Controlled human infection models as a tool for malaria and schistosomiasis vaccine research

Langenberg, M.C.C.

CHAPTER 4

A double-blind, placebo-controlled phase 1/2a trial of the genetically attenuated malaria vaccine PfSPZ-GA1

Meta Roestenberg*, Jona Walk*, Saskia C. van der Boor**, **Marijke C. C. Langenberg****, Marie-Astrid Hoogerwerf, Jacqueline J. Janse, Mikhael D. Manurung, Xi Zen Yap, Amanda Fabra-García, Jan Pieter R. Koopman, Pauline Meij, Els Wessels, Karina Teelen, Youri M. van Waardenburg, Marga van de Vegte-Bolmer, Geert-Jan van Gemert, Leo G. Visser, André J. A M van der Ven, Quirijn de Mast, KC Natasha, Yonas Abebe, Tooba Murshedkar, Peter F. Billingsley, Thomas L. Richie, B. Kim Lee Sim, Chris J. Janse, Stephen L. Hoffman, Shahid M. Khan***, Robert W. Sauerwein***

, **, * These authors contributed equally*

Sci Transl Med. 2020 May 20;12(544):eaaz5629. doi: 10.1126/scitranslmed.aaz5629

Abstract

Immunization with attenuated *Plasmodium* sporozoites can induce protection against malaria infection, as shown by *Plasmodium falciparum* (Pf) sporozoites attenuated by radiation in multiple clinical trials. As alternative attenuation strategy with a more homogeneous population of Pf sporozoites (PfSPZ), genetically engineered *Plasmodium berghei* sporozoites (SPZ) lacking the genes *b9* and *slarp* induced sterile protection against malaria in mice. Consequently, PfSPZ-GA1 Vaccine, a Pf identical double knockout (PfΔ*b9*Δ*slarp*), was generated as a genetically attenuated malaria parasite vaccine and tested for safety, immunogenicity, and preliminary efficacy in malaria-naïve Dutch volunteers. Dose-escalation immunizations up to 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine were well tolerated without breakthrough blood-stage infection. Subsequently, groups of volunteers were immunized three times by direct venous inoculation with cryopreserved PfSPZ-GA1 Vaccine (9.0×10^5 or 4.5×10^5 PfSPZ, $N = 13$ each), PfSPZ Vaccine (radiation-attenuated PfSPZ, 4.5×10^5 PfSPZ, $N = 13$), or normal saline placebo at 8-week intervals, followed by exposure to mosquito bite controlled human malaria infection (CHMI). After CHMI, 3 of 25 volunteers from both PfSPZ-GA1 groups were sterilely protected, and the remaining 17 of 22 showed a patency ≥9 days (median patency in controls, 7 days; range, 7 to 9). All volunteers in the PfSPZ Vaccine control group developed parasitemia (median patency, 9 days; range, 7 to 12). Immunized groups exhibited a significant, dose-related increase in anti-Pf circumsporozoite protein (CSP) antibodies and Pf-specific interferon-γ (IFN-γ)–producing T cells. Although no definite conclusion can be drawn on the potential strength of protective efficacy of PfSPZ-GA1 Vaccine, the favorable safety profile and induced immune responses by PfSPZ-GA1 Vaccine warrant further clinical evaluation.

Introduction

A recent resurgence in *Plasmodium falciparum (*Pf) malaria cases after years of control underscores the need for a highly efficacious vaccine for elimination (1). The Pf circumsporozoite protein (CSP) subunit vaccine RTS,S/AS01E (Mosquirix, GlaxoSmithKline) is the only malaria vaccine to move beyond phase 3 clinical trials, although it provides only short-term and partial clinical vaccine efficacy (2).

In the past decade, there has been a growing interest in attenuated whole Pf sporozoite (PfSPZ) vaccines based on the idea that this whole-organism immunization will be able to induce the protection needed against the breadth of antigens present in the parasite. The first approach to immunizing humans with radiation-attenuated PfSPZ was developed almost 50 years ago (3) and has now been translated to a vaccine, consisting of radiation-attenuated, metabolically active, aseptic PfSPZ that meet regulatory standards for direct venous inoculation (DVI). This product, Sanaria PfSPZ Vaccine, has shown an excellent safety profile in 1595 subjects aged 5 months to 65 years in 20 clinical trials in the United States, Europe, and Africa (4–6). Furthermore, it has provided protection against controlled human malaria infection (CHMI) and malaria infections in the field (4, 7, 8).

Radiation induces random DNA damage in the parasite genome, generating a heterogeneous nonreplicating population of PfSPZ. These PfSPZ invade hepatocytes, partially develop, and then arrest at an early stage in the liver (9). As an alternative to radiation-based attenuation, genetic modification generates a homogeneous formulation of PfSPZ, which stop development in the liver at a well-defined point (10). In rodent models, immunization with genetically attenuated malaria SPZ can induce similar, or even greater, protective immunity compared to radiation-attenuated malaria SPZ (11). The intrinsic and irreversible nature of the genetic attenuation greatly reduces safety risks during manufacturing of PfSPZ. Consequently, several liver-arresting genetically attenuated Pf parasites have been generated (12–14), two of which have been tested for safety in volunteers by mosquito bite (13, 15).

We engineered attenuated PfSPZ by deletion of two genes encoding *slarp* and *b9*, each governing independent and critical processes for successful liver-stage development (12). Pf double-knockout (Pf*Δb9Δslarp*) SPZ were capable of invading primary human hepatocytes *in vitro*, but arrested growth early after invasion and were not detected at days 2 to 7 after infection, similar to PfSPZ Vaccine. Pf*Δb9Δslarp* parasite development was fully abrogated in the liver of humanized mice (12). SPZ of the equivalent rodent *Plasmodium berghei*–attenuated parasite (Pb*Δb9Δslarp*) also showed aborted liver-stage development while retaining the capacity to induce fully protective immunity in both the BALB/c and C57BL/6 mouse models (12). These preclinical data justified formulation and clinical assessment of Pf*Δb9Δslarp*.

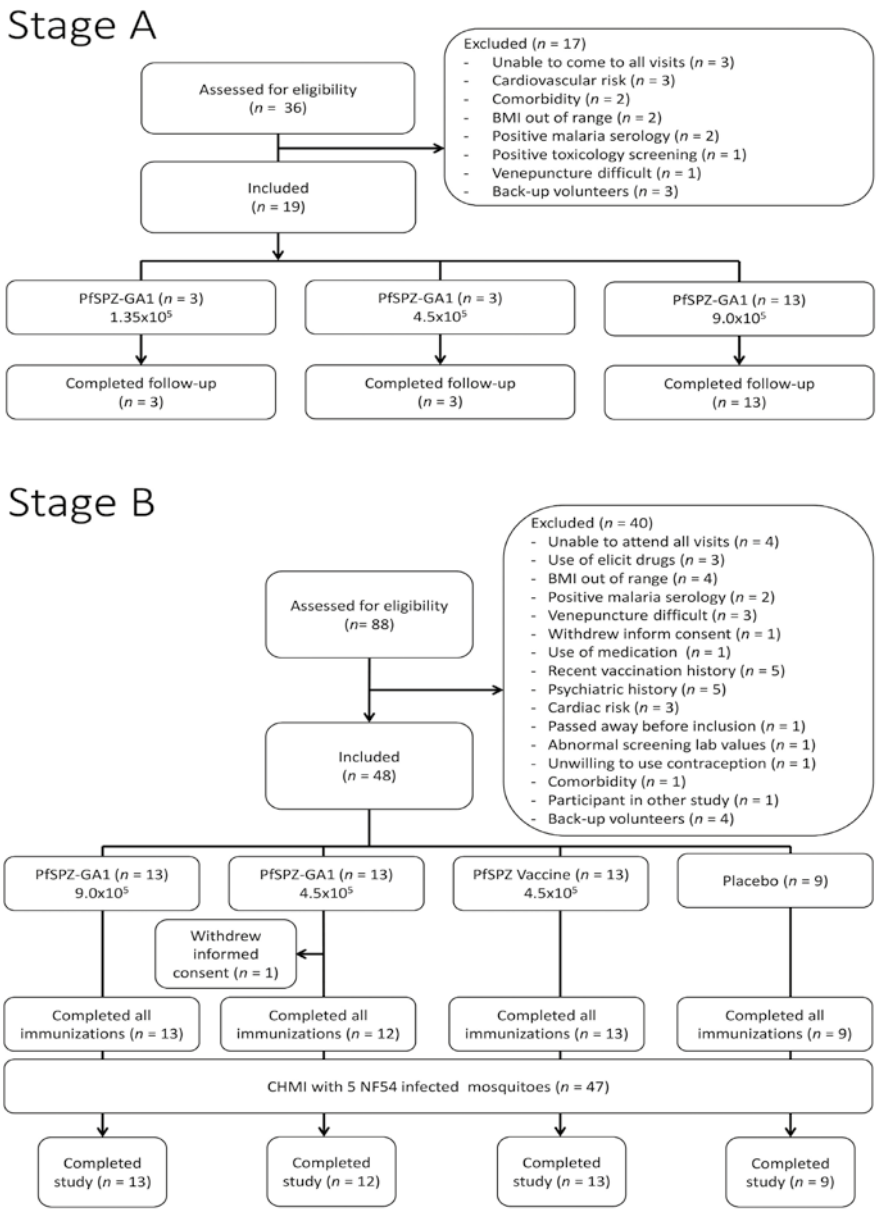

Figure 1. Study flow chart. Manufacture of aseptic, purified, and cryopreserved PfΔ*b9*Δ*slarp* PfSPZ (Sanaria PfSPZ-GA1 Vaccine) was performed in compliance with Good Manufacturing Practice (16). We report the first-in-human evaluation of PfSPZ-GA1 Vaccine (NCT0316121). We tested safety and immunogenicity of PfSPZ-GA1 Vaccine and subsequently examined the protective vaccine efficacy against a homologous CHMI with wild-type (WT) Pf (NF54) and compared this to a previously tested regimen of PfSPZ Vaccine.

Results

Study population

In total, 124 malaria-naïve adults were screened for participation in the study from 1 May to 28 November 2017. Nineteen volunteers were selected as volunteers in the safety dose-escalation stage A of the study, and 48 were selected for the immunogenicity and preliminary efficacy stage B. In addition, six backup volunteers were enrolled in stage B to replace any dropouts before immunization. One volunteer withdrew informed consent after the second immunization in stage B of the trial for reasons unrelated to the trial; all others completed follow-up (Fig. 1). In total, 34 of 67 (51%) were males. Mean age of the volunteers was 23 years old (SD, 4; range, 18 to 34), and mean body mass index (BMI) was 23.5 kg/m2 (SD, 3.0; range, 18 to 30) (Table 1). Volunteers in stage A were immunized once with escalating doses up to 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine, whereas in stage B volunteers were randomized double blind to receive three doses of 4.5×10^5 or 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine, 9.0×10^5 PfSPZ of PfSPZ Vaccine, or saline placebo at 8-week intervals.

Table 1. Volunteer demographics.

		STAGE A			STAGE B	Total
		Group A1	Group A2	Group A3		
Number of volunteers		3	3	13	48	67
Age (years)	Mean	23	25	23	23	23
	SD	3	3	5	3	4
	Median	23	26	23	23	23
	Min, Max	20, 26	20, 29	18, 34	18, 33	18, 34
Sex	Male	1	2	5	26	34
	Female	2	1	8	22	33
BMI	Mean	25.3	23.9	23.6	23.3	23.5
	SD	0.7	2.7	2.9	3.1	3.0
	Median	25.1	24.9	23.6	22.5	23.4
	Min, Max	25, 26	18, 26	19, 29	18, 30	18, 30

Safety results

No serious adverse events occurred during this trial. None of the blood samples taken for blood-stage infection at any time point after DVI of 1.35×10^5, 4.5×10^5, or 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine in stage A and after any of three immunizations with 4.5×10^5 or 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine in stage B were positive for parasite DNA. Blood samples were tested for erythrocytic-stage parasites by quantitative polymerase chain reaction (qPCR) every day from day 6 to day 21 after immunization and on day 28 in stage A and on day 14 after each immunization in stage B (Fig. 2).

All immunizations with PfSPZ-GA1 Vaccine and PfSPZ Vaccine were well tolerated, and there were no significant differences in incidence or severity of adverse events between vaccine and placebo groups in stage B. A total of 66 related adverse events were reported after immunization (table S1). DVI was successful after a single needle stick in 93% of injections (151 of 162 injections), and after three attempts, all but one DVI was successful. Volunteers reported no or mild pain during injection; only one volunteer reported severe pain once for a few seconds during needle insertion. Bruising after DVI was the most commonly reported local adverse event, occurring in 7 of 67 (10%) of volunteers. Headache and fatigue/malaise were the most frequently reported systemic adverse events (reported by 31 and 14 volunteers, respectively) in both intervention and placebo groups, of which three events were severe. One severe unsolicited adverse event probably related to immunization occurred when a volunteer experienced a vasovagal reaction during immunization. There were no clinically significant laboratory abnormalities.

The most common adverse events after CHMI with Pf WT NF54 were headache (52% of volunteers) and fatigue (51%). One volunteer (placebo group) reported severe chills 2 days after atovaquone/proguanil treatment for blood test–positive Pf malaria. Two volunteers (placebo group and PfSPZ Vaccine group) reported severe dizziness on the first and third day of atovaquone/proguanil treatment. All adverse events resolved without sequelae.

There were two cases of mild (grade 1) highly sensitive troponin T elevation to a maximum of 19 ng/ml (reference, <14 ng/ml) 10 to 12 days after CHMI at the time when blood samples were positive for Pf by qPCR: one (9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine group) deemed probably related and one (4.5×10^5 PfSPZ of PfSPZ-GA1 Vaccine) deemed possibly related to CHMI. Both volunteers were asymptomatic, and electrocardiogram did not show abnormalities. Both volunteers were treated with atovaquone/proguanil on the first day of troponin elevation, at which time blood samples were positive for Pf. The highly sensitive troponin T concentration decreased to normal range within a day, and volunteers experienced no sequelae.

Protective efficacy against CHMI

To obtain a preliminary measure of PfSPZ-GA1 vaccine efficacy (VE), the immunized volunteers in the stage B study underwent CHMI with Pf NF54 WT parasites by mosquito bite 3 weeks after the final immunization. The volunteers were monitored on a daily basis, and blood samples were tested for the presence of parasites by qPCR (Fig. 2). Although the primary endpoint of proportion protected was not significantly different between any vaccine groups and the placebo control group, all vaccine groups showed a significant delayed time to positive qPCR as compared to the placebo (Fig. 3; log-rank test, $P = 0.0003$). All volunteers in the placebo group developed parasitemia, with a median of 7 days after CHMI (seven volunteers at day 7, one at day 8, and one at day 9). All 13 volunteers immunized with the control 4.5×10^5 PfSPZ of PfSPZ Vaccine developed parasitemia, with a median delay of 2 days (median, 9; range, 7 to 12 days, compared with placebo Mann-Whitney, $P = 0.0078$). After CHMI, 3 of 25 volunteers from both PfSPZ-GA1 groups were sterilely protected. Immunization with 4.5×10^5 PfSPZ of PfSPZ-GA1 Vaccine resulted in 11 of 12 volunteers developing blood-stage parasitemia, with a median delay of 2 days (median, day 9; range, 7 to 12 days, compared with placebo Mann-Whitney, $P = 0.0005$). In the highest-dose PfSPZ-GA1 group, 11 of 13 volunteers became qPCR positive, with a median 4-day delay (median prepatent period, 11 days; range, 7 to 12 days, compared with placebo Mann-Whitney, $P = 0.0018$). This study was not powered to detect significant differences in time to positive qPCR between vaccine groups.

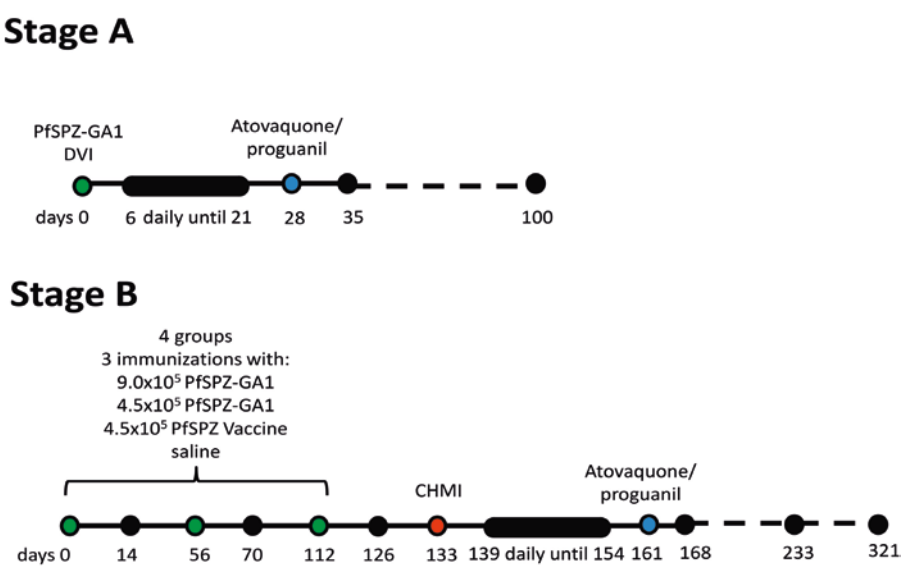

Figure 2. Study design. In stage A study, volunteers were immunized by direct venous inoculation (DVI) with either 1.35 × 10^5, 4.5 × 10^5, or 9.0 × 10 PfSPZ of PfSPZ-GA1 Vaccine (*n* = 3, 3, or 13, respectively; green circle), after which blood samples were taken on a daily basis from day 6 until day 21 (black circles). At day 28 (blue circle), all volunteers were treated with a curative regimen of atovaquone/proguanil. Final visits were at days 35 and 100. In stage B study, four groups of volunteers were immunized three times (green circles) with either 4.5 × 10^5 (*n* = 13) or 9.0 × 10^5 PfSPZ (*n* = 13) of PfSPZ-GA1 Vaccine or 4.5 × 10^5 PfSPZ of PfSPZ Vaccine (*n* = 13) or saline placebo *(n* = 9) by DVI, with blood samples taken for blood-stage parasitemia at day 14 after every immunization. CHMI by mosquito bite with WT Pf NF54 (red circle) was performed 3 weeks after the final immunization, after which daily follow-up was performed from day 139 to day 154. All volunteers received curative treatment with atovaquone/proguanil (blue circle) and came for three final follow-up visits.

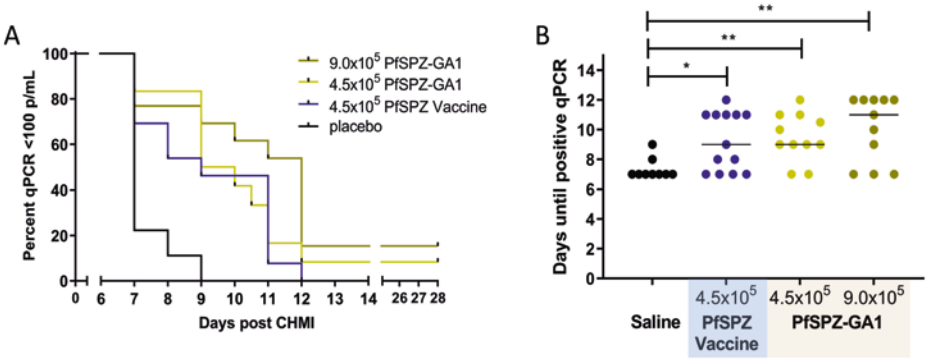

Figure 3. Parasitemia after CHMI. (**A**) Kaplan-Meier showing number of volunteers without blood-stage parasitemia as measured by qPCR between 0 to 28 days after CHMI (log-rank, *P* = 0.0003) and (**B**) days until patency [qPCR > 100 p/ml, for the 9.0 × 10^5 PfSPZ-GA1 Vaccine (dark green), 4.5 × 10^5 PfSPZ-GA1 Vaccine (green), 4.5 × 10^5 PfSPZ Vaccine (blue), and placebo group (black)]. Lines indicate median. (B) shows significance by Mann-Whitney test: **P* < 0.05 and ***P* < 0.01.

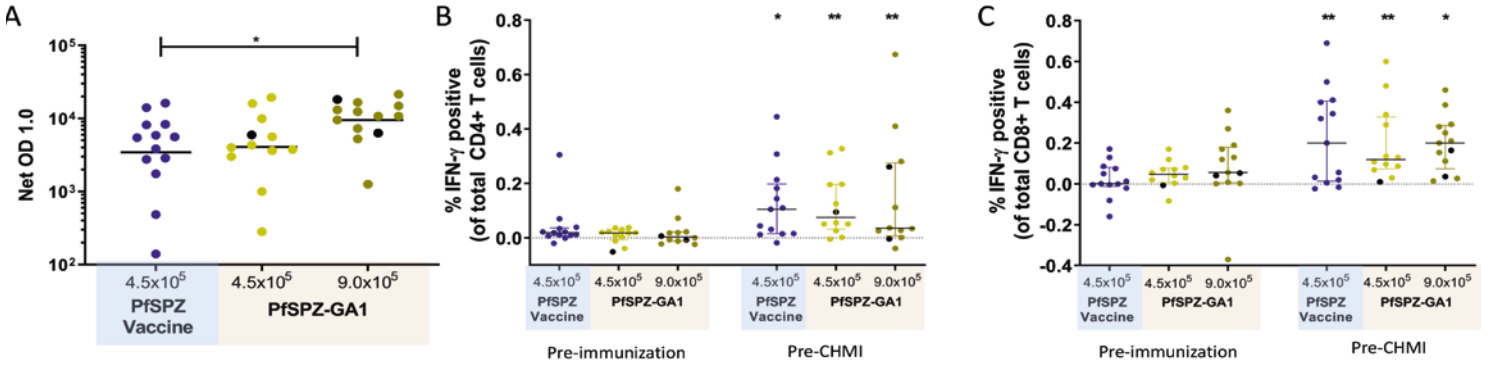

Figure 4. Immune responses after three vaccine doses. (**A**) Anti-PfCSP antibody titers as determined by ELISA for the 9.0 × 10^5 PfSPZ-GA1 Vaccine (dark green), 4.5 × 10^5 PfSPZ-GA1 Vaccine (green), and 4.5 × 10^5 PfSPZ Vaccine (blue) groups 14 days after the final immunization. Fully protected volunteers shown in black lines indicate geomeans. One-way ANOVA post hoc Tukey: **P* < 0.05. Number of (**B**) IFN-γ–producing $CD4^+$ and (**C**) $CD8^+$ T cells determined by flow cytometry after stimulation with infected RBC before immunization and the day before CHMI for the 9.0 × 10^5 PfSPZ-GA1 Vaccine (dark green), 4.5 × 10^5 PfSPZ-GA1 Vaccine (green), 4.5 × 10^5 PfSPZ Vaccine (blue), and placebo group (black). Fully protected volunteers are displayed in black. Lines indicate medians with interquartile range, and dotted line indicates zero response. Paired *t* test of pre-CHMI data with pre-immunization data: **P* < 0.05 and ***P* < 0.01.

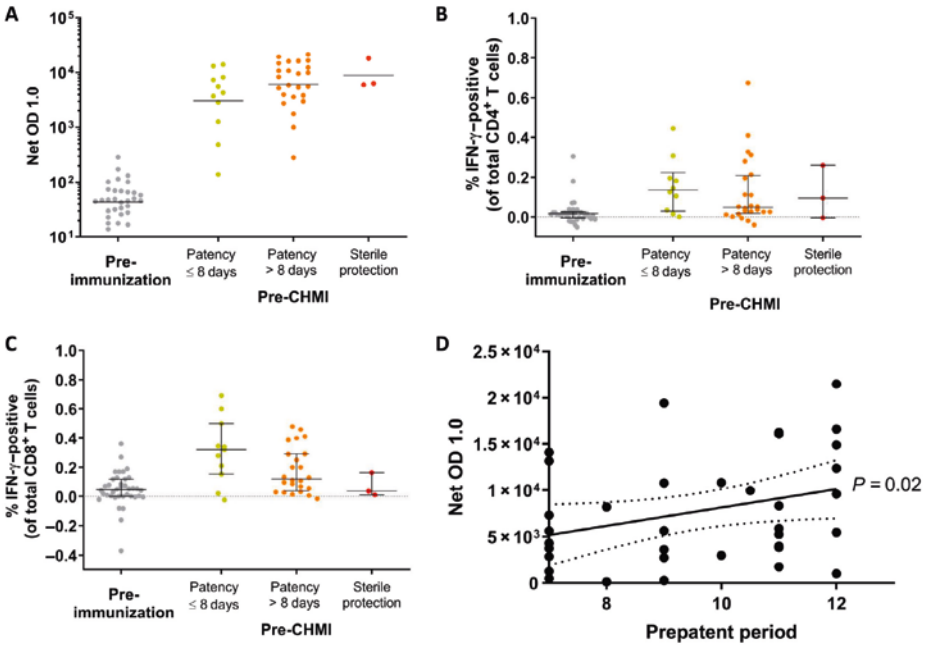

Figure 5. Relationship between immune parameters and protection. (**A**) Relationship between anti-PfCSP antibody titers (lines indicate geomean), % of (**B**) IFN-γ–producing $CD4^+$, or (**C**) $CD8^+$ T cells (lines indicate median and interquartile ranges) and the protection status of volunteers. Protection grouped by day of positive qPCR (patency) ≤8 or >8 or "sterile" if qPCR negative until day 28. One-way ANOVA, P = 0.05. (D) Correlation of anti-PfCSP antibody titer 14 days after the final immunization with prepatent period. Pearson correlation P = 0.02 and r = 0.32.

Immunogenicity

All immunized groups showed a significant increase in antibody titers against PfCSP between pre-immunization and pre-CHMI time points ($P < 0.0001$, paired t test overall; Fig. 4A). Immunization with 4.5×10^5 PfSPZ of PfSPZ Vaccine and PfSPZ-GA1 Vaccine induced similar anti-PfCSP antibody titers, whereas immunization with 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine produced significantly higher anti-PfCSP titers [Fig. 4A; one-way analysis of variance (ANOVA) Tukey post hoc mean difference, 5573; 95% confidence interval (CI), 332 to 30,823; $P = 0.04$].

Peripheral blood cells from all immunized groups exhibited a significant increase in $CD4^+$ and $CD8^+$ T cells producing interferon-γ (IFN-γ) upon stimulation with Pf-infected red blood cells after three immunizations (PfRBC; Fig. 4, D and E; $P < 0.03$) as compared to baseline. In total, 35 and 32% of all immunized volunteers were IFN-γ responders for CD4+ or CD8+ T cells, respectively, with 46% of volunteers showing an increase in at least one subset.

To examine whether there was an association between an increase in antibody or cellular responses and protection, data from immunized individuals were segregated on the basis of protection status (Fig. 5A). The anti-PfCSP antibody titers correlated significantly with time until

positive qPCR-based blood-stage patency (Pearson correlation $r = 0.32$; 95% CI, −0.01 to 0.59; $P = 0.02$, $R^2 = 0.1$; Fig. 5D). However, cellular responses did not correlate with protection (one-way ANOVA for patency ≤8 days, >8 days, and full protection, $P = 0.05$).

We thus demonstrate that immunization with three doses of 4.5×10^5 PfSPZ of PfSPZ Vaccine and PfSPZ-GA1 induced similar anti-PfCSP antibody responses and that there was a dose-dependent increase in anti-PfCSP responses after immunization with 9.0×10^5 PfSPZ of PfSPZ-GA1. Moreover, anti-PfCSP antibody responses correlated with protection.

Discussion

Here, we report the first-in-human administration and efficacy data of the live, injectable, nonreplicating, genetically attenuated PfSPZ, PfSPZ-GA1 Vaccine. PfSPZ-GA1 Vaccine was safe and well tolerated, and no blood-stage infections were observed in 45 volunteers after 97 injections, totaling more than 6×10^7 PfSPZ administered by DVI. PfSPZ-GA1 Vaccine was immunogenic and induced both antibody and $CD4^+$ and $CD8^+$ T cell responses, with a potency analogous to the comparator PfSPZ Vaccine. Homologous CHMI through the bites of WT (Pf NF54)–infected mosquitoes 3 weeks after the last immunization resulted in three fully protected individuals and delays in time to patency in 17 of 25 volunteers at both dosages of PfSPZ-GA1 Vaccine. The delay in time to patency correlated with increased anti-PfCSP antibody responses. This study shows that an injectable, double gene deletion attenuated parasite vaccine is safe and immunogenic in humans. Previously, two genetically attenuated parasites (GAPs) have undergone safety evaluation in healthy volunteers in an experimental setting through the bites of infected *Anopheles* mosquitoes (13, 15). One GAP, lacking the two genes *p52* and *p36*, showed a blood-stage infection in a single volunteer after being exposed to 200 infectious bites (15). The breakthrough blood parasites were confirmed as having the *p52* and *p36* gene deletion genotype, indicating that deletion of these two genes was not sufficient to result in a complete growth arrest in the liver stage. This same incomplete attenuation phenotype (at high infection doses) had also been observed in rodent malaria parasites lacking the same genes (17). In a subsequent study, another GAP was analyzed, which additionally included a deletion of the *slarp* gene, also referred to as *sap1*. This triple-knockout GAP (Pf*Δp52Δp36Δsap1*) was administered to healthy volunteers through the bites of 150 to 200 infectious *Anopheles* mosquitoes, and no breakthrough blood infections were observed (13). No protective efficacy studies of the triple-knockout GAP have been reported yet. In contrast to Pf*Δp52Δp36Δsap1*, PfSPZ-GA1 Vaccine was administered as an injectable vaccine.

We found a delay in patency up to day 12 after CHMI as compared to a 7- to 9-day patency in controls, reflecting a 2-log reduction in parasites released from the liver in volunteers vaccinated with both doses of PfSPZ-GA1. However, the interpretation of the vaccine efficacy data is complicated by the unexpected low efficacy of the PfSPZ Vaccine reference group. The dose of the control PfSPZ Vaccine was chosen on the basis of a previous study in which three doses of 4.5×10^5 PfSPZ of PfSPZ Vaccine by DVI protected 13 of 15 volunteers from mosquito bite CHMI 3 weeks after the last immunization (18). The same dose was selected for PfSPZ-GA1 in group 1 to enable a comparison of the two vaccines' immunogenicity. As anticipated when designing the trial, the PfSPZ Vaccine group allows us to put the vaccine efficacy results in perspective of

previous trials with the PfSPZ Vaccine as reference. This difference in vaccine efficacy of PfSPZ Vaccine between the study in the United States and our study may be explained by either (i) differences in the stringency of the CHMI or (ii) differences in the immunogenicity of PfSPZ Vaccine in the two studies. With regard to the first possibility, both studies used mosquito bite–based CHMI at 3 weeks after the last immunization. Mosquito bite CHMI is more similar to natural infections as it includes the possibly relevant SPZ skin stage, where antibodies against SPZ may have an effector function (19, 20). However, in the Epstein *et al*. study (18), the 3D7 clone of the NF54 strain of Pf was used in the CHMI, whereas in this study we used the NF54 strain of Pf, which is not clonal. Small genotypic differences between these two strains have been identified (21), but it remains unclear whether these are relevant and result in differences in prepatent period as observed in independent clinical trials (22). Unfortunately, direct comparisons have not been performed. Because PfSPZ-GA1 was created in an NF54 background, we would expect the NF54 CHMI to be more homologous as compared to the 3D7 CHMI. In addition, the primary parasitological outcome variable differed between the trials (qPCR in The Netherlands and thick smear in the United States), so prepatent periods cannot be directly compared, making it difficult to assess challenge stringency. However, for both strains, five mosquito bites are needed to achieve a virtually 100% infection rate and ultimately cannot account for the lower than expected vaccine efficacy. Thus, we do not think that differences in stringency of CHMI can explain the difference in VE between the two studies. Moreover, we have broad experience with this mosquito bite CHMI model, including studies in which we show 100% vaccine efficacy by the chemoprophylaxis with SPZ approach against homologous Pf NF54 CHMI (23–25).

Both PfSPZ Vaccine and PfSPZ-GA1 Vaccine were immunogenic and induced anti-PfCSP antibodies and PfRBC-specific T cells. There was a positive association between anti-PfCSP antibodies and the protection status of the volunteers as measured by prepatent period. However, less than half of volunteers had significant induction of T cell responses, and $CD8^+$ responses were inverse related to prepatency. Whether this reflects a compartmental shift of $CD8^+$ T cells to nonlymphoid tissues, as observed in animal models (26), will require further study. This is in contrast with other PfSPZ Vaccine studies (7, 8), in which typically most volunteers show induction of $CD4^+$ T cell responses, although $CD8^+$ T cell responses have been variable. Possibly a difference in the *in vitro* cell stimulus (PfRBC versus PfSPZ) could also explain this difference, and therefore, in future studies with GAP vaccines, it would be of importance to also compare these two stimuli. However, anti-PfCSP antibody titers in our study were also significantly lower than those found in the Epstein *et al*. study (18) after immunization with PfSPZ Vaccine using the same schedule and dose of 4.5×10^5 PfSPZ (median level [net optical density (OD), 1.0] at 2 weeks after the third dose of 19,044 versus 5465).

On the basis of these data, we consider a lower vaccine immunogenicity of PfSPZ Vaccine compared to the Epstein *et al*. study (18) to be a likely explanation for the decreased vaccine efficacy of PfSPZ Vaccine observed in our study. However, the true cause of the decreased immunogenicity remains unclear. Retrospective evaluation did not reveal any procedural complications or deviations from established protocols in vaccine transport, storage, or administration. In addition, the trial in our study was performed in two centers, with different teams performing vaccine preparation and administration and yet both showed similar immunogenicity results. Vaccine lot–specific problems also do not seem a likely explanation, given experiences in other sites with parallel trials. Although we observed that PfSPZ-GA1 Vaccine appeared to be as immunogenic as PfSPZ Vaccine, at an equivalent dose, the unexpected low vaccine efficacy of the PfSPZ Vaccine comparator limits our ability to draw firm conclusions on

the VE of PfSPZ-GA1 Vaccine. However, given the suboptimal vaccine efficacy of PfSPZ-GA1, the next-generation genetically attenuated PfSPZ vaccines should aim at enhanced potency either by increasing dose or potentially through an arrest later in the liver stage.

An alternative to Pf gene deletion mutants as whole SPZ vaccine is the use of *Plasmodium* species that are nonpathogenic for humans expressing selected Pf target proteins. Transgenic murine *P. berghei* SPZ expressing the Pf CSP were recently tested in a clinical trial for safety, immunogenicity, and protective efficacy against a CHMI (27).

This study demonstrates that a genetically attenuated, live parasite vaccine, PfSPZ-GA1, can be safely administered to malaria-naïve volunteers by DVI. Genetically attenuated PfSPZ have advantages over other whole PfSPZ vaccination strategies, because they can improve the safety and consistency of manufacturing. Although the potential protective efficacy of PfSPZ-GA1 Vaccine cannot be fully appreciated in this trial, the data show clear immunogenicity combined with a favorable safety and tolerability profile. The current trial underscores the clinical potential of genetically attenuated vaccines, boosting further development of such malaria vaccine strategies.

Materials and Methods

Study design

The study was designed as a multicenter phase 1, open-label, dose-escalating trial to assess safety, tolerability, and immunogenicity of PfSPZ-GA1. In the initial, open-label safety stage of the trial (stage A), single escalating doses of PfSPZ-GA1 were administered by DVI to three groups of healthy adults at the Leiden University Medical Center (LUMC). Group A1 (n = 3) was inoculated with 1.35×10^5 PfSPZ of PfSPZ-GA1 Vaccine, group A2 (n = 3) was inoculated with 4.5×10^5 PfSPZ of PfSPZ-GA1 Vaccine, and group A3 (n = 13) was inoculated with 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine. For this initial proof-of-concept study, a dose of 1.35×10^5 PfSPZ of PfSPZ-GA1 Vaccine was chosen because this is the lowest dose at which PfSPZ Vaccine has shown to induce protective immunity (18), whereas after three doses of 9.0×10^5 PfSPZ Vaccine >90% VE was to be expected. This is based on a study where three doses of 4.5×10^5 PfSPZ induced >80% protection (18). In the follow-on efficacy stage of the trial (stage B), a total of 48 volunteers were included at LUMC (n = 24) and Radboud University Medical Center (RUMC) (n = 24), with double-blind randomization over four study groups according to a randomization list prepared by the study head pharmacist. Randomization was stratified per study site. The investigator, site personnel, and the sponsor were masked to treatment assignment. The site pharmacist or qualified employees were not masked and prepared the assigned vaccines. Groups 1 and 2 received three immunizations with PfSPZ-GA1 Vaccine at doses of 9.0×10^5 (n = 13) and 4.5×10^5 (n = 13) PfSPZ. Group 3 received three immunizations with the control PfSPZ Vaccine at a dose of 4.5×10^5 PfSPZ (n = 13), and group 4 was injected three times with normal saline as placebo (n = 9). All immunizations in stage B were administered at 8-week intervals. Three weeks after the final immunization, all stage B volunteers were exposed to five bites of Pf NF54–infected *Anopheles stephensi* mosquitoes according to previously described procedures to assess vaccine efficacy (28). The primary objective of the study was to investigate the safety and tolerability of PfSPZ-GA1 Vaccine, by analysis of (i) the presence of blood-stage parasites after inoculation and (ii) the frequency and magnitude of adverse events. A

secondary objective was the VE of PfSPZ-GA1 Vaccine against mosquito bite CHMI with Pf NF54 SPZ, as assessed by the presence or absence of parasitemia after CHMI. The presence of blood-stage parasites after inoculation with PfSPZ-GA1 Vaccine and the frequency and magnitude of adverse events after immunization were primary endpoints. The presence of blood-stage parasites after immunization was a stopping criterium.

We calculated a sample size of 13 immunized subjects per group and 9 infectivity controls for the first CHMI to show with a power of 80% that a 50% blood-stage parasite positive rate in the immunized group and 100% in the control group are significantly different ($\alpha < 0.05$, two-tailed), assuming that one subject drops out from the immunized group (final $N = 12$).

The clinical trial was conducted under a U.S. Food and Drug Administration (FDA) Investigational New Drug (IND) application and was approved by the central committee for research involving human subjects in The Hague [Centrale Commissie Mensgebonden Onderzoek (Central Committee on Research Involving Human Subjects) (CCMO; NL56657.000.16)]. It was performed in The Netherlands under a license from the Dutch Ministry of Infrastructure and Environment (Ministerie van Infrastructuur en Milieu) for deliberate release of genetically modified organisms (IM-MV 15-004 and IM-MV 15-009). The study was registered at ClinicalTrials.gov (NCT03163121). Primary data are reported in data file S1.

Production of PfSPZ-GA1

The genetically attenuated Pf NF54 parasite, PfΔ*b9*Δ*slarp* (12), lacks two genes, *b9* and *slarp*, which are vital for liver-stage development (12). Master and working cell banks were generated from the clone Pf NF54 parasite, PfΔ*b9*Δ*slarp*, filed under an FDA Master File and IND application, resulting in the product referred to as PfSPZ-GA1 Vaccine. PfSPZ-GA1 parasites were tested sensitive to the anti-malarial drugs chloroquine, mefloquine, artemether/lumefantrine, atovaquone/proguanil, and pyrimethamine.

Manufacture of PfSPZ-GA1 Vaccine bulk product followed the identical manufacturing schema of PfSPZ (NF54) Vaccine (16) except for several tests of vialed final products that were specific to PfSPZ-GA1 Vaccine. These tests included a PCR test for identity that confirmed the genetic signature of PfΔ*b9*Δ*slarp* (12), the potency assay that documented 3-day parasites that were developed in HCO4 cells *in vitro*, and the 6-day safety assay that confirms the absence of late-stage developing parasites *in vitro*. The manufacturing process generated aseptic *A. stephensi* mosquitoes that were infected with PfΔ*b9*Δ*slarp* (12). PfSPZ were harvested, purified, vialed, cryopreserved, and shipped in liquid nitrogen vapor phase at −150° to −196°C. On the day of administration, vials of PfSPZ-GA1 Vaccine were thawed and diluted using phosphate-buffered saline and 25% human serum albumin (CSL Behring) to the correct dose in a sterile environment.

Participants

A total of 67 healthy malaria-naïve male and female volunteers aged 18 to 35 years were recruited for the study. All included volunteers were in good health as assessed by medical history, physical examination, general chemistry and hematology evaluation, and an electrocardiogram. All included volunteers provided informed consent, and females were counseled to use adequate

contraception. A detailed list of inclusion and exclusion criteria is provided in the Supplementary Materials.

Procedures

Volunteers were immunized by a trained nurse administering 0.5 ml of the vaccine by DVI through a 25-gauge needle. Volunteers were observed for 30 min after every immunization. Local adverse events and pain scores were assessed immediately. In stage A, volunteers visited the trial facility daily from day 6 to day 21 after every immunization to report adverse events and to collect blood samples for assessment of parasites by qPCR. During the immunization period, all volunteers were treated with a curative regiment of atovaquone/proguanil when qPCR was positive for malaria or at day 28 after immunization. Complete blood counts and general chemistry laboratories were performed on days 6, 14, 21, 30, and 35 after immunization. Platelet counts, lactate dehydrogenase, and highly sensitive troponin T tests were performed daily to detect possible myocarditis in an early stage, in line with previously established protocols (29, 30). Blood samples for immunological assays were taken at baseline and at days 6, 14, 21, 28, 35, 100, and 188. In stage B of the clinical trial, visits were on day 14 after every immunization and the day before immunization for safety assessments. Three weeks after the third and final immunization, volunteers were exposed to the bites of five mosquitoes infected with the homologous Pf NF54 strain (CHMI). All mosquitoes were checked for a blood meal and infectivity by dissection (28). Further details on the CHMI with Pf are reported in table S2. After CHMI, volunteers visited the trial center on a daily basis from day 6 to day 21. All volunteers were treated with a curative regimen of atovaquone/proguanil if they were qPCR positive or, alternatively, at day 28 after CHMI. Final visits took place at days 35, 100, and 188 after CHMI. Blood samples for immunological analysis were taken before and 14 days after each immunization, before CHMI, and at days 6, 14, 21, 35, 100, and 188 after CHMI.

Adverse events

Solicited and unsolicited adverse events after DVI were recorded at every visit until 35 days after immunization. Solicited local adverse events were tenderness, induration, bruising/extravasated blood, erythema, swelling, pain, and pruritis. Solicited systemic adverse events were fever, rash, urticaria, pruritis, edema, headache, fatigue, malaise, chills, myalgia, and arthralgia. All volunteers were instructed to fill out a diary card, listing daily temperature and any adverse events up to day 35 after immunization. Causality of all adverse events was assessed by the investigators as definitely related, probably related, possibly related, unlikely related, or not related to the study procedures. In dichotomous analysis, the latter two were regarded as “unrelated” and the first three categories as “related.” All adverse events were graded as mild (grade 1), moderate (grade 2), severe (grade 3), or serious (grade 4). Review of all safety data by an independent safety monitoring committee was performed at 28 days after each immunization in stage A, before continuing dose escalation to the next group and on day 28 after CHMI in stage B.

Blood-stage parasitemia

To examine whether PfSPZ-GA1 were fully attenuated and incapable of establishing a blood-stage infection, blood samples were monitored for parasites by qPCR (31). Blood samples were considered negative if no signal was detected in 50 cycles or the Pf load was <100 Pf/ml. Any sample with a load of >100 Pf/ml was considered positive. Parasite densities were determined with the use of a trendline of standardized control samples between 20 and 10^6 Pf/ml.

Immunology

Exploratory endpoints included immune responses after immunization with PfSPZ-GA1 Vaccine. Antibodies were detected by enzyme-linked immunosorbent assay (ELISA) against PfCSP (32). Cellular immune responses were analyzed using peripheral blood mononuclear cell (PBMC) samples obtained 1 day before the first immunization and 21 days after the third immunization. Cells were isolated using heparin cell preparation tubes according to previously published protocols (32). After thawing, cells were stimulated, as described previously (33). In short, PBMCs were cultured at 2.5×10^6 cells/ml in a final volume of 200µl per well in RPMI 1640 (Dutch Modification; Gibco) with gentamicin (5 mg/ml; Centraform), 100 mM pyruvate (Gibco), 200 mM GlutaMAX (Gibco), and 10% heat-inactivated pooled human A+ serum (Sanquin, Nijmegen, The Netherlands). Cells were stimulated with purified NF54 schizonts (PfRBC) or uninfected red blood cells (uRBC) at a concentration of 2.5×10^6 RBC/ml for 24 hours. Brefeldin A (10 µg/ml; Sigma-Aldrich) and monansin (2 µM; eBioscience) were added during the last 4 hours of stimulation. Cells were stained with fixable viability dye labelled with eFlour780 (eBioscience), CD3–phycoerythrin (PE)–Dazzle549 (BioLegend; clone OKT3), CD4–fluorescein isothiocyanate (FITC) (BioLegend; clone OKT4), CD8-Alexa Fluor 700 (BioLegend; clone HIT8A), pan-γδTCR-PE (Beckman Coulter; clone IMMU510), and CD56 peridinin chlorophyll protein (PerCP)–Cy5.5 (BioLegend; clone HCD56) for 30 min at 4°C. Cells were subsequently permeabilized using Foxp3 fixation/permeabilization buffer (eBioscience) and stained for intracellular cytokines with IFN-γ–PE-Cy7 (BioLegend; clone 4S.B3), interleukin-2 (IL-2)–BrilliantViolet510 (BioLegend; clone MQ1-17H12), and tumor necrosis factor–α (TNF-α)–Alexa Fluor 647 (BioLegend; clone MAb11). Analysis was performed using a Gallios flow cytometer (Beckman Coulter) and FlowJo software (version 10.0.8 for Apple OS). Background cytokine production after stimulation with uRBC was subtracted from PfRBC responses. On an individual level, we defined IFN-γ responders as those volunteers with a percentage increase in IFN-γ–producing cells greater than twice the SD of all pre-immunization samples.

Statistical analysis

Adverse events were evaluated by tabulating according to intention to treat analysis. The proportion of volunteers in each group who reported mild, moderate, or severe adverse events was calculated, and analysis was primarily descriptive. The secondary endpoint of the study was the presence of parasitemia (by qPCR) after CHMI with the (WT) Pf NF54 strain in stage B of the study. Differences between groups were evaluated by log-rank test.

Differences in immunological parameters between groups were assessed by comparing mean values between the groups using one-way ANOVA when comparing several groups or a two-tailed Student's *t* test or nonparametric equivalents. Paired tests were used if pre-exposure values were compared with post-exposure values, and unpaired tests were used if comparisons were made between groups. For discrete variables, the $\chi 2$ test or Fisher's exact test was used (two-tailed). All statistical analyses were performed with SPSS version 23.

Acknowledgments

This article is dedicated to the memory of S. Khan. We thank R. Stoter, W. Graumans, R. Heutink, J. Klaassen, L. Pelser-Posthumus, J. Kuhnen, and A. Pouwelsen for excellent technical assistance with generation of infected mosquitoes and with performing the malaria challenge infection. We thank W. Graumans for parasite molecular characterization and B. Winkel, R. van Schuijlenburg, Y. Kruize, and B. van Rooij for their assistance with the immunology assays. We thank J.-P. Koopman, P. Verbeek-Menken, K. Suijk, R. Nijhuis, L. van Lieshout, J. Fehrmann, and G. Hardeman for their clinical support and K. Bos, R. Hendrikx, A. de Boer, M. Ganesh, C. Feijt, F. Lin, N. al Sader, M. Jore, and R. de Jong for the preparation of the vaccine. We are grateful for the regulatory expertise and support of W. Graumans, G. van Willigen, R. Verbeek, and P. le Brun. We thank the Sanaria Manufacturing Team for PfSPZ-GA1 Vaccine, the Pharmaceutical Operations and Logistics Team and the Clinical Team who collaborated with the clinical sites, and the Sanaria Regulatory Team for their support.

References

1. World Health Organization, *WHO Malaria Report 2019* (World Health Organization, 2019).

2. RTS,S Clinical Trials Partnership, Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: Final results of a phase 3, individually randomised, controlled trial. Lancet 386, 31–45 (2015).

3. T. L. Richie, P. F. Billingsley, B. K. Sim, E. R. James, S. Chakravarty, J. E. Epstein, K. E. Lyke, B. Mordmuller, P. Alonso, P. E. Duffy, O. K. Doumbo, R. W. Sauerwein, M. Tanner, S. Abdulla, P. G. Kremsner, R. A. Seder, S. L. Hoffman, Progress with *Plasmodium falciparum* sporozoite (PfSPZ)-based malaria vaccines. Vaccine 33, 7452–7461 (2015).

4. M. S. Sissoko, S. A. Healy, A. Katile, F. Omaswa, I. Zaidi, E. E. Gabriel, B. Kamate, Y. Samake, M. A. Guindo, A. Dolo, A. Niangaly, K. Niare, A. Zeguime, K. Sissoko, H. Diallo, I. Thera, K. Ding, M. P. Fay, E. M. O'Connell, T. B. Nutman, S. Wong-Madden, T. Murshedkar, A. J. Ruben, M. Li, Y. Abebe, A. Manoj, A. Gunasekera, S. Chakravarty, B. K. L. Sim, P. F. Billingsley, E. R. James, M. Walther, T. L. Richie, S. L. Hoffman, O. Doumbo, P. E. Duffy, Safety and efficacy of PfSPZ Vaccine against *Plasmodium falciparum* via direct venous inoculation in healthy malaria-exposed adults in Mali: A randomised, double-blind phase 1 trial. Lancet Infect. Dis. 17, 498–509 (2017).

5. S. A. Jongo, S. A. Shekalaghe, L. W. P. Church, A. J. Ruben, T. Schindler, I. Zenklusen, T. Rutishauser, J. Rothen, A. Tumbo, C. Mkindi, M. Mpina, A. T. Mtoro, A. S. Ishizuka, K. R. Kassim, F. A. Milando, M. Qassim, O. A. Juma, S. Mwakasungula, B. Simon, E. R. James, Y. Abebe, N. Kc, S. Chakravarty, E. Saverino, B. M. Bakari, P. F. Billingsley, R. A. Seder, C. Daubenberger, B. K. L. Sim, T. L. Richie, M. Tanner, S. Abdulla, S. L. Hoffman, Safety, immunogenicity, and protective efficacy against controlled human malaria infection of *Plasmodium falciparum* sporozoite vaccine in Tanzanian adults. Am. J. Trop. Med. Hyg. 99, 338–349 (2018).

6. A. Olotu, V. Urbano, A. Hamad, M. Eka, M. Chemba, E. Nyakarungu, J. Raso, E. Eburi, D. O. Mandumbi, D. Hergott, C. D. Maas, M. O. Ayekaba, D. N. Milang, M. R. Rivas, T. Schindler, O. M. Embon, A. J. Ruben, E. Saverino, Y. Abebe, N. Kc, E. R. James, T. Murshedkar, A. Manoj, S. Chakravarty, M. Li, M. Adams, C. Schwabe, J. L. Segura, C. Daubenberger, M. Tanner, T. L. Richie, P. F. Billingsley, B. K. L. Sim, S. Abdulla, S. L. Hoffman, Advancing global health through development and clinical trials partnerships: A randomized, placebo-controlled, double-blind assessment of safety, tolerability, and immunogenicity of PfSPZ vaccine for malaria in healthy Equatoguinean men. Am. J. Trop. Med. Hyg. 98, 308–318 (2018).

7. A. S. Ishizuka, K. E. Lyke, A. DeZure, A. A. Berry, T. L. Richie, F. H. Mendoza, M. E. Enama, I. J. Gordon, L. J. Chang, U. N. Sarwar, K. L. Zephir, L. A. Holman, E. R. James, P. F. Billingsley, A. Gunasekera, S. Chakravarty, A. Manoj, M. Li, A. J. Ruben, T. Li, A. G. Eappen, R. E. Stafford, N. K C, T. Murshedkar, H. DeCederfelt, S. H. Plummer, C. S. Hendel, L. Novik, P. J. Costner, J. G. Saunders, M. B. Laurens, C. V. Plowe, B. Flynn, W. R. Whalen, J. P. Todd, J. Noor, S. Rao, K. Sierra-Davidson, G. M. Lynn, J. E. Epstein, M. A. Kemp, G. A. Fahle, S. A. Mikolajczak, M. Fishbaugher, B. K. Sack, S. H. Kappe, S. A. Davidson, L. S. Garver, N. K. Bjorkstrom, M. C. Nason, B. S. Graham, M. Roederer, B. K. Sim, S. L. Hoffman, J. E. Ledgerwood, R. A. Seder, Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. Nat. Med. 22, 614–623 (2016).

8. R. A. Seder, L. J. Chang, M. E. Enama, K. L. Zephir, U. N. Sarwar, I. J. Gordon, L. A. Holman, E. R. James, P. F. Billingsley, A. Gunasekera, A. Richman, S. Chakravarty, A. Manoj, S. Velmurugan, M. Li, A. J. Ruben, T. Li, A. G. Eappen, R. E. Stafford, S. H. Plummer, C. S. Hendel, L. Novik, P. J. Costner, F. H. Mendoza, J. G. Saunders, M. C. Nason, J. H. Richardson, J. Murphy, S. A. Davidson, T. L. Richie, M. Sedegah, A. Sutamihardja, G. A. Fahle, K. E. Lyke, M. B. Laurens, M. Roederer, K. Tewari, J. E. Epstein, B. K. Sim, J. E. Ledgerwood, B. S. Graham, S. L. Hoffman; VRC 312 Study Team, Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 341, 1359–1365 (2013).

9. R. Chattopadhyay, S. Conteh, M. Li, E. R. James, J. E. Epstein, S. L. Hoffman, The effects of radiation on the safety and protective efficacy of an attenuated *Plasmodium yoelii* sporozoite malaria vaccine. Vaccine 27, 3675–3680 (2009).

10. S. M. Khan, C. J. Janse, S. H. Kappe, S. A. Mikolajczak, Genetic engineering of attenuated malaria parasites for vaccination. Curr. Opin. Biotechnol. 23, 908–916 (2012).

11. N. S. Butler, N. W. Schmidt, A. M. Vaughan, A. S. Aly, S. H. Kappe, J. T. Harty, Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. Cell Host Microbe 9, 451–462 (2011).

12. B. C. L. van Schaijk, I. H. J. Ploemen, T. Annoura, M. W. Vos, L. Foquet, G.-J. van Gemert, S. Chevalley-Maurel, M. van de Vegte-Bolmer, M. Sajid, J.-F. Franetich, A. Lorthiois, G. Leroux-Roels, P. Meuleman, C. C. Hermsen, D. Mazier, S. L. Hoffman, C. J. Janse, S. M. Khan, R. W. Sauerwein, A genetically attenuated malaria vaccine candidate based on gene-deficient sporozoites. eLife 3, e03582 (2014).

13. J. G. Kublin, S. A. Mikolajczak, B. K. Sack, M. E. Fishbaugher, A. Seilie, L. Shelton, T. VonGoedert, M. Firat, S. Magee, E. Fritzen, W. Betz, H. S. Kain, D. A. Dankwa, R. W. Steel, A. M. Vaughan, S. D. Noah, S. C. Murphy, S. H. Kappe, Complete attenuation of genetically engineered *Plasmodium falciparum* sporozoites in human subjects. Sci. Transl. Med. 9, e03582 (2017).

14. S. A. Mikolajczak, V. Lakshmanan, M. Fishbaugher, N. Camargo, A. Harupa, A. Kaushansky, A. N. Douglass, M. Baldwin, J. Healer, M. O'Neill, T. Phuong, A. Cowman, S. H. Kappe, A next-generation genetically attenuated *Plasmodium falciparum* parasite created by triple gene deletion. Mol. Ther. 22, 1707–1715 (2014).

15. M. Spring, J. Murphy, R. Nielsen, M. Dowler, J. W. Bennett, S. Zarling, J. Williams, P. de la Vega, L. Ware, J. Komisar, M. Polhemus, T. L. Richie, J. Epstein, C. Tamminga, I. Chuang, N. Richie, M. O'Neil, D. G. Heppner, J. Healer, M. O'Neill, H. Smithers, O. C. Finney, S. A. Mikolajczak, R. Wang, A. Cowman, C. Ockenhouse, U. Krzych, S. H. Kappe, First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of *Anopheles* mosquitoes to adult volunteers. Vaccine 31, 4975–4983 (2013).

16. S. L. Hoffman, P. F. Billingsley, E. James, A. Richman, M. Loyevsky, T. Li, S. Chakravarty, A. Gunasekera, R. Chattopadhyay, M. Li, R. Stafford, A. Ahumada, J. E. Epstein, M. Sedegah, S. Reyes, T. L. Richie, K. E. Lyke, R. Edelman, M. B. Laurens, C. V. Plowe, B. K. Sim, Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. Hum. Vaccin. 6, 97–106 (2010).

17. T. Annoura, I. H. Ploemen, B. C. van Schaijk, M. Sajid, M. W. Vos, G. J. van Gemert, S. Chevalley-Maurel, B. M. Franke-Fayard, C. C. Hermsen, A. Gego, J. F. Franetich, D. Mazier, S. L. Hoffman, C. J. Janse, R. W. Sauerwein, S. M. Khan, Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. Vaccine 30, 2662–2670 (2012).

18. J. E. Epstein, K. M. Paolino, T. L. Richie, M. Sedegah, A. Singer, A. J. Ruben, S. Chakravarty, A. Stafford, R. C. Ruck, A. G. Eappen, T. Li, P. F. Billingsley, A. Manoj, J. C. Silva, K. Moser, R. Nielsen, D. Tosh, S. Cicatelli, H. Ganeshan, J. Case, D. Padilla, S. Davidson, L. Garver, E. Saverino, T. Murshedkar, A. Gunasekera, P. S. Twomey, S. Reyes, J. E. Moon, E. R. James, N. Kc, M. Li, E. Abot, A. Belmonte, K. Hauns, M. Belmonte, J. Huang, C. Vasquez, S. Remich, M. Carrington, Y. Abebe, A. Tillman, B. Hickey, J. Regules, E. Villasante, B. K. Sim, S. L. Hoffman, Protection against *Plasmodium falciparum* malaria by PfSPZ Vaccine. JCI Insight 2, e89154 (2017).

19. B. K. Sack, S. A. Mikolajczak, M. Fishbaugher, A. M. Vaughan, E. L. Flannery, T. Nguyen, W. Betz, N. M. Jane, L. Foquet, R. W. J. Steel, Z. P. Billman, S. C. Murphy, S. L. Hoffman, S. Chakravarty, B. K. L. Sim, M. Behet, I. J. Reuling, J. Walk, A. Scholzen, R. W. Sauerwein, A. S. Ishizuka, B. Flynn, R. A. Seder, S. H. I. Kappe, Humoral protection against mosquito bite-transmitted *Plasmodium falciparum* infection in humanized mice. NPJ Vaccin. 2, 27 (2017).

20. G. J. Keitany, B. Sack, H. Smithers, L. Chen, I. K. Jang, L. Sebastian, M. Gupta, D. N. Sather, M. Vignali, A. M. Vaughan, S. H. Kappe, R. Wang, Immunization of mice with live-attenuated late liver stage-arresting *Plasmodium yoelii* parasites generates protective antibody responses to preerythrocytic stages of malaria. Infect. Immun. 82, 5143–5153 (2014).

21. E. M. Bijker, G. J. H. Bastiaens, A. C. Teirlinck, G. J. van Gemert, W. Graumans, M. van de Vegte-Bolmer, R. Siebelink-Stoter, T. Arens, K. Teelen, W. Nahrendorf, E. J. Remarque, W. Roeffen, A. Jansens, D. Zimmerman, M. Vos, B. C. L. van Schaijk, J. Wiersma, A. J. A. M. van der Ven, Q. de Mast, L. van Lieshout, J. J. Verweij, C. C. Hermsen, A. Scholzen, R. W. Sauerwein, Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. Proc. Natl. Acad. Sci. U.S.A. 7, 7862–7867 (2013).

22. K. A. Moser, E. F. Drábek, A. Dwivedi, E. M. Stucke, J. Crabtree, A. Dara, Z. Shah, M. Adams, T. Li, P. T. Rodrigues, S. Koren, A. M. Phillippy, J. B. Munro, A. Ouattara, B. C. Sparklin, J. C. D. Hotopp, K. E. Lyke, L. Sadzewicz, L. J. Tallon, M. D. Spring, K. Jongsakul, C. Lon, D. L. Saunders, M. U. Ferreira, M. M. Nyunt, M. K. Laufer, M. A. Travassos, R. W. Sauerwein, S. Takala-Harrison, C. M. Fraser, B. K. L. Sim, S. L. Hoffman, C. V. Plowe, J. C. Silva, Strains used in whole organism *Plasmodium falciparum* vaccine trials differ in genome structure, sequence and immunologic potential. Genome Med. 8, 6 (2020).

23. M. Roestenberg, M. McCall, J. Hopman, J. Wiersma, A. J. Luty, G. J. van Gemert, M. van de Vegte-Bolmer, B. van Schaijk, K. Teelen, T. Arens, L. Spaarman, Q. de Mast, W. Roeffen, G. Snounou, L. Rénia, A. van der Ven, C. C. Hermsen, R. Sauerwein, Protection against a malaria challenge by sporozoite inoculation. N. Engl. J. Med. 361, 468–477 (2009).

24. J. Walk, R. Schats, M. C. Langenberg, I. J. Reuling, K. Teelen, M. Roestenberg, C. C. Hermsen, L. G. Visser, R. W. Sauerwein, Diagnosis and treatment based on quantitative PCR after controlled human malaria infection. Malar. J. 15, 398 (2016). 25. E. M. Bijker, R. W. Sauerwein, Enhancement of naturally acquired immunity against malaria by drug use. J. Med. Microbiol. 61, 904–910 (2012).

25. E. M. Bijker, R. W. Sauerwein, Enhancement of naturally acquired immunity against malaria by drug use. J. Med. Microbiol. 61, 904–910 (2012).

26. J. Walk, J. E. Stok, R. W. Sauerwein, Can patrolling liver-resident T cells control human malaria parasite development? Trends Immunol. 40, 186–196 (2019).

27. I. J. Reuling, A. M. Mendes, G. M. de Jong, A. Fabra-García, H. Nunes-Cabaço, G.-J. van Gemert, W. Graumans, L. E. Coffeng, S. J. de Vlas, A. S. P. Yang, C. Lee, Y. Wu, A. J. Birkett, C. F. Ockenhouse, R. Koelewijn, J. J. van Hellemond, P. J. J. van Genderen, R. W. Sauerwein, M. Prudêncio, An open-label phase 1/2a trial of a genetically modified rodent malaria parasite for immunization against *Plasmodium falciparum* malaria. Sci. Transl. Med. 12, eaay2578 (2020).

28. D. F. Verhage, D. S. Telgt, J. T. Bousema, C. C. Hermsen, G. J. van Gemert, J. W. van der Meer, R. W. Sauerwein, Clinical outcome of experimental human malaria induced by *Plasmodium falciparum*-infected mosquitoes. Neth. J. Med. 63, 52–58 (2005).

29. M. P. van Meer, G. J. Bastiaens, M. Boulaksil, Q. de Mast, A. Gunasekera, S. L. Hoffman, G. Pop, A. J. van der Ven, R. W. Sauerwein, Idiopathic acute myocarditis during treatment for controlled human malaria infection: A case report. Malar. J. 13, 38 (2014).

30. A. E. Nieman, Q. de Mast, M. Roestenberg, J. Wiersma, G. Pop, A. Stalenhoef, P. Druilhe, R. Sauerwein, A. van der Ven, Cardiac complication after experimental human malaria infection: A case report. Malar. J. 8, 277 (2009).

31. C. C. Hermsen, D. S. Telgt, E. H. Linders, L. A. van de Locht, W. M. Eling, E. J. Mensink, R. W. Sauerwein, Detection of *Plasmodium falciparum* malaria parasites *in vivo* by real-time quantitative PCR. Mol. Biochem. Parasitol. 118, 247–251 (2001).

32. B. Mordmuller, G. Surat, H. Lagler, S. Chakravarty, A. S. Ishizuka, A. Lalremruata, M. Gmeiner, J. J. Campo, M. Esen, A. J. Ruben, J. Held, C. L. Calle, J. B. Mengue, T. Gebru, J. Ibanez, M. Sulyok, E. R. James, P. F. Billingsley, K. C. Natasha, A. Manoj, T. Murshedkar, A. Gunasekera, A. G. Eappen, T. Li, R. E. Stafford, M. Li, P. L. Felgner, R. A. Seder, T. L. Richie, B. K. Sim, S. L. Hoffman, P. G. Kremsner, Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature 542, 445–449 (2017).

33. J. Walk, I. J. Reuling, M. C. Behet, L. Meerstein-Kessel, W. Graumans, G.-J. van Gemert, R. Siebelink-Stoter, M. van de Vegte-Bolmer, T. Janssen, K. Teelen, J. H. W. de Wilt, Q. de Mast, A. J. van der Ven, E. D. Benavente, S. Campino, T. G. Clark, M. A. Huynen, C. C. Hermsen, E. M. Bijker, A. Scholzen, R. W. Sauerwein, Modest heterologous protection after *Plasmodium falciparum* sporozoite immunization: A double-blind randomized controlled clinical trial. BMC Med. 15, 168 (2017).

Supplementary

Inclusion and exclusion criteria

Inclusion 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 or within reasonable travelling distance from the Radboudumc from day -1 till day +28 after each parasite exposure. After CHMI, subjects have to be reachable by phone (24/7) from day -1 until day 35.

5. Subject agrees to inform his/her general practitioner (GP) about participation in the study and to sign a request to release by the GP, and medical specialist when necessary, any relevant medical information concerning possible contra-indications for 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 females of reproductive potential (i.e., have a uterus and are neither surgically sterilized nor post-menopausal) should agree to use adequate contraception and not to 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 has signed informed consent.

Exclusion criteria

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, hematological, infectious, immune-deficient, psychiatric or other disorders, which could compromise the health of the volunteer 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/m2 at screening

b. A heightened risk of cardiovascular disease, defined as:

i. An estimated ten-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 non-febrile seizure at any time 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 immune modifying drugs within three months prior to study onset (excluding inhaled and topical corticosteroids and incidental use of oral anti-histamines) 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 five 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 or amphetamines at screening or prior to infection or positive urine toxicology test for cannabis prior to infection.

2. For female subjects: breastfeeding, or positive urine pregnancy test at screening or prior to immunization or prior to CHMI.

3. Any history of malaria, positive serology for *P. falciparum*, 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.

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.

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 Medical Microbiology or Infectious Diseases of the Radboudumc or the LUMC.

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.

Table S1. Number and severity of adverse events after immunization. Number of volunteers reporting solicited local and systemic adverse events possibly, probably or definitely related to immunization. Percentages are given between parenthesis. Data collected until 35 days after each immunization. No rash, induration, edema, chills or arthralgia were reported.

			PfSPZ-GA1 Vaccine			**PfSPZ Vaccine**	**Placebo**
			1.35x10^5	**4.5x10^5**	**9.0x10^5**	**4.5x10^5**	
		Grade	***n*=3**	***n*=16**	***n*=26**	***n*=13**	***n*=9**
Local	Tenderness	1	0 (0)	0 (0)	2 (8)	1 (8)	0 (0)
	Bruising	1	1 (33)	0 (0)	3 (12)	3 (23)	0 (0)
	Erythema	1	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)
	Swelling	1	0 (0)	0 (0)	1 (4)	2 (15)	0 (0)
	Pain	1	0 (0)	0 (0)	2 (8)	1 (8)	1 (11)
	Pruritis	1	0 (0)	0 (0)	2 (8)	0 (0)	1 (11)
Systemic	Fever	1	0 (0)	1 (6)	1 (4)	1 (8)	0 (0)
		2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		3	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)
	Headache	1	0 (0)	5 (31)	11 (42)	4 (31)	3 (33)
		2	1 (33)	1 (6)	2 (8)	2 (15)	1 (11)
		3	0 (0)	0 (0)	0 (0)	1(8)	0 (0)
	Fatigue/malaise	1	1 (33)	2 (13)	3 (12)	2 (15)	1 (11)
		2	0 (0)	1 (6)	1 (4)	0 (0)	1 (11)
		3	0 (0)	0 (0)	1 (4)	0 (0)	1 (11)
	Myalgia	1	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)
		2	0 (0)	0 (0)	2 (8)	0 (0)	1 (11)
		3	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)

Table S2. Bite numbers used for CHMI for the different study arms. Mosquitoes were 100% infected with a mean of 106,000 sporozoites per mosquito.

	Infection		
	Number of sessions median (range)	Number of Infected bites median (range)	Number of Uninfected bites median (range)
PfSPZ-GA1 9 x10^5	1 (1-3)	5	0 (0-2)
PfSPZ-GA1 4.5 x10^5	1 (1-2)	5	0 (0-2)
PfSPZ Vaccine 4.5 x10^5	1 (1-2)	5	0 (0-1)
Placebo	1 (1-2)	5	0 (0-3)