated samples after subtraction of the same gate on the same sample stimulated with unRBC is reported for analysis. Frequencies of cytokine-positive CD4+ and γδ+ Vδ2 cells were calculated as a percentage of CD3+ cells by using the frequencies of total CD4+ and γδ+ Vδ2 cells, respectively. Statistical analysis Baseline characteristics of participants are reported as both means with standard deviations and medians with range for continuous variables and as frequencies with percentages for categorical variables. The incidence of AEs is reported as frequencies with percentages (risk). Time-to-parasitemia is reported as a Kaplan–Meier graph and the difference between groups is evaluated using a log-rank test. Antibody concentrations are reported in µg ml−1 and the frequency of responding cells as a percentage of the indicated population, both as arithmetic means with standard error of means. Antibody concentrations and cell populations are compared between groups with a two-tailed Mann–Whitney test. Figures were produced in GraphPad Prism (v.9.3.1) and RStudio (v.4.2.1).

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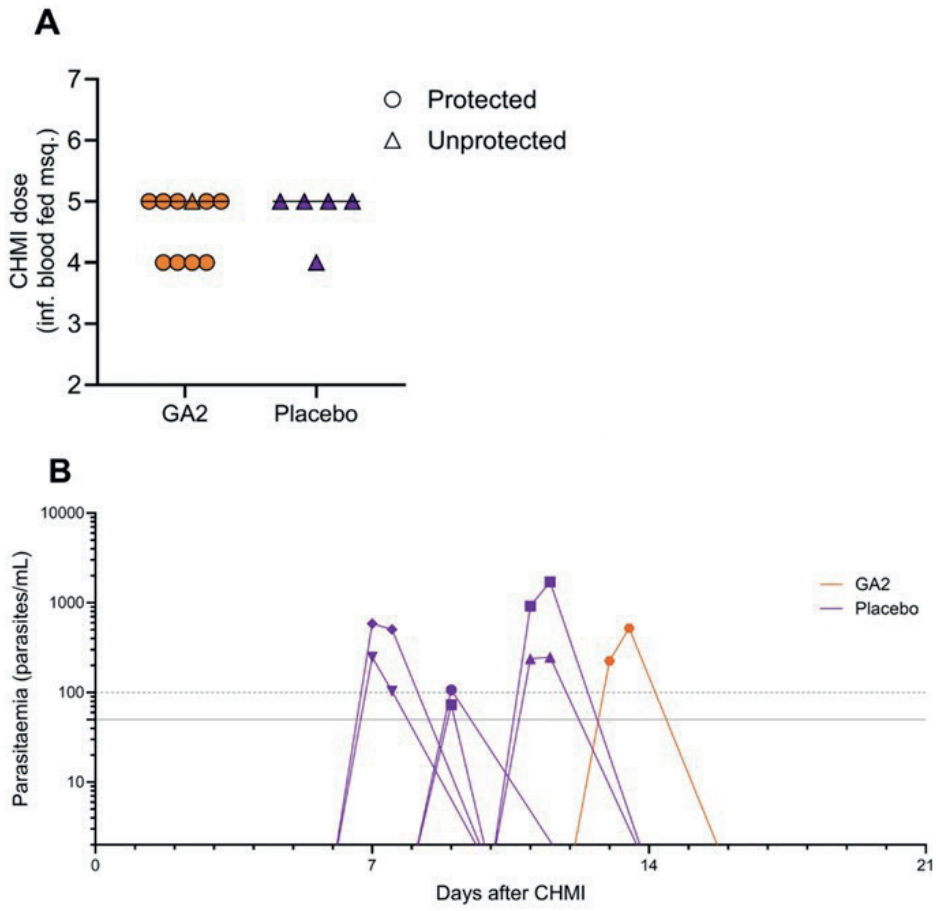
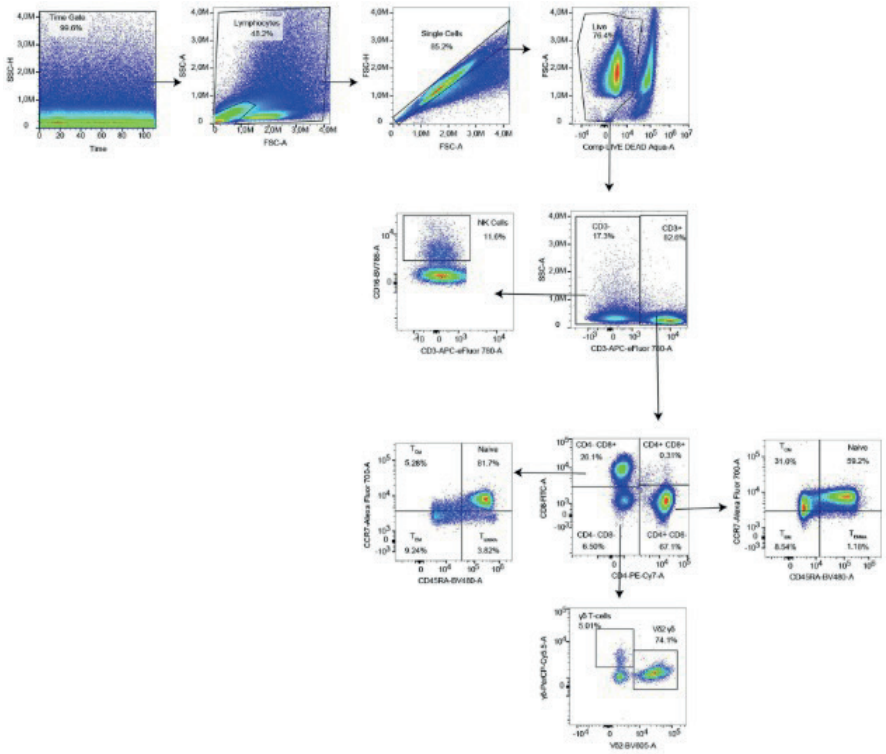
Supplementary appendix

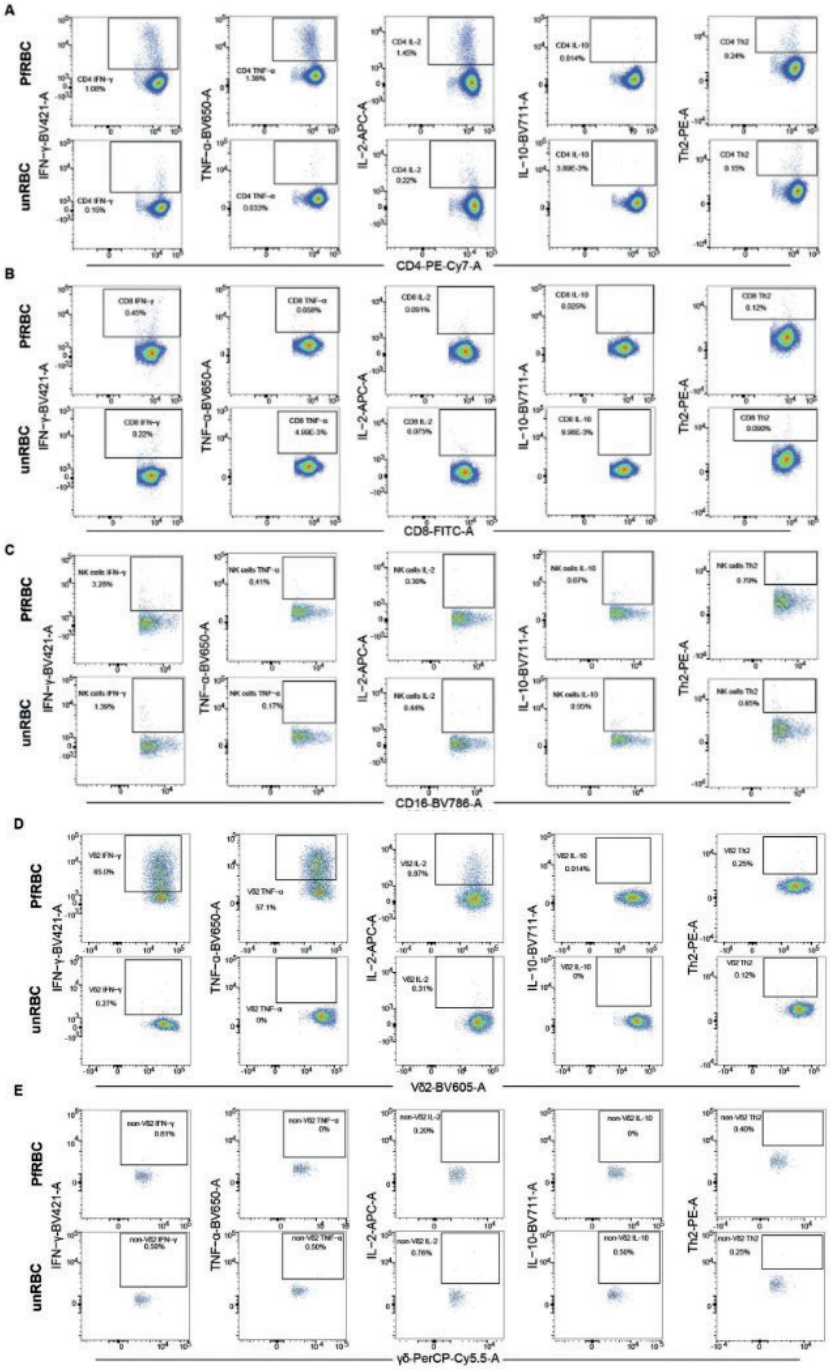
Supplementary figures



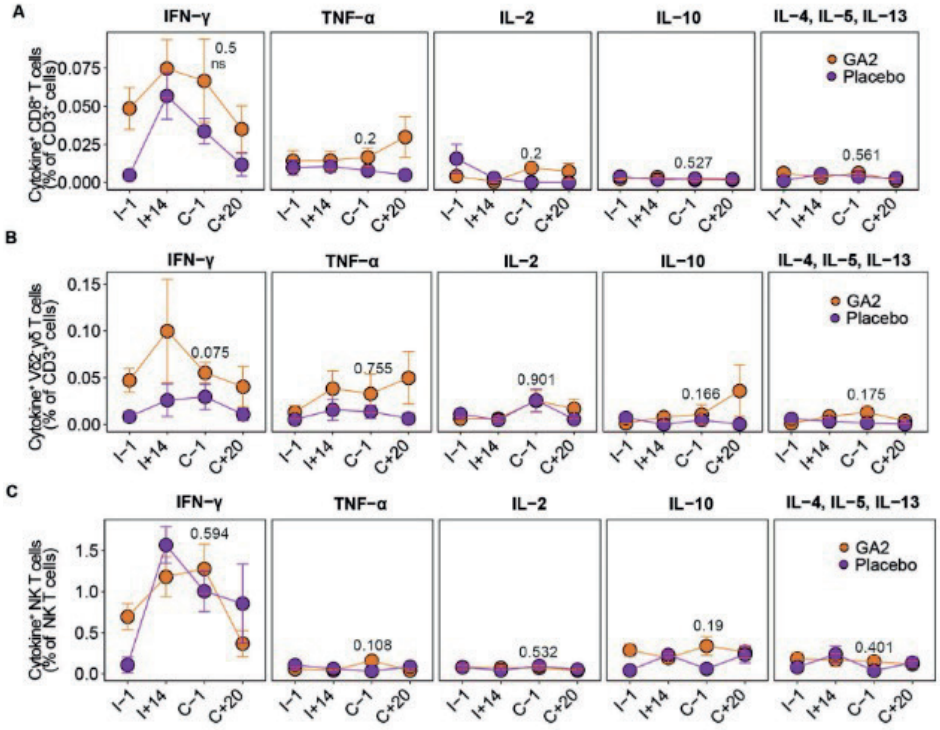
Supplementary Figure 1. CHMI dose and time-to-parasitaemia.
A. Number of infected blood fed mosquitoes per participant at the controlled human malaria infection (CHMI). A black horizontal line represents the median.
B. Development of parasitaemia per day after CHMI. Lines with different symbols represent different participants. The horizontal dotted grey line represents the cut-off for parasitaemia (100 parasites/mL blood) at which treatment is given. The horizontal continuous grey line represents the lowest limit of detection of the assay (50 parasites/mL blood).



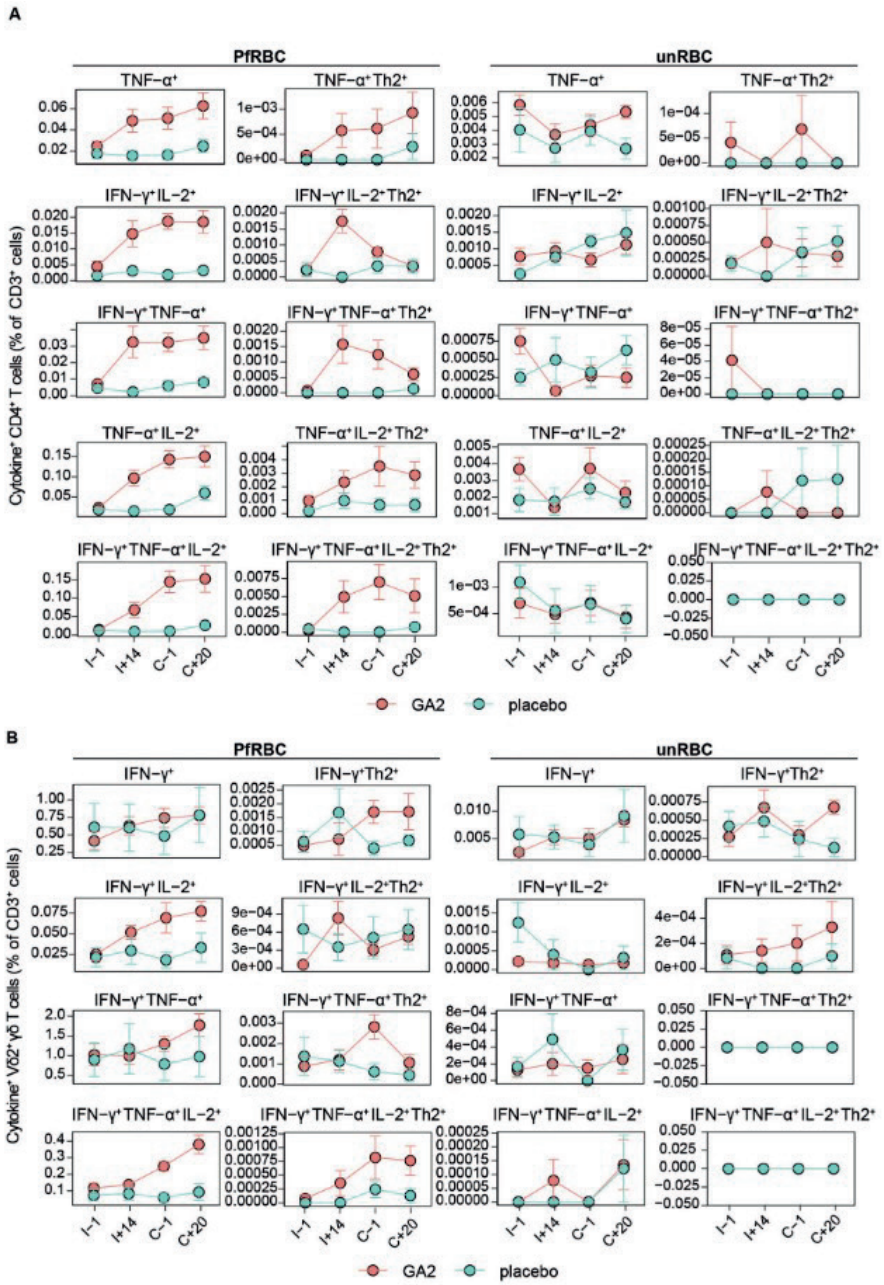
Supplementary Figure 2. Representative gating strategy to define T cell subsets
Live single cells were defined as NK cells ($CD3^{-}CD56^{+}$), CD4 or CD8 effector ($CCR7^{-}CD45RA^{-}$) and central ($CCR7^{+}CD45RA^{-}$) memory T cells and $V\delta2^{+}$ or $V\delta2^{-}$ γδ T cells, based on the surface marker expression.



Supplementary Figure 3. Representative cytokine expression gating strategy.
Gates defining the indicated cytokine expressing CD4⁺ (**A**), CD8⁺ (**B**), NK (**C**), Vδ2⁺ γδ (**D**) and Vδ2⁻ γδ (**E**) T cells upon stimulation with *Pf* infected (*Pf*RBC; top) and uninfected RBC (unRBC).

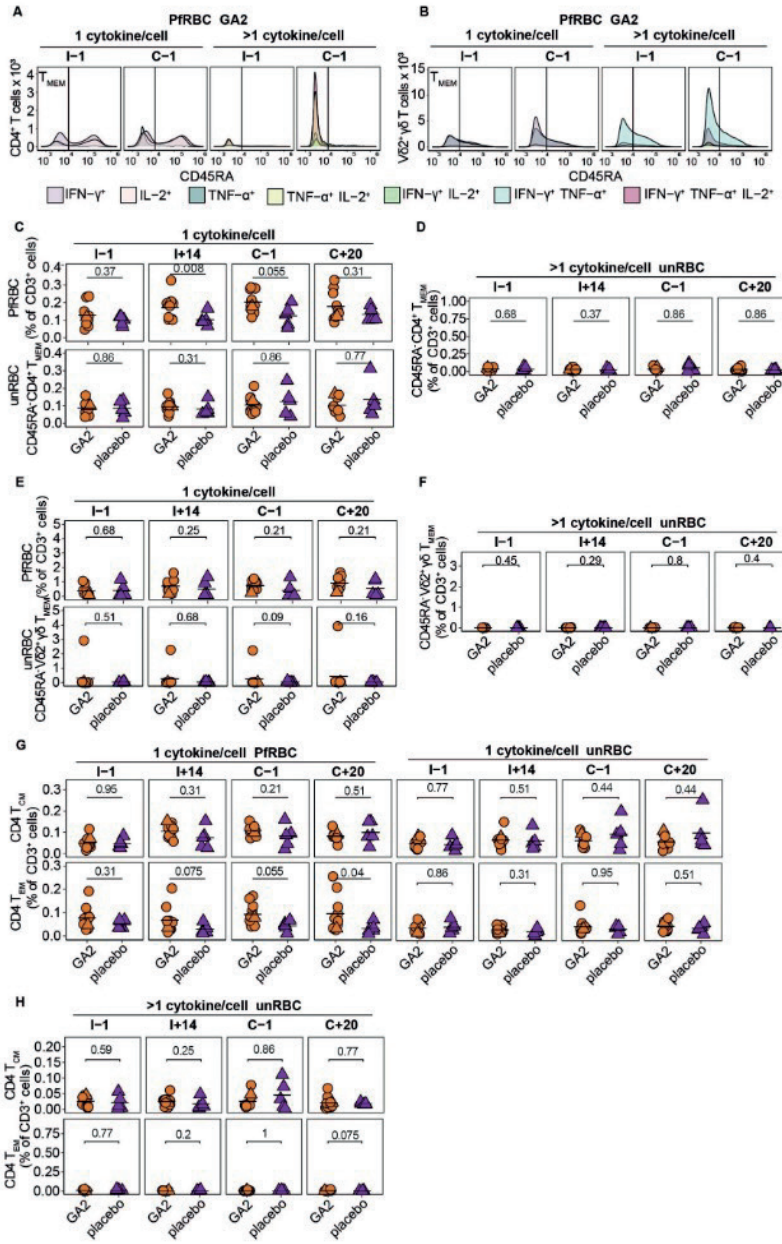


Supplementary 4. Cytokine expression in T cell subsets.
$CD8^+$ (**A**), $V\delta2^-$ γδ (**B**) and, NK (**C**) T cells expressing the indicated cytokines upon stimulation with *Pf* infected RBC (*Pf*RBC) reference corrected for uninfected RBC (unRBC) stimulation. Data corresponding to GA2-MB and placebo groups are indicated in orange and purple, respectively (**A–C**). Filled circles and error bars indicate arithmetic mean and standard error mean, respectively. Two tailed Mann-Whitney test (at C-1).



Supplementary Figure 5. Mono and polyfunctional T cells moderately co-expressing Th2 cytokines

Frequency of CD4+ (**A**) and Vδ2+ γδ (**B**) T cells expressing the indicated cytokines in combination with or without type-2 cytokines (Th2: IL-4, IL-5 and IL-13) among GA2-MB and mock-immunised participants upon *Pf*RBC (left) and unRBC (right) stimulation. Filled circles and error bars indicate arithmetic mean and standard error of mean of both intervention groups, respectively.



Supplementary Figure 6. Memory status of T cell subsets.
A,B. CD45RA expression in CD4⁺ (**A**) and Vδ2⁺ γδ (**B**) T cells among cytokine expressing cells at baseline (I-1) and C-1 upon *Pf*RBC stimulation.
C-F. Frequency of CD45RA⁻ mono⁻ (**C**,**E**) and polyfunctional (**D**,**F**) CD4⁺ (**C**,**D**) and Vδ2⁺ γδ (**E**,**F**) memory T cells (T_{MEM}) upon *Pf*RBC and unRBC stimulation.
G,H. Frequency of central (T_{CM}; top) and effector (T_{EM}; bottom) memory cells among monofunctional and polyfunctional in CD4⁺ (**G**) and Vδ2⁺ γδ (**H**) T cells. Each data point represent individual participant and data corresponding to GA2-MB and placebo groups are indicated in orange and purple, respectively (**C-H**). Horizontal lines indicate arithmetic mean (**C-H**). Two tailed Mann-Whitney test.

Supplementary tables

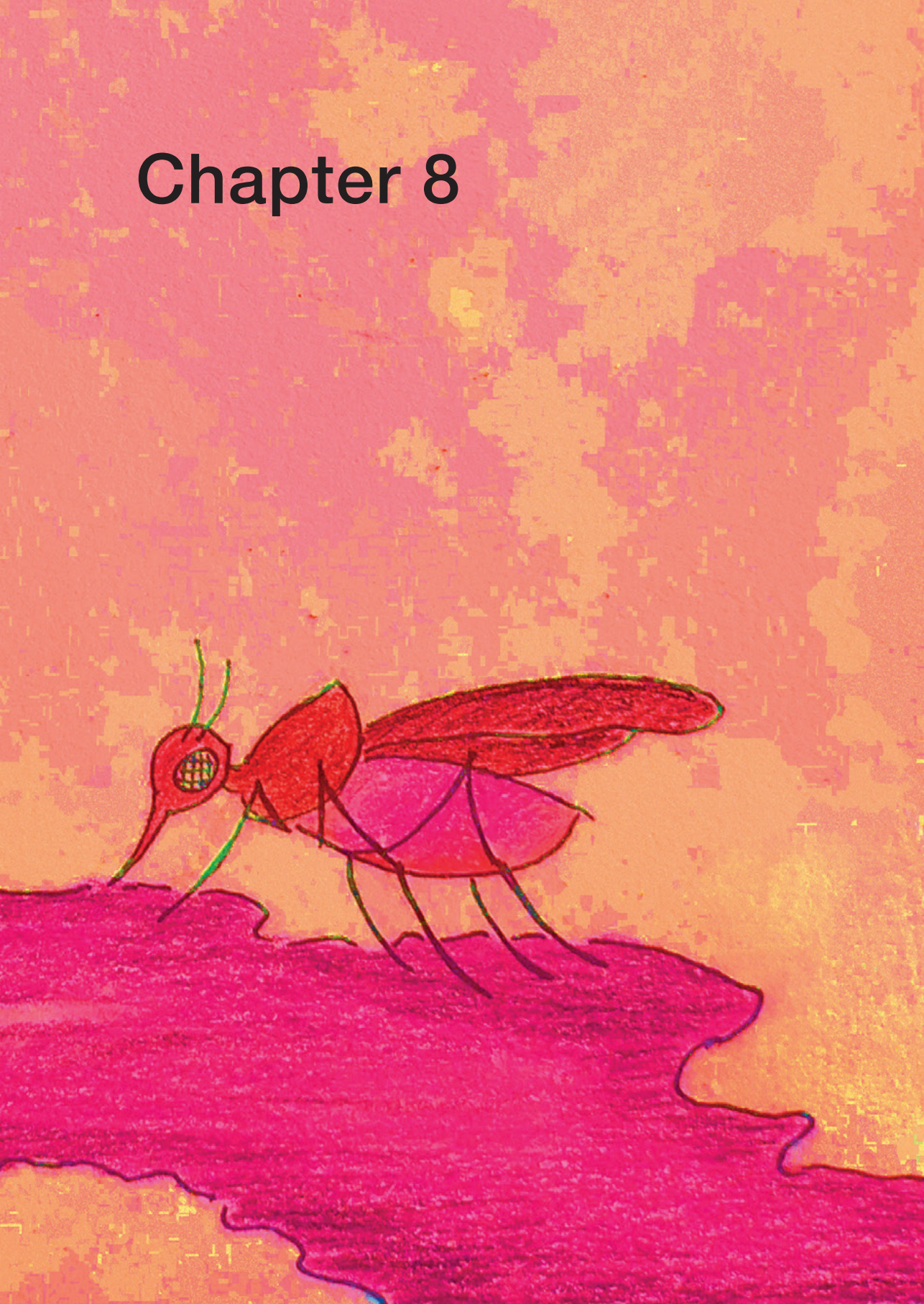
Supplementary Table 1. Risk of study related adverse events

n (%)	GA2 (n=10)			Placebo (n=5)		
	Mild	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**
Mosquito bites – immunization (45 – 55 blood fed mosquitoes)						
Bullous skin reaction	1 (10.0)	-	-	-	-	-
Swelling	-	1 (10.0)	-	-	-	-
Pain	1 (10.0)	1 (10.0)	-	-	-	-
Itch	5 (50.0)	-	1 (10.0)	3 (60.0)	-	-
Insect bite reaction	8 (80.0)	1 (10.0)	-	5 (100)	-	-
Immunization						
Increased liver transaminase	1 (10.0)	-	-	-	-	-
Chills	-	1 (10.0)	-	-	-	-
Myalgia	-	1 (10.0)	-	-	-	-
Malaise	-	1 (10.0)	-	-	-	-
Diarrhea	-	1 (10.0)	-	1 (20.0)	-	-
Fever	2 (20.0)	-	-	-	-	-
Headache	1 (10.0)	2 (20.0)	-	1 (20.0)	-	-
Mosquito bites – CHMI (5 infected blood fed mosquitoes)						
Itch	5 (50.0)	1 (10.0)	-	3 (60.0)	1 (20.0)	-
Insect bite reaction	6 (60.0)	1 (10.0)	-	5 (100.0)	-	-
CHMI						
Thrombocytopenia	-	-	-	1 (20.0)	-	-
Nausea or vomiting	1 (10.0)	-	-	-	-	-
Malaise	1 (10.0)	-	-	1 (20.0)	-	-
Fever	1 (10.0)	-	-	-	-	1 (20.0)
Myalgia	-	1 (10.0)	-	-	-	-
Abdominal pain	-	1 (10.0)	-	-	-	-
Chills	-	-	1 (10.0)	-	-	-
Headache	3 (30.0)	1 (10.0)	-	1 (20.0)	2 (40.0)	-
Venepuncture						
Pain	1 (10.0)	-	-	-	-	-
Hematoma	4 (40.0)	-	-	1 (20.0)	1 (20.0)	-
Anti-malarial treatment						
Dizziness	-	-	-	-	1 (20.0)	-
Abdominal pain	-	-	-	-	1 (20.0)	-
Flatulence	1 (10.0)	-		-	-	
Diarrhea	1 (10.0)	-	-	-	-	-
Headache	1 (10.0)	-	-	1 (20.0)	-	-
Nausea or vomiting	-	3 (30.0)	-	-	-	-

Supplementary Table 2. Staining antibody panel used in multi-parameter spectro-flow cytometry

Marker	Fluorophore	Dilution	Manufacturer	Cat. Number	Clone
TCR γδ	PerCP-Cy5.5	1:50	Biolegend	331224	B1
CD56	BV786	1:200	BD	744222	R19-760
TCR γδ Vδ2	BV605	1:200	BD	743751	B6
CCR7	AF700	1:100	Biolegend	353244	G043H7
CD3	APC-ef780	1:800	Invitrogen	47-0038-42	UCHT1
CD4	PE-Cy7	1:800	BD	557852	SK3
CD25	PE-fire 640	1:200	Biolegend	356148	M-A251
CD11c	AF532	1:100	Invitrogen	58011641	ITGAX
CD45RA	BV480	1:100	BD	746799	5H9
INF-γ	BV421	1:500	Biolegend	502532	4S.B3
TNF-α	BV650	1:500	Biolegend	502938	MAB11
IL-10	BV711	1:125	BD	564050	JES-9D7
IL-2	APC	1:100	Biolegend	500310	MQ1-17H12
IL-4	PE	1:20	BD	340451	3.010.211
IL-5	PE	1:250	Biolegend	504304	TRFK5
IL-13	PE	1:100	Biolegend	501903	JES10-5A2
FoxP3	PE-CF594	1:50	BD	562421	259D/C7
CD8	FITC	1:400	BD	555366	RPA-T8

Chapter 8



Discussion

This thesis describes the development and clinical testing of the late arresting genetically attenuated malaria parasite (GAP) GA2: after confirming its attenuation phenotype in humans, the preliminary protective efficacy of the GAP was assessed against controlled human malaria infection (CHMI) with the homologous parasite strain *Pf3D7*. Additionally, the factors influencing the motivation and decision-making process of medical volunteers taking part in early drug and vaccine studies were analysed, specifically focussing on SARS-CoV-2 vaccine trial participants and analysing the differences between phase 1 clinical trials and controlled human infection (CHI) studies.

GAPs that arrest late in the liver stage of malaria have the potential to increase immunogenicity and protective efficacy. GA2, the first late-arresting GAP to be developed and tested in humans, has shown to be both safe and well-tolerated with high rates of protection in malaria-naïve individuals. While further research is needed to confirm these preliminary findings, GA2 could represent a new tool in the fight against malaria.

The investigation of malaria vaccine candidates relies on clinical testing and hence on the participants who volunteer their time to help the advancement of science. The ethical implications of research involving humans are far-reaching and have been extensively discussed. One of the concerns is that the monetary compensation phase 1 participants receive, may cause them to overlook the risks and dangers of research. However, as outlined in this thesis, medical volunteers have a variety of motivations for participating in clinical trials, which are not all financial in nature. Rather than providing a definitive answer to this complex ethical debate, we would like to highlight the importance of being aware and respecting the value of clinical trial participants' commitment to science.

In this final chapter, we will summarize answers to the following questions:

- Are late-arresting whole sporozoites (WSp) the new frontier of malaria vaccinology?
- What is the importance of the liver stage for the development of immunity against malaria?
- How can a genetically attenuated malaria vaccine be implemented?
- Can the motivations and decision-making process of clinical trial participants be predicted?
- How do controlled human infection studies differ from other clinical research and what are the ethical implications hereof?

Are late-arresting whole sporozoites (WSp) the new frontier of malaria vaccinology?

The quest to develop an effective vaccine against malaria has been ongoing for more than half a century. While whole sporozoite (WSp) vaccines and, more specifically, radiation attenuated sporozoites (RAS), were the first immunization strategy against malaria tested in humans with some success[1,2], the long awaited breakthrough has not yet materialized. Significant advances have been made in the field, including the approval of two subunit vaccines (RTS,S and R21)[3,4] and the development of several alternate WSp vaccine strategies[5-7]. Live sporozoites administered in combination with chemoprophylaxis (CPS) have yielded excellent results, with up to 100% protection of malaria-naïve individuals at low doses[5,8]. The superior protection of CPS compared to RAS highlights the importance of a prolonged development of the attenuated parasite in the host. However, efficacy in endemic regions is variable and logistic challenges complicate the widespread administration of both subunit and WSp vaccines[9]. More specifically, protection by subunit vaccines is closely related to antibody titres and therefore wanes over times as titres decrease as well[4]. In contrast, CPS has shown to induce a more durable immune response[10]. However, CPS also bears the intrinsic risk of exposing healthy individuals to unattenuated parasites[10] and is therefore unsuitable for large-scale administration.

GAPs offer a new hope as they can be made to arrest at various timepoints in the developmental cycle, thereby mimicking the CPS phenotype, without sacrificing safety. To allow for sufficient exposure to the immune system, but avoid disease symptoms, genetic manipulation must occur in the pre-erythrocytic stages of development. Furthermore, parasites need to be attenuated without losing their capacity for sporozoite production and infectivity[11]. Whole genome sequencing of *Plasmodium* allowed for structured investigation and selection of genes[12,13] and for the generation of the first rodent GAPs[12], soon followed by equivalent *Pf* knock-out (KO) parasites[11]. Initially GAPs were early-arresting and, akin to RAS, interrupted development in the first 24-48 hours after administration: despite their safety, their efficacy was lower than desired[6,14-16]. The advent of late-arresting GAPs marks a new generation of WSp vaccine candidates with potentially increased potency, but without the safety concerns of CPS, such as the risks related to the administration of live sporozoites to healthy individuals.

GA2 (*PfΔmei2*), presented in **Chapter 1 and Chapter 5**, is one such late-arresting GAP lacking the *meiosis inhibited 2* (*mei2*) gene, which codes for an RNA binding protein expressed exclusively during the liver stage and involved in DNA segregation. *Mei2* mutants develop normally until the end of the liver stage (6-7 days), but fail to produce functional merozoites[17,18].

As outlined in **Chapter 2**, the development of GAPs for testing in humans was long and at times complicated. Prior to testing in humans, the safety of GA2 was extensively evaluated in pre-clinical models. In cultured human hepatocytes GA2 showed a similar development to wild-type (WT) parasites up until day 9. The GAP was then administered to humanised FRG huHep mice injected with human erythrocytes to assess safety. qPCR analysis on blood taken on days 7 and 9 showed the presence of small amounts of parasitic genetic material, yet in much lower quantities compared to wild type (WT). When blood samples were cultured for 28 days, no parasitic growth was detected. The very small amount of DNA and the lack of growth in culture suggests that the positive qPCR signal was due to the release of aberrant, non-viable merozoites from liver schizonts and not to a blood-stage infection[18].

The murine analogue *PyΔmei2* also induced high protection: immunization of BALB/cJ mice with 1 x 10^4 *PyΔmei2* parasites and subsequent challenge 46 days later with 1 x 10^4 WT parasite resulted in 100% protection, which was maintained when mice were challenged again 56 day later[17] (also see **Chapter 5).**

Given the promising results of the rodent model, safety and preliminary protective efficacy of GA2 were investigated in humans and compared to that of its predecessor GA1 (**Chapter 1**). Even though efficacy of GA1 had already been tested in one previous clinical trial and was known to be limited[6], the set-up of the clinical trial allowed for a direct comparison of an early- (GA1) and late-arresting (GA2) GAP. After safety testing of escalating doses of 15 and 50 mosquito bites in five and the 15 participants, protection by GA2 was assessed against homologous controlled human malaria infection (CHMI). For this purpose, participants were immunized three times, at 28-day intervals, with 50 GA2-or GA1-infected or uninfected mosquito bites. Protection induced by GA2 was 89% and by far exceeded that of its comparators (13% for GA1 and 0% for placebo) and that of previously tested GAPs, which ranged between 0% and 50%[6,16,19].

Subsequent research, described in **Chapter 6**, confirmed the safety of GA2 and investigated its efficacy when administered at lower doses. Ten healthy participants were exposed to a single immunization of 50 GA2-infected mosquito bites and then challenged with five unattenuated mosquito bites six week later. None of the exposed individuals experienced a breakthrough infection and 90% of them were protected against challenge.

These findings unequivocally confirm that late-arresting parasites induce higher protection at much a smaller dose than early-arresting ones. We have shown that the efficacy of WSp vaccines can be improved by prolonging their permanence in the host: extensive exposure to the immune system, wider antigenic variability and increased biomass of late-

arresting parasites are likely the cause of their enhanced immunogenicity. Considering that the duration of the hepatic stage is the most significant difference between early and late-arresting GAPs, our results highlights the importance of the liver for sterile protective immunity against malaria. Going forward, research on WSp vaccines should focus on candidates with a prolonged development, which induce higher protection rates without sacrificing safety and tolerability.

What is the importance of the liver stage for the development of immunity against malaria?

The high protective efficacy of GA2 (89%) described in **Chapter 1** and confirmed in **Chapter 6** provides hope for the future, yet also raises the question of which immunological mechanisms underlie these results. As mentioned previously, the superior efficacy of CPS and late-arresting GA2[5,8], compared to RAS[20] and early-arresting GAPs[6], highlights the importance of the liver stage in achieving immunity against malaria. Indeed, the liver has long been the focus of malaria research because it represents a population bottleneck: sporozoites are thought to spend a short time in the skin and it is only after the release of merozoites into the circulation that parasite replication becomes exponential[21]. Hence, it is not farfetched to assume that an immunological intervention would be most effective in the pre-erythrocytic stage and specifically in the hepatic stage of the *Plasmodium* life-cycle. The first indications that liver stage immunity may be involved in protection against malaria came from mouse experiments, which showed that the adoptive transfer of hepatic mononuclear cells from protected to unprotected animals also transferred protection[22]. Moreover, RAS-immunized non-human primates display high frequencies of $CD4^+$ and $CD8^+$ T cells in liver tissue, even when responses in peripheral blood remain undetectable[23]. Since then, much research has attempted to unravel the complex and often interrelated pathways that define hepatic immunity against malaria. In the following, we will summarize what is known on the topic and attempt to explain which immunological mechanisms could underlie GA2's protective efficacy.

Cellular immunity

T cells have received much attention in the field of malaria research. In **Chapter 1** we described our findings concerning the immunological response of participants immunized with early- and late-arresting GAPs. After stimulating peripheral blood nuclear cells (PBMCs) of participants with infected erythrocytes, we found an increase in *Pf*-specific $CD4^+$ T cells and $\gamma\delta$ T cells expressing the $V\delta2$ chain in GA2- but not GA1-immunized participants. On a single cell level, both $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells had an effector-memory phenotype,

producing multiple type 1 cytokines: $CD4^+$ T cells preferentially expressed tumor necrosis factor-α (TNF−α) and interlukin-2 (IL−2), whereas $V\delta2^+$ γδ T cells expressed interferon-γ (IFN−γ) and TNF−α. $CD8^+$, $V\delta2^-$ γδ and natural killer (NK) T cell compartments did not display any *Pf*-specific responses. These findings indicate that GA2 induces memory T cells with a Th1 type phenotype and were largely confirmed in **Chapter 6** as well.

$CD8^+$ T cells

$CD8^+$ T cells have long been hailed as key players in achieving protection against malaria and are thought to detect and eliminate infected hepatocytes[24].

The first experiments identifying a role for $CD8^+$ T cells against malaria date back to the eighties of the previous century: the administration of monoclonal antibodies to block $CD8^+$ T cell in protected mice, abolished such protection, yet this was not true for $CD4^+$ T cells[25]. Besides specificity, the number of $CD8^+$ T cells also matters, as, below a certain threshold, they are unable to confer protection[26].

A recent study found an association between protection in RAS-immunized individuals and a previously unidentified sub-population of $CCR6^+$ $CD8^+$ T cells[27]. Production of IFN-γ and TNFα and the cytotoxic mediators granzyme A and perforin mark these cells early effector memory rather that effector or central memory cells[28]. Similarly, a correlation between activated $CD8^+$ T cells producing granzyme B and protection has been reported after CPS immunization[29,30].

However, these results were difficult to replicate and there are many instances in which protection did not correlate with an increase in $CD8^+$ T cells[5,20,31]. As described in **Chapter 1**, we also did not detect changes in the $CD8^+$ T cell compartment after immunization with GA2. Thus, the idea arose that, upon malaria infection and priming by dendritic cells (DC) in the lymph nodes, $CD8^+$ T cells may relocate to the liver, where they act as tissue-resident memory cells. Additional priming of $CD8^+$ T cells is thought to occur in the liver itself through antigen-presentation by infected hepatocytes[32].

In murine models, the fact that $CD8^+$ T cells do not readily recirculate in parabiotic mice and that abolishing the function of liver-resident T cells also abolishes protection[33], suggests that this subset is liver-resident. Similarly, non-human primates (NHP) intravenously vaccinated with RAS displayed high frequencies of sporozoite-specific and INF-γ-producing $CD4^+$ and $CD8^+$ T cells and even when cellular immune responses were not detected in the periphery, they were abundantly present in the liver[23]. In yet another study with RAS-immunized NHP, the frequency of $CD8^+$ T cells was a hundred times higher in

the liver than in the peripheral circulation: 60% of these liver-resident lymphocytes with an IFN-γ recall response were $CD8^+$ T cells[34].

Given the invasiveness of biopsy procedures, few studies have attempted to directly study the immunological processes occurring in the human liver after malaria vaccination or infection. However, there has been one instance of fine needle aspirates collected from the livers of participants immunized three times with chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) encoding a multi-epitope string, followed by the pre-erythrocytic thrombospondin related adhesion protein (ME-TRAP), before undergoing CHMI[35]. ChAd63 and MVA encoding ME-TRAP are subunit vaccines designed to activate and attract T cells to the liver by means a prime-boost approach[36]. The researchers identified a population of $CD8^+$ memory T cells that was present in high frequency in hepatic tissue and in low frequency in the peripheral circulation. On the one hand, these results confirmed the liver-residency of malaria-specific $CD8^+$ T cells, on the other they suggested that this subset should also be detectable in the periphery[35]. Why the detection of $CD8^+$ T cells in blood has proven difficult[6,23,37,38] in practice is difficult to say, but could be related to insufficient sensitivity and specificity of the implemented assays. Expanding flow cytometry panels could be one method to increase the chances of detecting more specific cell subtypes.

In conclusion, the lack of $CD8^+$ responses in our samples is in line with findings from previous literature[5,20,31], supporting the idea of a compartmental shift of this cell subset from the circulation to the liver, where they recognize and kill infected hepatocytes. In the future, it would be advisable to use more sensitive techniques to detect $CD8^+$ T cells responses in the peripheral circulation.

$CD4^+$ T cells

$CD4^+$ T cells are thought to play an important role in protection against malaria by acting as direct cytotoxic effector cells, but also as helpers to $CD8^+$ and B cells[39].

Priming and activation probably occurs in lymph nodes upon antigen presentation by DCs, as there is no direct evidence for priming in the liver[40]. Cytokine production by DCs then determines the developmental fate of naïve $CD4^+$ T cells, resulting in a Th1, Th2 or T follicular helper (Tfh) phenotype.

Production of IL-12 by DCs induces Tbet expression by $CD4^+$ T cells and their subsequent development into Th1 cells: pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-2 have been shown to have anti-parasitic functions. $CD4^+$ T cells of the Th1 subtype may also directly act as effector cells through the production of cytotoxic granules[39].

The role of Th2 cells seems to be limited in malaria, but they are induced by IL-4 production and, in murine models, have been shown to be necessary for the sustained proliferation of $CD8^+$ T cells[41].

Tfh cells are another important subset: induced by IL-6, they are pivotal for the formation of germinal centers and the development of functional B cells. In humans, Tfh cell production is impaired in malaria and polarized towards a Th1 phenotype: T-bet induces atypical memory B cells, which are less efficient at producing antibodies[39,42].

The first studies conducted with RAS looked at cytokine production by PBMCs in general and did not distinguish between lymphocyte subsets. Proliferative responses were observed after incubation of PBMCs with recombinant CSP[43]. Since then, these results have been widely replicated: PBMCs isolated from immunized individuals and incubated with *Plasmodium* sporozoites or asexual blood stages were found to produce proinflammatory cytokines, such as IFN-γ, TNFα and IL-2[38,44,45].When PBMCs were incubated with immortalised cells pulsed with CSP, >95% of cytotoxic cells reacted with OKT4 antibody and were therefore recognized as $CD4^+$ T cells[46]. This was the first indication that $CD4^+$ T cells may play an important role in protection against malaria.

Subsequent studies isolating and focusing on $CD4^+$ T cells specifically found that when presented with unattenuated sporozoites after immunization with WSp, the recall responses of this lymphocyte subset were mostly pro-inflammatory and marked by IFN-γ production[14,23,34,37,47,48]. A recent paper investigating gene expression patterns across RAS-immunized protected and unprotected participants, found that protected participants showed a polarization towards Th1 cells, whereas non protected participants had increased Th2 cells responses[49]. Similar results have been replicated in another study, which found that TNF-α/IFN-γ secreting $CD4^+$ cells were associated with protection in RAS-immunized participants[27].

$CD4^+$ T cells with an effector memory phenotype, expressing IFN-γ, IL-2, or TNF-α, have previously been found to increase after immunization of participants with chemo-attenuated parasites (CPS)[5] and to strongly associate with protection[8,27]. In contrast, unprotected CPS-immunized participants more frequently display $CD4^+$ T cells producing a single cytokine[8,14]. $CD4^+$ T cells expressing the marker of cytotoxic degranulation CD107a with an effector memory phenotype and cytotoxic properties were correlated with protection in yet another CPS immunization study[29].

However, there are also studies that failed to establish a relationship between $CD4^+$ T cells and protection upon vaccination with WSp[6,14]. What accounts for the discrepancy between results is uncertain, but small sample size and low protection rates may have played a role.

Our results align with literature and largely confirm previous findings. The increase of $CD4^+$ T cells with a Th1 phenotype is associated with protection against the disease and thought have anti-parasitic properties. Hence it is likely that at least part of the GA2-induced production is mediated by $CD4^+$ T cells, although it is difficult to say whether that activity is due to the cytotoxic effector functioning or mediated by cytokine production and $CD8^+$ T cell stimulation.

γδ T cells

Another cell subset that has received much attention are γδ T cells. The distinctive feature of γδ T cells is that they have a bridging function between the innate and adaptive immune response, expressing professional antigen-presenting cell markers and even killing opsonized pathogens[50].

It is not known how and where γδ T cells are primed, but the $\delta 2^+$ subset is thought to interact with phosphoantigens produced by *Plasmodium*[51]. It has been further hypothesized that γδ T cells produce INF-γ, TNF-α and M-CSF, possibly activating $CD8^+$ T cells and inducing phagocytic activity by macrophages[52]. It seems that while liver-resident $CD8^+$ T cells induce protection against malaria by killing infected hepatocytes, γδ T cells are required for the activation of said $CD8^+$ T cells. Indeed, abrogation of γδ T cells in RAS-immunized mice led to a reduced accumulation of $CD8^+$ T cells in the liver and nullified protection. However, γδ T cells alone, where not, in the absence of $CD8^+$ T cells, sufficient for protection[53].

Several studies investigating both CPS and RAS immunization, reported that an increased frequency of $V\delta 2^+$ T cells correlated with protection[8,34,53,54]. In a clinical trial, which studied the difference between CPS immunization with a blood-stage active drug (chloroquine) and a liver-stage active drug (pyrimethamine), the frequency of $V\delta 2^+$ T cells was found to be lower in the only unprotected participant in the chloroquine group and dependent on the sporozoite-dose in the pyrimethamine group. Among the participants who had been administered pyrimethamine, expansion of $V\delta 2^+$ T cells in the immunization phase of the trial also correlated with protection[54].

Endemic studies conducted in children as well as adults, have also failed to find an association between γδ T cells and protection after immunization with PfSPZ Vaccine[37,48,55-57].

As to the explanation of these result, different hypotheses have been put forward, including a relative abundance of T regulatory cells and a more tolerogenic immunological microenvironment in these populations. Individuals in malaria endemic regions may have higher percentages of differentiated T cells, leaving behind a smaller pool of naïve T cells

capable of expanding after immunization. These findings align with studies that found that γδ T cells expand after primary infection with malaria, but contract with subsequent exposures[58].

Our results confirm previous findings, by showing that an increase in Vδ2$^+$ T cells in malaria-naïve individuals is correlated with protection against the disease. While their exact role remains unclear, γδ T cells may be important for the recruitment of liver-resident CD8$^+$ T cells.

Humoral immunity

As described in **Chapter 1**, antibody titers against the circumsporozoite protein (CSP) were significantly higher after three immunizations with GA2 compared to baseline, but did not correlate with protection. Titers of anti-apical merozoite antigen 1 (AMA1) and anti-merozoite surface protein 1 (MSP1) antibodies were not increased upon immunization.

A correlation between CSP-antibody titres and protection against malaria has been historically difficult to establish in an unequivocal manner[59]. Still, the sporozoite-neutralizing potential of antibodies has been confirmed *in vitro* and *in vivo*. Incubation of sporozoites with antibodies from immunized participants inhibits their capacity to invade hepatocytes *in vitro*[37,48,60,61]. Similarly, passive transfer of purified antibodies from immunized participants to naïve humanized mice, protected the latter from infection with *Plasmodium* and reduced liver load[34]. Even more to the point, recent studies with monoclonal antibodies have shown that they can prevent more than 80% of homologous infections in malaria-naïve individuals[62,63].

On the other side, it has been suggested that B cell activity and antibody production may be suboptimal in malaria. Only a small subset of antibodies isolated from RAS-immunized protected individuals living in endemic areas have both high affinity and efficacy against sporozoites[64]. The structure of CSP with its many repeat regions make it an unsuitable target for antibody production. CSP can cross-link multiple B cell receptors at the same time, activating B cells without the need of T cell help[65]. This mechanism impairs long-term memory by generating short-lived plasmablasts[66] instead of long-lived plasma cells. Indeed, anti-CSP antibodies produced in response to subunit malaria vaccines, such as RTS,S and R21, decrease more rapidly than those produced after administration of other subunit vaccines (e.g.: tetanus and diphtheria)[67,68]. Similarly, the ability of CSP to cross-link multiple B cell receptors, resulting in strong antigen recognition, may impair affinity maturation[59]. In yet another study it was shown that anti-CSP antibody responses plateau in RAS-immunized individuals, probably due to a negative feedback mechanism and epitope masking, making it impossible to reach protective levels[69].

While antibodies may contribute and, under specific circumstances (high quantity and affinity) may be sufficient to achieve protection[63,70], they are not a reliable correlate of immunity. The impaired production, affinity maturation and persistence of antibodies as well as the large inter-individual variability[59], make it difficult to predict protection based on serum titres alone.

An integrative summary of putative immune responses after GA2 administration

Protection against malaria is a complex multifactorial process that has been difficult to unravel: even though separate elements are known, the bigger picture still eludes us. Based on our current knowledge, we propose the following immunological mechanisms that lead to protection against malaria in general and may underlie the efficacy of GA2 in the current study (**Fig. 1**).

After administration of GA2, naïve $CD8^+$ and $CD4^+$T cells are primed in the skin-draining lymph nodes and the spleen. From here, $CD8^+$ T cells migrate to the liver under the influence of γδ T cells. Proliferation of $CD8^+$ T cells and their differentiation into tissue-resident memory cells is stimulated by further antigen presentation in the liver, which may be enhanced by $CD4^+$ T cells. Once established, liver-resident memory $CD8^+$ T cells exert a cytotoxic function, killing infected hepatocytes. $CD4^+$ T cells may have a similar role, also acting as effector cells. Finally, antibodies may contribute to protection against malaria as well, but for them to work, they need to be present in sufficient amounts and have good binding affinity for their target antigens. While GA2 immunization induced anti-CSP antibodies in our studies, the relatively short time that sporozoites spend in the circulation before moving to the liver suggests that it may be difficult to produce sufficient quality and quantity to fend off an infection. In conclusion, GA2 likely acts on all of the above delineated mechanisms, however, its prolonged permanence in the liver is specifically favorable to the activation of cellular immunity.

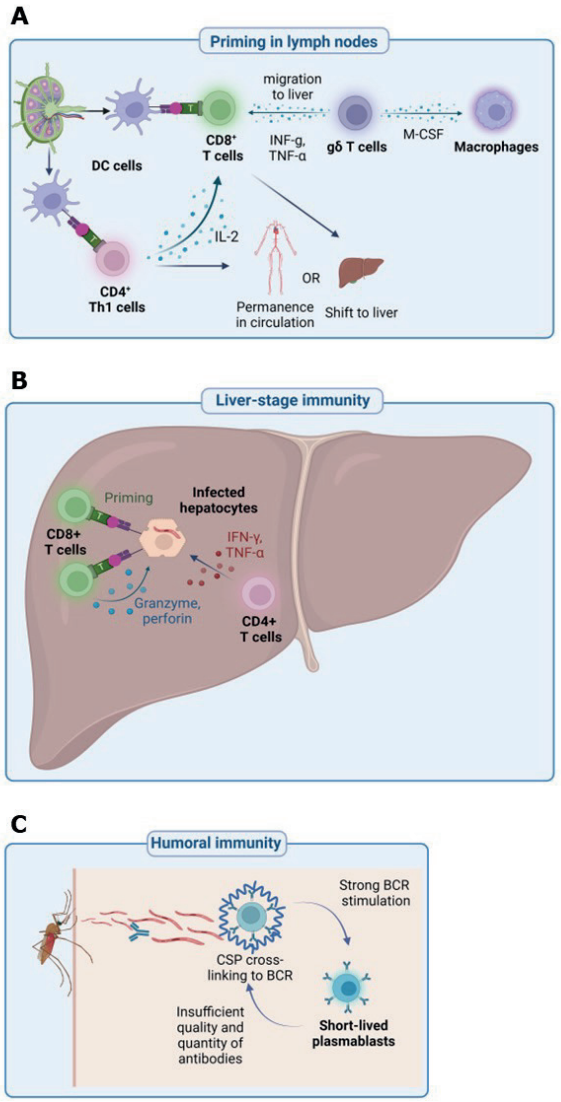


Figure 1. Overview of immunological mechanisms involved in protection against malaria
A. CD8⁺ and CD4⁺ T cells are thought to be primed in skin-draining lymph nodes by DCs. CD4⁺ T cells may act as helper cells to CD8⁺ T cells, producing IL-2 and stimulating proliferation. While it is not clear how and where γδ T cells are primed, they likely have a role in stimulating CD8⁺ T through INF-γ and TNF-α production, also affecting their migration to the liver. γδ T cells may also produce M-CSF, which stimulates macrophages' phagocytic activity .
B. Antigen presentation continues in the liver where infected hepatocytes further prime CD8⁺ T cells. CD8⁺ T cell-dependent killing of infected hepatocytes is perforin- and granzyme- mediated. CD4⁺ T cells are also thought to exert a direct effector function by killing infected hepatocytes.
C. While humoral immunity contributes to protection against malaria, the production of antibodies of sufficient quantity and quality is thought to be impaired. Cross-linking of antibodies instead of binding to target epitopes generates a strong B cell receptor signal which may lead to production of short-lived plasmablasts instead of long-live memory cells.
DC: dendritic cell; IL-2: interleukin 2, INF-γ: interferon-γ; TNF-α: tumor necrosis factor-α, M-CSF: macrophage colony stimulating factor.

How can a genetically attenuated malaria vaccine be implemented?

The good safety profile and relative lack of adverse events reported in **Chapter 1**, warrant the investigation of GA2 as a potential new vaccine candidate.

GA2 efficacy needs to be confirmed with larger numbers: while we did not observe any breakthrough infections of the GAP, it cannot be completely excluded that such an event could occur in a larger sample including more susceptible individuals.

It has been hypothesized that deleting an additional gene could improve GA2's safety: while it could be argued that knocking out more genes is always safer, this strategy does not guarantee success if the deletion targets are poorly chosen or if further genetic manipulation affects immunogenicity. In **Chapter 2** we briefly discuss the late liver-stage arresting replication competent (LARC2), a new GAP that lacks the *liver stage nuclear protein* (*LINUP*) gene in addition to *mei2*. Unlike the murine analogue of GA2 (*PyΔmei2*), administration of 2,5 x 10^5 PyLARC2 sporozoites to highly susceptible BALB/CyJ mice did not cause any breakthrough infections. Protective efficacy of PyLARC2 in mice is also promising and was tested across more conditions than the *PyΔmei2*. Immunization of 10 BALB/cJ mice with 1 x10^4 sporozoites twice at monthly intervals were protected against challenge with 1 x 10^4 WT sporozoites. Intramuscularly immunization of BALB/cJ mice with 2 x 10^4 PyLARC2 sporozoites two times one month apart and subsequent challenge with 1 x 10^4 WT sporozoites resulted in 70% protection and a delay to time to parasitaemia in the unprotected animals. To assess protection over time, BALB/cJ mice were immunized twice, one month apart, with 5 x 10^4 PyLARC2 sporozoites and then challenged six or 12 months with 1 x 10^4 WT sporozoites: protection was 90% in the first group and 60% in the latter[71].

Next the PyLARC2 was tested in outbred mice, which are less responsive to immunization. Twofold immunization at two-month intervals with 5 x10^4 sporozoites and subsequent challenge with 1 x10^3 WT sporozoites two months after the last immunization resulted in 70% protection[71].

Immunization with cryopreserved sporozoites was also attempted: administration of 5 x 10^4 sporozoites at two week intervals followed by challenge with 1 x 10^4 WT sporozoites left all mice protected. Finally, protection of *Py*LARC2 against blood stage infection was tested. After threefold immunization at monthly intervals of BALB/cJ mice with 1 x 10^4 sporozoites, they were injected with lethal *Py* blood stage parasites. All immunized animals were able to completely clear the infection within 12 days[71].

Similar to GA2, when *Pf*LARC2 was tested in FRG HuHep mice injected with human erythrocytes, it was shown to be safe and incapable of producing viable merozoites[71]. Clinical testing will determine its efficacy in humans.

Furthermore, GA2-induced protection needs to hold up in malaria-endemic areas, as almost every WSp vaccine candidate tested to date performed worse in pre-exposed populations[57,72] compared to malaria-naïve ones[5,8,73].

Generally, WSp-induced protection is less robust after heterologous challenge with different *Pf* strains, compared to homologous challenge with the same or a closely related one[5,72]. Genetic variability may allow parasites to evade immunosurveillance[74,75] and accounts for both the slow acquisition of natural immunity and episodes of severe malaria in semi-immune adults after infection with new strains[76].

Another factor influencing vaccine efficacy may be hypo-responsiveness, characterized by pre-existing yet counterproductive immunological mechanisms and chronic inflammation[77].

Pre-existing immunity may be harmful if it causes rapid clearance of the vaccine itself, thereby reducing its immunogenicity[77,78]. Repeated infections with *Plasmodium* or other pathogens may also lead to a state of chronic inflammation which eventually results in immune exhaustion and senescence, skewed immune responses and changes in the lymphoid organs[77]. Exhaustion and senescence refer to the gradual dysfunction and accelerated aging of the immune system, respectively. While these two processes can be mechanistically different, they eventually both result in the decreased reactivity and efficiency of several cell subsets, including NK, B and T cells[77]. In the presence of chronic inflammation, immunological tolerance may develop. While a skewing of the immune response towards a more regulatory type 2 phenotype is necessary to protect from excessive tissue damage, it is less effective at clearing infections[77,79]. Helminth co-infections, which are not uncommon in malaria-endemic regions and preferentially induce a regulatory type 2 immune profile, are negatively associated with vaccine performance[80]. Furthermore, it has been shown that severe malaria infections disrupt normal germinal centre functioning, thereby impairing effective humoral immune responses[81].

If GA2 were indeed to perform worse in pre-exposed populations, one could consider adding adjuvants to sporozoites to make them more immunogenic and boost both innate and adaptive immune responses. One possibility would be co-administer GAPs with compounds that serve as adjuvants and therefore enhance the immune reaction elicited by the vaccine. The drawback is that such substances will most likely be administered independently of the parasites and therefore prompt a systemic, possibly less specific reaction.

Another option would be to genetically modify parasites to express adjuvants on their own: this solution has the potential advantage of a targeted immune response and a more precise delivery of the adjuvant. However, this approach is not easy as it requires the sporozoites to remain viable despite additional manipulations and the immune response to be sufficient for protection yet not so strong as to cause irreversible damage to the host.

The current administration route, namely mosquito bites, represents yet another obstacle: it is not feasible for large-scale implementation and does not comply with the WHO preferred product characteristics[9].

Hence, the production of late-arresting GAPs as an injectable product, as the US-based biotech company Sanaria is currently doing, is a pre-requisite for further vaccine development[82]. Another debate is whether subcutaneous or intramuscular administration of WSp should be preferred over intravenous administration: while intravenous injection of sporozoites is more efficacious[34], subcutaneous and/or intramuscular administration would be more practical[9]. Finally, WSp vaccines require storage in liquid nitrogen at -140°C and careful preparation of the injectable product by trained personnel to avoid contamination and/or infection[83].

Another question that arises is how GA2 would fit in the current panorama of approved malaria-vaccines. Both RTS,S and R21 have been recommended by the WHO for implementation in Africa[84].

RTS,S is a subunit vaccine consisting of CSP expressed with fused and free hepatitis B surface antigen (HBsAg) with the addition of the AS01 adjuvant. In a phase 3 trial, vaccine efficacy at 12 months varied between 55.8% and 31.3% in children (aged 5-12 months) and infants (aged 6-12 weeks), respectively. After three years, vaccine efficacy had decreased to between 28.3%-36.3% in children and to between 25.9% and 18.3% in infants, depending on whether they received a booster vaccine after 20 months or not[4].

R21 is another subunit vaccines that closely resembles RTS,S, but contains less HBsAg and is formulated with the adjuvant Matrix M. The idea is that without the excess HBsAg more CSP would be presented to the immune system[85]. A 2021 study involving 450 children, aged 5-17 months, from a highly endemic region in Burkina Faso were vaccinated three times at four-week intervals followed by a booster 12 months later. All vaccinated children received 5 ug of R21, but two different doses of the adjuvant Matrix M were tested, either 25 ug or 50 ug. Vaccine efficacy was 74% and 77% at six months and 71% and 77% at 12 months in the high and low adjuvant groups, respectively[85]. Results were replicated in a subsequent trial, which enrolled 4644 children, aged five months to

three years, half of them living in regions with seasonal malaria and the other half living in regions with perennial malaria. Immunized participants all received the same dose consisting of 5 µg R21/50 µg Matrix-M and were vaccinated three times at four-week intervals, followed by a booster 12 months later. Vaccine efficacy at 12 months remained high and was 75% at seasonal and 68% at perennial malaria sites[3].

It has been argued that a direct comparison of RTS,S and R21 is difficult and that any judgement about the superiority of one or the other should be suspended. An often criticised point is the dosing: while RTS,S was administered based on child age, R21 was administered just prior to the malaria transmission season, therefore ensuring optimal protection during the most vulnerable period[86]. It should also be noted that only children, aged five months and up, were included in both R21 studies, while the RTS,S phase 3 trial also included infants aged 6-12 weeks. Protective efficacy of RTS,S was lower in the younger age group and the question of R21's efficacy in a similar cohort remains unanswered[87].

Despite the promising results and the enthusiasm with which the WHO-approval of RTS,S and R21 has been greeted, the long-term efficacy and the role of these vaccines in malaria eradication is uncertain[86]. If WSp vaccines were to be approved in the near future, one could hypothesize co-administration with subunit vaccine for optimal activation of humoral and cellular immune responses.

On the other hand, one could also imagine that a population primed by subunit vaccines would have high antibody titres against CSP, which cannot discriminate between WT and attenuated parasites: WSp delivery to the liver could be inhibited or possibly even blocked by high antibody titres. If this were the case, administration of WSp would have to take place instead of or prior to subunit immunization to avoid an antagonistic effect.

Finally, given the success in the fight against SARS-CoV-2 and the possibility of concomitantly presenting multiple antigens, the idea of developing an mRNA vaccine against malaria has recently gained popularity. The question then arises of how mRNA vaccines may compare to WSp vaccines in general and GAPs in particular. While mRNA alone is insufficient to induce $CD8^+$ liver-resident T cells, the formulation of an mRNA lipoplex of the highly conserved PRL6 gene with the modified NK cell-specific agonist α-galactosylceramide$_B$ (αGC_B) showed not only efficient recruitment of $CD8^+$ T cells to the liver but also up to 80% protection in mice. Interestingly, protection was maintained even in pre-exposed mice, signifying that this method can avoid the immune suppression that is often observed in the presence of blood-stage parasitaemia[88]. The complexity of *Plasmodium* favours a vaccination approach that can present multiple antigens and activate several immunological subsets at the same time. The unique possibility of

including many, yet specific, antigens into a single formulation, make mRNA an extremely versatile technology with a distinct advantage over subunit vaccines. The relative ease of production, storage and administration may also favour mRNA vaccines over WSp.

In conclusion, there are two ways to look at GA2 and WSp vaccines in general: either they are considered a viable alternative and addition to currently approved subunit vaccines or they are seen as scientific tools to understand the immune physiology of malaria. While the hope that GA2 may serve to finally strike back against malaria is not unfunded, the many uncertainties and intrinsic drawbacks related to WSp vaccines cannot be omitted either. Hence, the novelty of GA2 lays not only in its excellent protective efficacy but, and maybe more importantly, in its role in proving the importance of the liver stage to achieve protective immunity against malaria.

Can the motivations and decision-making process of clinical trial participants be predicted?

Clinical trials would not be possible without the participation of healthy individuals, who volunteer to be the first on whom new drugs are tested. Since participants of such trials experience no personal benefit except for a financial compensation, some have questioned the undue influence of economical remuneration on the participants' judgement[89,90]. In order to shed light on the matter, we investigated the motivations of participants of SARS-CoV-2 vaccination studies.

As outlined in **Chapter 3**, we found that SARS-CoV-2 participants were mostly motivated by the desire to advance science and help people in The Netherlands. While mixed motivations have been reported in the past[91], the altruistic tendencies were much more pronounced in the current case. The question then arises of what prompted SARS-CoV-2 study participants to behave differently than other medical volunteers. In order to answer this question, we developed a model, which delineates three factors that play a pivotal role in the decision-making process of clinical trial participants, namely rewards, the costs of inaction, and the risks of participation. In the case of SARS-CoV-2 vaccine trials, the rewards were both monetary and personal, as participants were vaccinated ahead of the rest of the population; the cost of inaction was high, because the pandemic affected every member of society; and finally the risks were deemed low by the participants (as assessed in a survey). The favorable combination of these three factors may explain the eagerness of SARS-CoV-2 vaccine trial participants and their relative disregard for financial incentives.

It may also be argued that participants were motivated by true altruism, that is, the genuine desire to help others in the absence of personal gain and even in the face of personal damage. However, the concept of altruism is a complicated one, and it has been shown that most cases of situational altruism are not truly so, as individuals experience some type of advantage from their actions[92]. To test whether the desire to help others and contribute to science is an intrinsic characteristic of SARS-CoV-2 vaccine trial participants, it would be interesting to see whether they maintain their altruistic inclinations in other circumstances as well or whether they were restricted to the pandemic alone.

Altruistic behavior can also be explained in terms of evolutionary advantages if it increases the chances of preserving direct offspring or related individuals[93]. Hence, the pandemic, a situation that threatened the survival of a large number of individuals, may have stimulated evolutionary altruism. This concept is much less applicable to "regular" phase 1 clinical trials, as any studied disease will only ever affect a subset of the population.

The presented model provides a method for assessing the motivations of clinical trial participants and their ethical implications, which may vary depending on the circumstances. The undue influence of financial compensation is an example of how the model could be useful: the importance of economic remuneration for an individual's decision to participate in a clinical trial may depend on what other factors are at play. Hence, while concerns about the undue influence of remuneration may be valid in a scenario where financial compensation is the main reward (i.e.: regular phase 1 clinical trial participants), they may not be applicable in others where participation is associated with other advantages (i.e.: SARS-CoV-2 vaccination trial).

In conclusion, the SARS-CoV-2 pandemic represented an exceptional situation, which made for a very interesting case study. While findings presented here may appear situational, the theoretical model we developed to explain the behavior of clinical trial participants transcends specific circumstances.

How do controlled human infection studies differ from other clinical research and what are the ethical implications hereof?

CHI studies may evoke a negative emotional response and therefore questions surrounding their social value, more so than phase 1 clinical trials[94]. Negatively influencing the perception of CHI studies is the fact that they are generally associated with potentially dangerous pathogens, whereas phase 1 clinical trials are more often referenced in the context of therapeutic drugs. This may lead to the misunderstanding that participants of phase 1 clinical trials may gain direct or indirect benefit from their participation,

whereas CHI study participants may only suffer the negative consequences. However, this therapeutic misconception has been addressed previously: clinical trial participants, whether it be a phase 1 or CHI study, do not by definition stand to gain benefit from their participation[95]. Additionally, the confluence of medical care and research has been debated in the past. Research ethics is distinct from medical ethics, because research has the scope of gaining knowledge rather than benefitting any (patient) population[96].

Finally, some might say that the social value of CHI studies is doubtful, as they are not strictly necessary for the development and approval of new drugs. Standard phase 3 field trials, such as those carried out to test efficacy of SARS-CoV2 vaccines[97], could replace CHI studies. In contrast, phase 1 clinical trials are indispensable for safety assessment of new products[98]. However, given the rising risk of new pandemics, CHI studies and the associated benefit of time- and cost-effectiveness, will become more relevant in the future. Additionally, CHI studies are often the only alternative for studying treatable diseases that cannot reliably be tested in animals first. Another instance in which CHI studies may be preferable to other types of clinical investigation is when disease incidence is too low for regular clinical testing[99].

It has also been argued that CHI studies should be judged by means of a different set of ethical and regulatory criteria than other types of clinical research. Since CHI studies involve the deliberate infection of healthy individuals with pathogens, risk to the participants themselves and to bystanders can be perceived as characteristics that set them apart from other clinical research. However, as delineated in **Chapter 4,** we argue that this is not the case and that CHI studies do not fundamentally differ from regular phase 1 clinical trials.

Phase 1 clinical trials generally involve a remote risk of unknown and potentially serious adverse events[100-102], whereas the risks related to CHI studies are predictable and treatable[103].

Linked to the perceived risk to the participants themselves is the bystander risk, meaning that someone who is not participating in a CHI study could inadvertently be infected as well: while possible in CHI studies, disease spread to third parties is not applicable to phase 1 clinical trials[104].

Hence, we identified risk to bystanders as a factor that may set CHI studies apart from phase 1 clinical trials and therefore requires additional attention. To facilitate the evaluation and assessment of CHI studies in the future, we provide a set of questions that researchers may ask themselves when designing such a trial. While these are not meant

as a replacement for the judgement of a medical ethical committee, they may provide additional guidance in study design.

Concluding remarks

This discussion provides an overview of the current state of GAPs, including the potential immunological mechanisms underlying protection, describing a way forward towards their implementation as malaria vaccines on a larger scale. It also focuses on the motivations of clinical trial participants and on the ethical difference between phase 1 clinical trials and CHI studies.

Late-arresting GAP GA2 combines the advantages of previous immunization methods employing attenuated parasites, namely the safety of RAS and the effectiveness of CPS. GA2's high protective efficacy of 89% warrants further investigation as a potential vaccine candidate. Yet GA2 is also interesting from an immunological perspective as it unequivocally demonstrates the importance of the liver stage to achieve protection against malaria.

None of the research contained in this thesis would have been possible without the participation of medical volunteers. Understanding the motivations of clinical trial participants is important to protect their rights and safeguard the ethical conduct of clinical research. We found that the SARS-CoV-2 pandemic stimulated behavior aimed at helping others and contributing to science, possibly because of the moral proximity of the issue at hand.

In conclusion, this thesis provides an integrated approach to the elimination of infectious diseases, such as malaria, from preclinical research to clinical testing and participant involvement.

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Non scientific part

Portfolio

The main project of this PhD has been the GA2 project, the clinical trial involving the administration of genetically attenuated malaria parasites to healthy human participants. Much time and effort went into this project as I was in charge of it from beginning to end. I helped redact the application for using the genetically modified parasite GA2 in clinical research and then wrote the application for the medical ethical committee. I was the contact person for other collaborating departments within the Leiden University Medical Center and for our collaborators in the Radboud Medical Center in Nijmegen. I recruited and screened potential participants for the clinical trial, coordinated the study visits and collected data. I then analyzed the clinical data and worked in the lab to analyze part of the immunological data as well. Finally, I wrote the first draft of the manuscript and many revisions after that (**Chapter 1**).

Besides the GA2, I worked on several side projects, which, unfortunately, have not all resulted in publication. Among these was the endeavor meant to analyze the movement of different types of genetically attenuated and wild-type sporozoites using the confocal microscope. However, due to the lack of results, i.e. no tangible difference in the movement patterns of said sporozoites, this project was abandoned.

To place the GA2 in a broader context and discuss potential implications for its large-scale implementation, I wrote a review about existing genetically attenuated parasites, the process that lead to their development and their current test status (**Chapter 2**).

After that I decided to continue the work of a previous colleague, investigating and comparing the motivations of clinical trial participants in SARS-CoV2 vaccination (**Chapter 3**). A second article about a further comparison between motivations of phase 1 and controlled human infection study participants is in the make, yet not included in this thesis. Finally, I wrote an opinion piece dissecting the conceptual and ethical differences between phase 1 trials and controlled human infections (**Chapter 4**).

I furthermore collaborated on several of my colleagues' projects, helping recruit participants, being the study physician for the participants and reviewing the final manuscripts.

I have decided to include two collaborative projects in this thesis because I believe that they provide additional information to better understand the position of the GA2 in the current scientific panorama. **Chapter 5** describes the development and pre-clinical evaluation of GA2 and therefore represents, if you will, the fundaments upon which this thesis is built. In **Chapter 6** the efficacy of a lower dose of GA2, namely a single immunization with 50 mosquito bites, is reported. This chapter is a continuation of my

work and confirms once more not only the safety, but also the high protection rates (90%) achieved by this parasite.

For an overview of all publications presented in this PhD thesis please refer to **Table 1** and for an overview of all publications please refer to **Table 2**.

I was allowed to present my work on a couple of occasions: I did an oral presentation at the Dutch Malaria Day in Leiden, the Spring Meeting of the Dutch Society for Parasitology in Utrecht, the BioMalPar in Heidelberg and the Dutch Internist Days in Maastricht; I had a poster and short pitch at the Spring Meeting of the British Society for Parasitology in Edinburgh (**Table 3**).

During my PhD I also completed all mandatory courses (**Table 4**) and was involved in teaching activities, giving several lectures to medical students and co-supervising biomedical students writing their thesis. Additionally, working on most of the papers included in this thesis allowed me to get acquainted with R for data organization, statistical analysis and figure generation.

Table 1. PhD candidate contributions to each article according to the CRediT methodology

Main Articles			
Article Title	**Year of publication**	**Journal**	**Contribution**
Safety and Efficacy of Immunization with a Late-Liver-Stage Attenuated Malaria Parasite	2024	New England Journal of Medicine	Data curation Formal analysis Investigation Methodology Project administration Validation Visualization Writing – original draft Writing – review & editing
The path from early- to late-liver stage arresting genetically attenuated parasites as a malaria vaccination strategy	2025	NPJ Vaccines	Conceptualization Data curation Investigation Methodology Visualization Writing – original draft Writing – review & editing
What motivates SARS-CoV-2 vaccine trial participants? A pre- and post-participation survey study	2024	Trials	Conceptualization Data curation Formal analysis Investigation Methodology Software Visualization Writing – original draft Writing – review & editing
Risk in healthy volunteer studies: does it matter if the agent is a drug or a bug?	2025	Submitted, under peer-review at Trials	Data curation Formal analysis Investigation Methodology Project administration Visualization Writing – original draft Writing – review & editing
Collaborative projects			
Creation and preclinical evaluation of genetically attenuated malaria parasites arresting growth late in the liver	2022	NPJ Vaccines	Writing – review & editing
Single immunization with genetically attenuated PfΔmei2 (GA2) parasites by mosquito bite in controlled human malaria infection: a placebo-controlled randomized trial	2025	Nature Medicine	Investigation Writing – review & editing

Table 2. Overview of all publications in chronological order

Title	Author	Journal	Year
Opioid-related beliefs and prescription modalities for postoperative pain of Dutch and American physicians.	Lamers OAC, Kuck K, Okifuji A, Stuart, AR, Johnson KB and Rijsdijk M	Pain Management	2019
Treatment strategies for GLILD in common variable immunodeficiency: a systematic review	Lamers OAC, Smits BM, Leavis HL, de Bree GJ, Cunningham-Rundles C, Dalm VA, Ho HE, Hurst JR, IJspeert H, Prevaes SM, Robinson A	Frontiers Immunology	2021
Creation and preclinical evaluation of genetically attenuated malaria parasites arresting growth late in the liver	Franke-Fayard BMD, Marin-Mogollon C, Geurten FJA, Chevalley-Maurel S, Ramesar J, Kroeze H, Baalbergen E, Wessels E, Baron L, Soulard V, Martinson T, Aleshnick M, Huijs ATG, Subudhi AK, Miyazaki Y, Othman AS, Kolli SK, Lamers OAC, Roques M, Stanway RR, Murphy SC, Foquet L, Moita D, Mendes AM, Prudêncio M, Dechering KJ, Heussler VT, Pain A, Wilder BK, Roestenberg M, Janse CJ	NPJ Vaccines	2022
Safety and infectivity of female cercariae in Schistosoma-naïve, healthy participants: A controlled human *Schistosoma mansoni* infection study	Koopman JPR, Houlder EL, Janse JJ, Casacuberta-Partal M, Lamers OAC, Sijtsma JC, de Dood C, Hilt ST, Ozir-Fazalalikhan A, Kuiper VP, Roozen GVT, de Bes-Roeleveld LM, Kruize YCM, Wammes LJ, Smits HH, van Lieshout L, van Dam GJ, van Amerongen-Westra IM, Meij P, Corstjens PLAM, Jochems SP, van Diepen A, Yazdanbakhsh M, Hokke CH, Roestenberg M	EBioMedicine	2023
Causal chemoprophylactic activity of cabamiquine against Plasmodium falciparum in a controlled human malaria infection: a randomised, double-blind, placebo-controlled study in the Netherlands	van der Plas JL, Kuiper VP, Bagchus WM, Bödding M, Yalkinoglu Ö, Tappert A, Seitzinger A, Spangenberg T, Bezuidenhout D, Wilkins J, Oeuvray C, Dhingra SK, Thathy V, Fidock DA, Smidt LCA, Roozen GVT, Koopman JPR, Lamers OAC, Sijtsma J, van Schuijlenburg R, Wessels E, Meij P, Kamerling IMC, Roestenberg M, Khandelwal A.	The Lancet Infectious Diseases	2023
Intradermal delivery of the third dose of the mRNA-1273 SARS-CoV-2 vaccine: safety and immunogenicity of a fractional booster dose	Roozen GVT, Prins MLM, Prins C, Janse JJ, de Gruyter HLM, Pothast CR, Huisman W, Koopman JPR, Lamers OAC, Kuijer M, Myeni SK, van Binnendijk RS, Hartog GD, Heemskerk MHM, Jochems SP, Feltkamp MCW, Kikkert M, Rosendaal FR, Roestenberg M, Visser LG, Roukens AHE	Clinical Microbiology and Infection	2024
Immunogenicity and reactogenicity of intradermal mRNA-1273 SARS-CoV-2 vaccination: a non-inferiority, randomized-controlled trial	Prins MLM, Roozen GVT, Pothast CR, Huisman W, van Binnendijk R, den Hartog G, Kuiper VP, Prins C, Janse JJ, Lamers OAC, Koopman JPR, Kruithof AC, Kamerling IMC, Dijkland RC, de Kroon AC, Azimi S, Feltkamp MCW, Kuijer M, Jochems SP, Heemskerk MHM, Rosendaal FR, Roestenberg M, Visser LG, Roukens AHE	NPJ Vaccines	2024

Title	Author	Journal	Year
Clinical tolerance but no protective efficacy in a placebo-controlled trial of repeated controlled schistosome infection	Koopman JPR, Houlder EL, Janse JJ, Lamers OAC, Roozen GVT, Sijtsma JC, Casacuberta-Partal M, Hilt ST, van der Stoep MYEC, van Amerongen-Westra IM, Brienen EA, Wammes LJ, van Lieshout L, van Dam GJ, Corstjens PL, van Diepen A, Yazdanbakhsh M, Hokke CH, Roestenberg M	The Journal of Clinical Investigations	2024
Safety and Efficacy of Immunization with a Late-Liver-Stage Attenuated Malaria Parasite	Lamers OAC, Franke-Fayard BMD, Koopman JPR, Roozen GVT, Janse JJ, Chevalley-Maurel SC, Geurten FJA, de Bes Roeleveld HM, Iliopoulou E, Colstrup E, Wessels E, van Gemert GJ, van de Vegte-Bolmer M, Graumans W, Stoter TR, Mordmüller BG, Houlder EL, Bousema T, Murugan R, McCall MBB, Janse CJ, Roestenberg M	New England Journal of Medicine	2024
What motivates SARS-CoV-2 vaccine trial participants? A pre- and post-participation survey study	Lamers OAC, Roestenberg M, de Vries MC, Hoogerwerf MA	Trials	2024
Single immunization with genetically attenuated PfΔmei2 (GA2) parasites by mosquito bite in controlled human malaria infection: a placebo-controlled randomized trial	Roozen GVT, van Schuijlenburg R, Hensen ADO, Koopman JPR, Lamers OAC, Geurten FJA, Sijtsma JC, Baalbergen E, Janse JJ, Chevalley-Maurel S, Naar CM, Bezemer S, Kroeze H, van de Stadt HJF, de Visser B, Meij P, Tihaya MS, Colstrup E, Iliopoulou E, de Bes-Roeleveld HM, Wessels E, van der Stoep MYEC, Janse CJ, Murugan R, Franke-Fayard BMD, Roestenberg M	Nature Medicine	2025
The path from early- to late-liver stage arresting genetically attenuated parasites as a malaria vaccination strategy	Lamers OAC, Franke-Fayard BMD, Roestenberg M, Krol JMM	NPJ Vaccines	2025
Correlative humoral and cellular immunity to genetically attenuated malaria parasites in humans	Colstrup E, Nakajima R, Krol JM, Lamers OAC, de Assis RR, Jain A, Jasinskas A, Iliopoulou E, de Bes-Roeleveld HM, Franke-Fayard BMD, Roestenberg M.	iScience	2025

Table 3. Overview of courses completed

Course	Year
BROK	2020, requalification in 2024
Leiden University Onboarding Programme Inform & Connect	2022
Basic methods and reasoning in Biostatistics	2022
Communication training	2022
Job orientation training	2024

Table 4. Overview of conferences attended

Conference	Year	Presentation
British Society of Parasitology Spring Meeting	2023	Poster and pitch
Dutch Society for Parasitology Spring Meeting	2023	Presentation
Dutch Malaria Day	2023	Presentation
BioMalParXX	2024	Presentation
Travelers' medicine	2024	Not applicable
Regional training evening on infectious diseases	2024	Not applicable
Dutch internist days	2025	Presentation

Nederlandse samenvatting

Dit proefschrift beschrijft de ontwikkeling en het testen van de genetisch verzwakte malariaparasiet GA2. Daarnaast werden de motivatie en het besluitvormingsproces van vrijwilligers, die aan vaccinonderzoek deelnamen, onderzocht.

Malaria

Malaria is een ernstige ziekte die door de *Plasmodium* parasiet wordt veroorzaakt en door vrouwelijke *Anopheles* muggen wordt overgedragen.

Er zijn vijf soorten *Plasmodia* die bij mensen tot malaria kunnen leiden: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* en *Plasmodium knowlesi*. *Plasmodium falciparum* (*Pf*) is veruit de meest voorkomende en virulente soort en dus ook het onderwerp van deze scriptie.

De parasieten worden door muggen in de huid geïnjecteerd, migreren vervolgens naar de lever waar ze na ongeveer zeven dagen merozoïten produceren Deze komen dan in de bloedbaan waar ze rode bloedcellen infecteren. Terwijl het leverstadium asymptomatisch is, wordt het bloedstadium door de typische symptomen van malaria, zoals koorts en malaise, gekenmerkt.

Ondanks de vele pogingen om de ziekte uit te roeien, komt malaria nog steeds veel voor: in 2023 alleen waren er 263 miljoen ziekte- en 597.000 sterfgevallen. Kinderen onder de vijf jaar en zwangere vrouwen zijn het meest getroffen. Hoewel de incidentie van malaria over de laatste decennia geleidelijk is gedaald, komt het sinds 2019 weer vaker voor. De negatieve effecten van de SARS-CoV-2-pandemie op preventieve maatregelen, in combinatie met de toenemende resistentie van parasieten tegen antimalaria medicatie en van muggen tegen insecticiden, zijn mogelijke verklaringen voor deze bevindingen.

Als het ambitieuze WHO-doel om malariasterfte met 90% terug te dringen in 2030 bereikt moet worden, zijn er nieuwe en effectievere bestrijdingsstrategieën nodig. Een goedwerkend vaccin zou niet alleen de symptomen van malaria kunnen verminderen, maar ook de transmissie van de ziekte kunnen onderbreken. Op deze manier zou een vaccin de doorslaggevende stap kunnen zijn voor de uitroeiing van malaria.

Vaccins tegen malaria

De enige twee WHO-goedgekeurde malaria vaccinkandidaten zijn RTS,S/A01 (merknaam Mosquirix) en R21, allebei gebaseerd op het grootste en meest voorkomende eiwit op het oppervlak van de parasiet. De bescherming van RTS,S/A01 varieert tussen 30% en 50% bij zuigelingen en kinderen en neemt in de loop van de tijd af. De werkzaamheid van R21 is iets hoger en ligt na één jaar tussen 68% en 75%. Ondanks deze veelbelovende resultaten is er momenteel echter onvoldoende bewijs om te concluderen dat R21 op de lange termijn beter werkt dan RTS,S/A01.

Een ander type vaccin tegen malaria zijn levend verzwakte parasieten, die in onderzoekssettings tot 100% bescherming kunnen bieden. Levende parasieten kunnen op verschillende manieren verzwakt worden, zoals door straling, toediening van medicijnen of genetische modificatie.
Door straling verzwakte parasieten overleven 24-48 uur in de mens en zijn veilig, maar hebben hoge doseringen nodig om effectief te zijn en de resultaten blijven suboptimaal in endemische gebieden. Parasieten die samen met medicijnen worden toegediend hebben tot nu toe de hoogste effectiviteit bereikt, maar hebben ook het nadeel dat ze wel degelijk symptomen kunnen veroorzaken aangezien ze meestal pas in het bloedstadium door de medicatie gedood worden.
Het verschil in effectiviteit tussen door bestraling of door medicatie verzwakte parasieten, laat zien dat hoe langer de levenscyclus van de parasiet in de gastheer is, hoe sterker de reactie van het immuunsysteem en hoe beter de bescherming tegen malaria zal zijn. Genetisch verzwakte parasieten zijn een derde soort levend vaccin dat potentieel de voordelen van de eerder genoemde immunisatie strategieën kan combineren zonder de nadelen ervan. Het idee is dat, door genetische verzwakking, parasieten zo gemaakt kunnen worden dat de levenscyclus op elk moment onderbroken kan worden, dus ook aan het einde van het leverstadium en voor het bereiken van het bloedstadium.

Gecontroleerde humane infecties

Het testen van zowel nieuwe antimalaria middelen als ook vaccinkandidaten tegen de ziekte gebeurt vaak door middel van gecontroleerde humane malaria infecties. Hierbij worden gezonde vrijwilligers aan de beten van vijf geïnfecteerde malariamuggen blootgesteld en vervolgens nauwlettend gecontroleerd om ze tijdig te behandelen en symptomen te beperken.
Door de effectiviteit van een nieuw middel in een vroeg stadium door een gecontroleerde humane infectie te onderzoeken, is het mogelijk om tijd en geld te besparen. Ook kunnen gecontroleerde humane infecties nuttig zijn wanneer geen adequate dierlijke modellen bestaan of wanneer de incidentie van een ziekte te laag is om conventioneel onderzoek uit te voeren.

GA2 parasiet

GA2 is een nieuwe genetisch verzwakte parasiet, die in het late leverstadium dood gaat: hij mist het *mei2* gen, waardoor de vorming van merozoïten aan het einde van het leverstadium voorkomen wordt. Na uitgebreide preklinische testen (**Hoofdstuk 5**), is GA2 voor het eerst in mensen onderzocht, zoals in **Hoofdstuk 1** beschreven. De beschermende werking van GA2 werd ook met die van een eerder ontwikkelde parasiet, GA1, en een placebo, oftewel nep-immunisatie, vergeleken. GA2 bleek veilig en had

weinig bijwerkingen. Ook de beschermende werking van GA2 was veelbelovend. Na drie toedieningen van 50 GA2-geinfecteerde muggenbeten, waren 8/9 (89%) van de GA2-geimmuniseerde deelnemers tegen een gecontroleerde humane malaria infectie beschermd, terwijl dit maar in 1/8 (13%) en 0/3 (0%) van de GA1- en nep-geïmmuniseerde deelnemers het geval was. Deze resultaten zijn later ook door een tweede studie, beschreven in **Hoofdstuk 6**, bevestigd. Zelfs wanneer een lagere dosering van de GA2 parasiet, namelijk een eenmalige blootstelling van 50 muggenbeten, wordt gebruikt om gezonde deelnemers te vaccineren blijft de effectiviteit tegen malaria 90%.
Op immunologisch gebied, hadden GA2-geimmuniseerde deelnemers significant meer cytokine-producerende $CD4^+$ en $V\delta2$ $\gamma\delta$ T cellen, wat wijst op een rol van deze cellen in bescherming tegen malaria.
In **Hoofdstuk 2** wordt de GA2 dan in een breder perspectief geplaatst waarbij de ontwikkeling van genetisch verzwakte parasieten wordt samengevat. Het grootste verschil tussen GA2 en zijn voorlopers is dat deze parasiet zijn ontwikkeling aan het einde van het leverstadium onderbreekt. Toekomstig onderzoek zal zich dan ook met name op genetisch verzwakte parasieten richten die een langere ontwikkeling doormaken.

Het belang van deelnemers voor klinisch onderzoek

Geen van het dusver beschreven onderzoek zou mogelijk geweest zijn zonder gezonde vrijwilligers. Er is veel ethische discussie rondom de deelname van gezonde vrijwilligers aan klinisch onderzoek omdat zij geen voordeel hiervan ervaren met uitzondering van een vergoeding. Het argument wat vaak aangedragen wordt is dat deelnemers financieel gemotiveerd zijn en dus de risico's van het onderzoek mogelijk over het hoofd zouden kunnen zien. Om deze redenen zijn in **Hoofdstuk 3** de motivaties en het besluitvormingsproces van deelnemers van klinisch onderzoek geanalyseerd. De focus lag specifiek op de motivaties van deelnemers van SARS-CoV-2 vaccinatie studies. Het bleek dat deze doelgroep vooral aan onderzoek deelnam om anderen te helpen en aan de wetenschap bij te dragen. Hoewel in eerdere studies soortgelijke motivaties ook al naar voren waren gekomen, was dit nooit in deze mate. We hebben een model ontwikkelen dat deze verschillen probeert te verklaren. Er zijn drie factoren die van invloed kunnen zijn op iemands bereidwilligheid om aan een klinische studie deel te nemen: de beloning, de kosten van niets doen en het risico van het onderzoek. Zolang de eerste twee opwegen tegen de derde, zal de beslissing om aan een studie deel te nemen positief uitvallen, negatief als het omkeerde waar is. De pandemie was een uitzonderlijke situatie, waarbij de beloning van deelname niet alleen financieel was, maar ook het toegevoegde voordeel had dat mensen eerder gevaccineerde konden worden. Tevens waren de kosten van niets doen hoog, gezien de hele maatschappij onder de beperkingen van lockdowns leed.

Het risico van deelname werd over het algemeen als laag ingeschat. De opsomming van bescherming tegen de ziekte in de vorm van een vaccinatie en het perspectief op het opheffen van lockdowns woog dus op tegen het relatief lage risico van participatie.
Concluderend, biedt dit model een hulpmiddel om de motivaties van deelnemers van klinische studies te analyseren die gebaseerd op de externe omstandigheden kunnen variëren.

Hoofdstuk 4 is een opiniestuk over de ethische verschillen tussen gecontroleerde human infecties en fase 1 studies. We beargumenteren dat er ethisch gezien geen verschil is tussen deze soorten onderzoek. Hoewel deelnemers van gecontroleerde humane infecties soms meer symptomen kunnen ervaren, zijn deze makkelijker te voorspellen en beter te behandelen. First-in-human fase 1 studies behouden, ondanks uitgebreid preklinisch onderzoek, altijd een mate van onzekerheid en de kans op ernstige bijwerkingen.
Wat gecontroleerde humane infectie studies van fase 1 klinische studies onderscheid, is dat er een kans op besmetting van niet-deelnemende derden is. Om deze redenen moeten vrijwilligers van gecontroleerde humane infecties soms in quarantaine blijven totdat ze behandeld zijn.
We identificeren de kans op besmetting van derden dus als een mogelijk verschil tussen gecontroleerde humane infectie studies en traditionele fase 1 klinische studies. Derhalve presenteren wij ook een samenvatting van de meest belangrijke vragen die beantwoord moeten worden om een gecontroleerde humane infectie op een veilige manier te laten verlopen.

Curriculum vitae

Olivia Aurelia Catherina Lamers was born on the 18th of January 1994 in Freiburg im Breisgau, Germany. She went to school in Germany and Italy, graduating from high school in 2012. She then moved to The Netherlands where she enrolled in a Liberal Arts and Sciences bachelor with a focus on life sciences at Maastricht University College, The Netherlands. As part of the programme she spent a semester at the University of Sydney, Australia. She graduated in 2015.

Given her desire to combine basic research with medical care, she pursued the Selective Utrecht Medical Master (SUMMA), from which she graduated in 2020. Her master thesis focussed on post-operative opioid use in Netherlands and the United States of America, the findings of which were published in *Pain Management* and *.Anesthesia & Analgesia.* During her master's, Olivia took part in a global health course, which ignited her interest for neglected tropical diseases and specifically malaria. She then proceeded to do a Ph.D. on the topic of malaria in Prof. Dr. Meta Roestenberg's group at Leiden University Medical Center in Leiden, The Netherlands, the results of which are presented in this thesis.

List of publications

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14. Colstrup, Emil, et al. “Correlative humoral and cellular immunity to genetically attenuated malaria parasites in humans.” iScience 28.6 (2025).

Acknowledgements

Thank you Meta, for you gave me the opportunity to do my PhD in your group and introduced me to clinical research. It has been a rollercoaster of emotions at times, but I cannot deny that this experience has taught me so much. From the importance of following my intuition to being resilient, from asking questions to digging below the surface, this and more I owe to you.

Thank you Blandine and Gopal for the supervision and teaching me the importance of taking responsibility.

Thank you Ed, Anna, Jelte and Marie-Astrid for your guidance and support, I will forever be grateful.

Thanks to everyone who contributed to the GA2 clinical trial, first and foremost the participants. Thanks to Ellen and Joost for giving me a place to stay in Leiden. Thanks to Jos, Kitty and Petra for teaching me how to draw blood and always helping with the clinical trials. Thank you Vincent (the first) for your efficiency and professionality and for introducing me to the world of controlled human malaria infections. Thank you Jacqueline for being the clinical trial manager. Thanks to the team in Nijmegen, especially Matthew, Geert-Jan and Benjamin.

Thank you Victor and Ingrid for the friendly, pleasant collaboration at the CHDR.

Thank you Emma, Cynthia, Emil, Eveline, Laura (Pattacini), Vincent (the second), Mirte, Chris, Sev, Lili, Annie, Claudia, Hans, Nik: we might not have worked together much, but you were always nice to me.

Thanks to my students, Eveline, Pauline and Stefanie for teaching me to teach.

Thank you Chanel, Roos and Fiona for your enthusiasm, for it is truly contagious.

Thank you Clarize for patiently showing me how to dissect mosquitoes and to use the confocal microscope (to no avail in the end, I fear).

Thank you Els for indulging my love for bees, bumbles, snails and all the small wonders of nature.

Thank you Miriam for your kindness and generosity, you always made me feel welcome.

Thank you Geert for showing me the nuances, not everything is black, red and green after all.

Thank you Joseph for stimulating my creativity and teaching me how to deal with mice.

Thank you Jeroen and Laura (de Bes-Roeleveld), for providing sporozoites and designing FACS panels, without you much of the research in this thesis would not have been possible. Thank you for conversations about life and teaching me all there is to know about small kids. Thank you for being the most cheerful and wonderful colleagues.

Thank you Eva, JP, Annefleur and Sascha for making everything better. Eva you truly are the best neighbour I could have wished for, smart and wise and silly in just the right way. JP, I am not sure where I would be without your unwavering intellectual and moral support. Annefleur thank you for your stories (you really do tell the best stories), for putting fierceness and passion in everything you do and for helping me navigate the uncertainties in life. Sascha, our conversations about literature and cinematography always cheered me up and your kind words were patches to my soul in times of need.

Thanks to my parents for the terrible advice and for the endless support.

Thank you Erik. Even though I have tried many times, I don't think that my love and gratitude can be put into words. You are my safe haven in the storms of life, my compass when I have lost direction, you bring peace to the turmoil of my mind. In everything I create there is a little bit of you and this thesis is no different.